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Review

Combining enzyme and metabolic engineering for microbial supply of therapeutic phytochemicals☆

Maxence Holtz, Carlos G Acevedo-Rocha and Michael K Jensen

The history of pharmacology is deeply intertwined with plant-derived compounds, which continue to be crucial in drug development. However, their complex structures and limited availability in plants challenge drug discovery, optimization, development, and industrial production via chemical synthesis or natural extraction. This review delves into the integration of metabolic and enzyme engineering to leverage micro-organisms as platforms for the sustainable and reliable production of therapeutic phytochemicals. We argue that engineered microbes can serve a triple role in this paradigm: facilitating pathway discovery, acting as cell factories for scalable manufacturing, and functioning as platforms for chemical derivatization. Analyzing recent progress and outlining future directions, the review highlights microbial biotechnology's transformative potential in expanding plant-derived human therapeutics' discovery and supply chains.

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Introduction

Natural products and their derivatives play an important role in pharmacology, with an estimation that between 1981 and 2019, more than half of approved small-molecule drugs were based on natural product scaffolds [1]. Plants in particular produce a wide diversity of bioactive secondary metabolites, including important chemical scaffolds, such as alkaloids, terpenoids, and phenolics, which have been used for millennia to treat human pathologies [2,3].

Although their effectiveness and necessity for human health are undeniable, supplying safe, efficient, and sufficient amounts of active pharmaceutical ingredients based on therapeutic phytochemicals for the manufacturing of medicines is a major challenge. In most cases, plant bioactives display structures considerably too complex for cost-efficient manufacturing via total chemical synthesis, and thus, supply chains still rely on extraction from plant materials, optionally followed by chemical derivatization [4]. As an example, the anticancer drugs irinotecan and topotecan are currently produced chemically from the plant-extracted alkaloid camptothecin in a semisynthetic process [5]. Extraction from plants is, however, made difficult by low metabolite accumulation *in planta*, difficulty in cultivating certain plants at large scale, slow growth, presence of multiple structurally closely related metabolites in raw starting materials, as well as inefficient and energy-consuming extraction processes [2,4]. Additional climatic and political variations destabilize the supply chains of therapeutic phytochemicals leading to frequent shortages of essential medicines [6]. To give an example, for the anticancer alkaloids vinblastine and vincristine 500 kg and 2 tons, respectively, of dried Madagascar periwinkle *Catharanthus roseus* leaves are necessary to obtain 1 g of product [7]. Total chemical synthesis is not feasible at industrial scale owing to the highly complex structure of these drugs containing multiple stereocenters [8]. Plant extraction–based supply for these essential leukemia medicines leads to 20–100.000 $/kg price, and shortages have been recurring over the last years [7,9].

Over the last 20 years, metabolic engineering of microbial cell factories has emerged as an alternative manufacturing route for therapeutic phytochemicals, promising to be more decentralized, sustainable, scal-

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able, and therefore more reliable by fermentation of relatively cheap feedstocks. This was first illustrated in the pioneering work by Ro et al. for the construction of a yeast cell factory producing artemisinic acid, a precursor of the antimalarial medicine artemisinin [10,11]. Their final optimized strain produced > 25 g/l of artemisinic acid from glucose and was licensed to the pharmaceutical company Sanofi for commercialization [12]. Since then, tremendous technical progress has been made in omics-based plant pathway discovery, tools for genetic engineering in microbes, and enzyme engineering, which has fostered a multitude of seminal metabolic engineering studies on the production of therapeutic phytochemicals with clinical relevance [13–19].

In this review, we focus on recent efforts in the construction and optimization of microbial cell factories to produce and derivatize plant bioactives. Although we aim to cover different product chemical families, particular attention is given to alkaloids due to their high pharmacological importance.

Engineering microbes as biosynthetic pathway discovery platforms

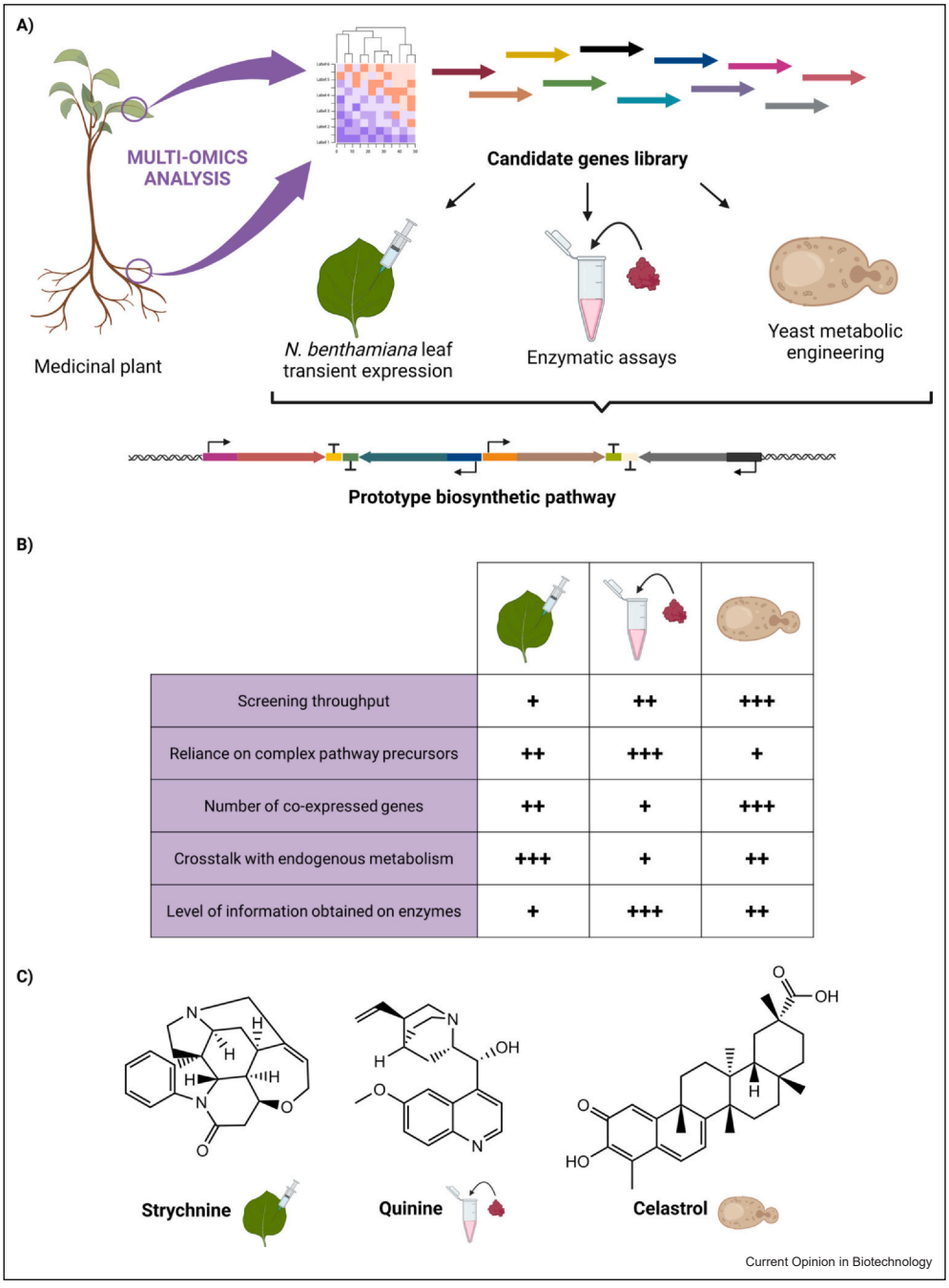
The first critical factor to assess when starting a metabolic engineering project aiming at producing a therapeutic phytochemical is the knowledge on the biosynthetic pathway needed to produce it. While progress has been made in applying and integrating multiomics methods in plants to identify candidate genes in a pathway of interest [20,21], efficient discovery of biosynthetic pathways is hampered by limitations in both the screening throughput of gene candidates encoding biosynthetic pathway enzymes and the limited accessibility of complex pathway intermediates. The three main methods employed are (1) *in planta* testing by transient gene expression in *Nicotiana benthamiana* leaves, (2) *in vitro* assays of purified proteins, and (3) yeast metabolic engineering (Figure 1).

Through *Agrobacterium tumefaciens*–mediated transfection, it is possible to co-express as well as downregulate multiple gene candidates in *N. benthamiana*, and if necessary, co-infiltrate pathway substrates followed by assessment of production profiles by high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) or nuclear magnetic resonance. Recently, the biosynthetic pathway for the neurotoxic alkaloid strychnine from the poison fruit *Strychnos nux-vomica* was uncovered with this method [22]. Using combinatorial testing of candidate genes selected based on spatial- and co-expression transcriptomic analysis as well as putative enzyme function, the authors identified the nine enzymes responsible for strychnine, brucine, and diaboline biosynthesis from geissoschizine [22].

In vitro enzymatic assays performed with purified proteins obtained from heterologous hosts (usually *Escherichia coli* or *Saccharomyces cerevisiae*) are another way to obtain information on plant biosynthetic pathways. Such an approach makes detailed biochemical characterization accessible, including information on kinetic parameters, substrate, cofactor preference, regio-/stereoselectivity as well as potential inhibitors. With this approach, the biosynthesis of quinine, the antimalarial, and bitter flavoring alkaloid was investigated by Trenti et al. using purified proteins produced both in *E. coli* and *S. cerevisiae*, resulting in the discovery of three enzymes of the pathway: an alcohol dehydrogenase (*Cp*DCS), an esterase (*Cp*DCE), and an O-methyltransferase (*Cp*OMT1) from red cinchona *Cinchona pubescens* [23]. *In vitro* experiments combined with site-directed mutagenesis can also shed light on the structure-to-function relationships of enzymes of interest especially regarding catalytic activity, specificity, and stability. The stereospecificity of various medium-chain dehydrogenase/reductase from *C. roseus* enabling the production of different stereochemical profiles of bioactive heteroyohimbine alkaloids was carried out this way [24]. After resolving the crystal structures of the specialized tetrahydroalstonine synthase *Cro*THAS1 and its promiscuous heteroyohimbine synthase homolog *Cro*HYS, producing three different products, the authors conducted point mutations and loop grafting between the two enzymes gaining insight into their catalytic mechanism and the structural determinants controlling their stereoselectivity. It should be noted that the adoption of *in vitro* assays, compared with *in vivo* studies, often is limited by the price and availability of intermediates in plant biosynthetic pathways used as substrates for testing candidate enzymes.

Engineered yeast can also be leveraged as platforms for pathway elucidation. High-throughput engineering methods, either based on CRISPR-Cas9 genomic integration or plasmid-based screening, hold the promise to increase the speed of enzyme discovery. Yeast can be engineered to produce *de novo* complex and rare intermediates through refactoring of long pathways, which is tedious, or even impossible, when using *N. benthamiana* assays [25]. In addition, yeast usually presents less metabolic crosstalk compared with tobacco plants, which contain many endogenous hydroxylases and glycosyltransferases [25,26]. This was exemplified recently by the discovery of 20 new enzymes involved in the biosynthetic pathway of tropane alkaloid biosynthesis in coca plant *Erythroxylum coca* employing a yeast platform engineered for the overproduction of the precursor spermidine [25]. Their sequential and combinatorial yeast engineering approach complemented by *in vitro* and *in planta* assays provides an excellent illustration of the complementarity of the methods currently adopted for pathway discovery. Other examples were recently provided by the Kampranis laboratory combining yeast and tobacco platforms to elucidate the biosynthesis of a

Figure 1



General workflow for elucidating plant biosynthetic pathways. **(a)** Medicinal plants of interest are subjected to multiomics analysis to select a library of candidate pathway genes. These candidates are tested by expression in either tobacco leaves, *in vitro* enzymatic assays, or yeast. (**b)** Comparison of these three plant candidate gene screening methods (for each criterion, + = poor, ++ = medium, and +++ = good). Choice of method mainly depends on the throughput required and accessibility to complex pathway intermediates. (**c**) Examples of the structure of some therapeutic phytochemicals whose biosynthetic pathways have recently been fully or partially elucidated. These examples and corresponding references are detailed in the main text.

neuromodulatory ginkgolide terpenoid in *Ginkgo biloba* [27] and antiobesity compound celastrol from *Tripterygium wilfordii* [28].

With respect to engineered microbial cells for pathway discovery, it is worth mentioning that the throughput of microbe-based pathway discovery is today still largely

limited by the price of DNA synthesis. As this cost is still set to decrease in the coming years [29], we envision the possibility of ordering hundreds to thousands of codon-optimized gene fragments at large scale, cloning them in expression vectors using automatable techniques such as Golden Gate Assembly [30] and therefore reaching genome scale–level screening of particular enzyme classes from medicinal plants.

Combining metabolic and enzyme engineering for cell factory optimization

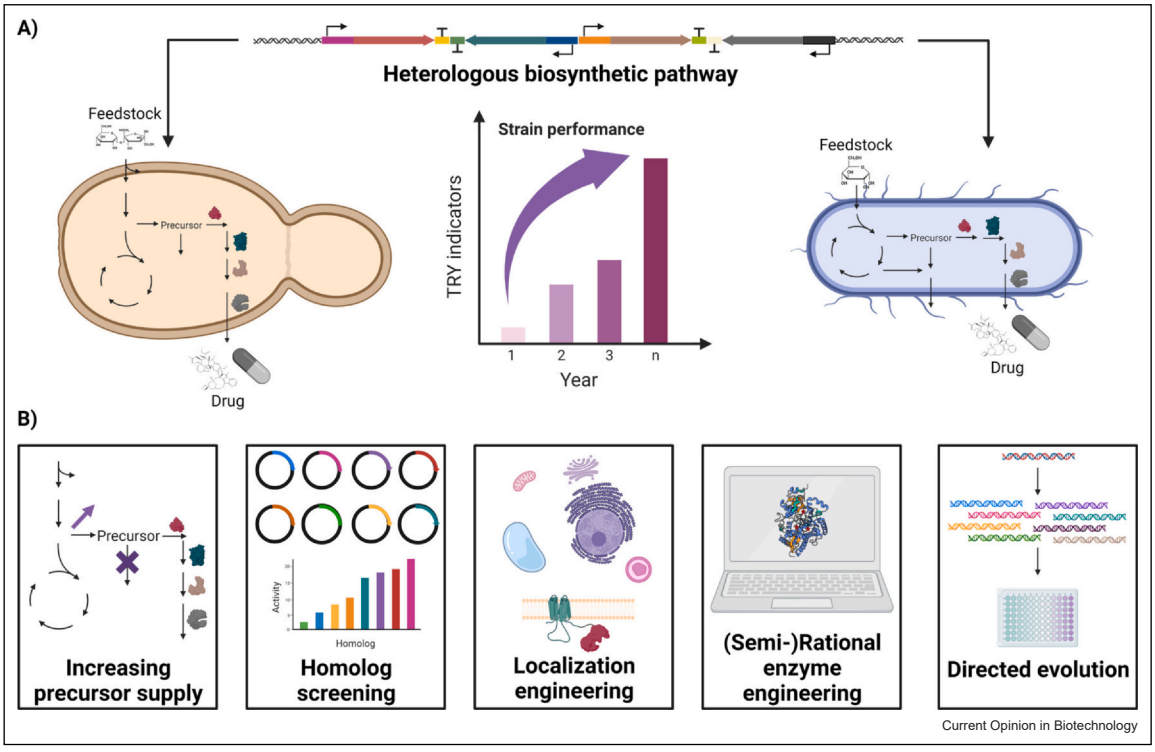
With fully elucidated biosynthetic pathways from plants available, the first prototypic microbial cell factories can be built and tested. Given the magnitude of the engineering work required to refactor and optimize long plant secondary metabolism pathways, most studies rely on industrial biotechnology workhorses *E. coli* and *S. cerevisiae*, both of which benefit from highly developed synthetic biology toolboxes, fast growth, well-characterized metabolisms, easy handling, and scalable cultivation setups. For heterologous expression of biosynthetic pathways for phytochemical production, yeast is currently the preferred choice, thanks to its eukaryotic cell architecture shared with plants, enabling complex post-translational protein modifications, compartmentalized metabolism, as well as expression of endomembrane-anchored enzymes such as cytochrome P450s that are prevalent in plant secondary metabolism [31–33]. Importantly, the choice of chassis organism depends on the compound family (e.g. derived from shikimate mevalonate pathways), the type of enzymes (e.g. membrane capacity for P450 enzymes), and cofactors involved, as well as potential toxicity of pathway intermediates or products.

The first proof-of-concept strains obtained upon refactoring the prototype pathway usually produce low titers in the µg/l to low mg/l range [2]. Strain optimization to achieve commercially relevant titer, rate, and yield (TRY) is a time-consuming procedure that involves several years of costly consecutive engineering rounds [34–36] (Figure 2a). Coming back to the previous vinblastine example, technoeconomic analysis concluded that high titer > 1 g/l was required for rentability of yeast-based tabersonine (vinblastine precursor) and 10–50 mg/l for yeast-based vinblastine supply, meaning subsequent strain improvement still needs to be carried before reaching a competitive minimal selling price on the market [9]. This type of analysis is important to carry in the early stages of process development to identify the target strain TRY to reach for industrial application.

An important parameter to solve when refactoring plant biosynthetic pathways in microbial cells is to increase and balance the supply of precursors and cofactors. This includes overexpression of endogenous genes, deletion of competing pathways, regulator engineering, as well as transplant of superior heterologous pathways modules (Figure 2b). Chen et al. combined these methods in *S. cerevisiae* to improve caffeic acid and ferulic acid production, important precursors for bioactive plant phenolics [37]. Upon using feedback-insensitive shikimate enzyme variants and deleting competing pathways to optimize precursor p-coumaric acid supply, they found that redox and methyl-transfer cofactors suborganelle distribution compromised the natural product biosynthesis and engineering cofactor colocalization led to the production of 5.5 g/l caffeic acid and 3.8 g/l ferulic acid in glucose fed–batch fermentation. Examples of platform strains producing significant amounts of the different plant natural product precursors are widely available in the literature (Table 1). Speeding up this precursor supply optimization will entail significant effort to increase the size of the libraries of designs, including the use of metabolic modeling pipelines as well as involvement of automated biofoundries to build and test strains in high-throughput. This was illustrated recently by the presentation of Amyris's automated scientist named Lila, used to design and optimize both *E. coli* and *S. cerevisiae* cell factories producing 454 different target molecules [35]. Using a design of experiment framework, Lila carried out combinatorial strain designs for every target compound by sampling various combinations of promoter, terminator, enzyme homolog, N-terminal truncations, codon optimization, genomic integration site, copy number of pathway, and competing pathway knockout. Using their high-throughput strain engineering, fermentation, and analytical chemistry platforms, the authors built and tested > 32 000 strains and reached impressive performances for many compounds, including the first > 1.5 g/l naringenin producer from glucose, paving the way to industrial flavonoid production for health applications.

In addition to precursor supply challenges, the activity profiles of the enzymes involved in therapeutic phytochemicals pathways often greatly limit cell factory performance. These enzymes originate from plant secondary metabolism and have neither evolved to support high fluxes, nor been optimized in terms of resource efficiency [46]. Other challenges arise because of their heterologous expression, especially in terms of stability, making enzyme screening and engineering a cornerstone of phytochemical-producing cell factory optimization (Table 2). One common strategy aimed at mitigating this issue is to screen libraries of enzyme homologs for each reaction step to identify the best performing enzyme. This strategy was widely used in the refactoring of the 30-enzymatic step biosynthetic pathway for anticancer drug vinblastine in yeast [7]. In particular, for the step catalyzed by strictosidine-β-d-glucosidase (SGD), a gateway enzyme for the production of monoterpene indole alkaloids (MIAs), 46 homologs from different plants were assessed. The best performing candidate, *Rse*SGD from *Rauwolfia serpentina*,

Figure 2



Strategies for optimization of microbial cell factories for producing plant natural products. **(a)** Obtaining cell factories that meet commercial TRY is a long and costly process involving both metabolic and enzyme engineering campaigns**. (b)** Common approaches include rewiring host metabolism to optimize precursor supply, screening for superior enzyme homologs, altering enzyme localization, and engineering key bottleneck enzymes.

was used as a basis for further rational engineering through domain swapping, and the best variant used in the final production strain enabled > 100-fold higher MIA titers compared with *Cro*SGD from vinblastine-producing *C. roseus* [47].

Plants use natural products for specialized functions, such as defense against predation and drought, and thus spatiotemporally regulate their production [48]. This includes compartmentalizing individual enzyme reactions or modules of enzyme cascades in distinct subcellular compartments as exemplified recently for vinblastine [49]. Therefore, consideration of subcellular localization is also necessary for the functional expression of plant enzymes in heterologous hosts. Cytochrome P450s, for example, usually need to be localized to the endoplasmic reticulum membrane with their reductase to be functional [7,50]. Some enzymes might require specific biochemical environments to be functional, to go back to the previous SGD example, this enzyme has been shown to localize in the nucleus in plants. Zhang et al. observed that in yeast too, the only active homologs were the ones located in the nucleus. Addressing enzymes in different organelles using known tags in yeast can also be used to increase the production of target compounds. Recently, toxic effects of cytosolic expression of norcoclaurine synthase (NCS) in *S. cerevisiae*, the enzyme that catalyzes the first committed step in benzylisoquinoline alkaloids (BIAs) biosynthesis, were observed [32]. Through localizing this enzyme in the peroxisome and increasing peroxisome capacity, increased norcoclaurine titers and growth rates were obtained. Improving the spatial proximity of enzymes in a pathway through the construction of fusion enzymes or use of scaffolding platforms is another strategy to promote metabolic channeling. Production of α-ionone, a violet fragrance terpene with multiple bioactivities, was improved > 2.5-fold in *E. coli* by fusing the last two enzymes of the pathway lycopene ε-cyclase (LcyE) and carotenoid cleavage dioxygenase 1 (CCD1) [51]. By doing so, they increased accessibility of CCD1 to its hydrophobic substrate ε-carotene produced by membrane-anchored enzyme LcyE while decreasing side product formation due to CCD1 promiscuity. This strategy was also applied to kaurenoic acid 13α-hydroxylase (KAH) and ent-kaurene oxidase (KO), two cytochrome P450s and their reductase CPR1 to improve production of the sweetener rubusoside in yeast. The best design involving the fusion enzyme KAH-GGGGS$_3$-trCPR1 and a scaffolding peptide tag construct KO-RIDD/KAH-RIAD yielded an eightfold increase in precursor steviol titer over the base strain [52].

Table 1

Examples of platform microbial strains to produce pharmaceutically relevant plant natural products.

Target compound	Chassis	Titer	Utility	Reference
Alkaloids				
Strictosidine	*S. cerevisiae*	25.2 mg/l	Precursor of >3000 MIAs, many of which are FDA-approved drugs or have documented bioactivities	[7]
Reticuline	*S. cerevisiae*	4.6 g/l	Precursor of tetrahydroisoquinoline BIAs with >3000 structures and many bioactive natural products and semisynthetic analogs	[38]
Scopolamine and tropine	*S. cerevisiae*	30 µg/l scopolamine and 3 mg/l tropine	Entry point to produce tropane alkaloids such as atropine or cocaine and derivatives with important pharmaceutical interest	[39]
Terpenoids				
Farnesene	*S. cerevisiae*	130 g/l	Platform strain with rewired central metabolism for optimal farnesyl pyrophosphate (FPP) supply used to produce sesquiterpenes (C15) and triterpenes (C30) with multiple health applications	[40]
α-humulene	*Y. lipolytica*	21.7 g/l	High-level production of FPP-derived terpenoids in oleaginous yeast *Yarrowia lipolytica*	[41]
Lycopene	*S. cerevisiae*	1 g/l	Strain rewired with 374-fold increased geranylgeranyl pyrophosphate supply of interest for diterpene (C20) and tetraterpene (C40) production	[42]
Geraniol	*E. coli*	2.1 g/l	Platform for geranyl pyrophosphate (GPP)–derived monoterpenes (C10) with important bioactivities	[43]
Limonene	*E. coli*	3.6 g/L	Platform for GPP-derived monoterpenes (C10) with important bioactivities	[44]
Phenolics				
Caffeic acid and ferulic acid	*S. cerevisiae*	5.5 g/l caffeic acid and 3.8 g/l ferulic acid	Common precursors for many industrially relevant plant polyphenols for health applications	[37]
Naringenin	*S. cerevisiae*	1.5 g/l	Platform strain for production of >9000 flavonoids with multiple bioactivities	[35]
Resveratrol	*Y. lipolytica*	12.4 g/l	Potential host for production of other stilbenoids of clinical interest	[45]

FDA, US Food and Drug Administration.

Table 2

Recent examples of enzyme screening and engineering to improve therapeutic phytochemicals production in a microbial host.

Target compound	Chassis	Enzyme(s)	Optimization method	Biological effect	Reference
Alkaloids					
4-OMe-Norbelladine	*E. coli*	Nb4OMT	Machine learning–guided semirational engineering based on an Alphafold2 structural model combined with an engineered transcription factor–based biosensor for screening	60% improvement in product titer, 17-fold reduced remnant substrate, and threefold lower off-product regioisomer formation	[56]
Vinblastine	*S. cerevisiae*	SGD	1. Enzyme homolog screening 2. Domain swapping	1. Validation of strictosidine aglycone production 2. > 100-fold increase in tetrahydroalstonine production with chimeric enzyme compared to CroSGD (from vinblastine-producing plant *Catharanthus roseus*)	[7]
THP	*E. coli*	GfOMT1 (*Glaucium flavum* O-methyltransferase 1)	1. Directed evolution using error-prone PCR (epPCR) combined with an engineered transcription factor–based biosensor for screening	Obtention of a multifunctional GfOMT1 variant with six mutations capable of methylating norlaudanosoline at four positions yielding THP with one enzyme instead of four. This variant produced 1.48 mg/l from 2.88 mg/l norcoclaurine in whole-cell bioconversion experiment	[58]
THP	*S. cerevisiae*	1. EcNMCH (N-methylcoclaurine hydroxylase) 2. TfS9OMT (*Thalictrum flavum* soulerine 9-O-methyltransferase)	Combination of directed evolution, structure-guided site-saturated mutagenesis and DNA shuffling	Top performing EcNMCH^{L203S} and TfS9OMT^{OPT} variants identified with, respectively, 40-fold and 35-fold higher activity toward non-native N-demethylated substrates enabling functional synthetic pathway for *de novo* THP in yeast reaching 121 µg/l	[67]
Norcoclaurine	*S. cerevisiae*	NCS	1. Enzyme homolog screening 2. Truncation of best performing homolog Relocation of tNCS to the peroxisome	1. 20-fold higher norcoclaurine titer with truncated version of best performing NCS homolog compared with initial strain 2. 78% improvement of growth rate and 54% higher norcoclaurine titer upon truncated NCS peroxisome relocation reaching 1.5 mg/l	[32]
THP/tetrahydropalmatrubine (THPB)	*S. cerevisiae*	TfS9OMT (*Thalictrum flavum* soulerine 9-O-methyltransferase)	1. Rational engineering based on a crystal structure	1. Identification of mutants with altered substrate and regioselectivity enabling a synthetic route for *de novo* production of THP and THPB in yeast	[68]
Guattegaumerine/berbamunine	*S. cerevisiae*	1. DRS-DRR (dehydroreticuline synthase–dehydroreticuline reductase)	1. Splitting bifunctional DRS-DRR enzyme in separate domains and investigation of split and chimeric	Split PrDRS and PbDRR V2 constructs enabled production of 109 mg/l guattergaumerine in yeast	[69]

Table 2 (*continued*)

Target compound	Chassis	Enzyme(s)	Optimization method	Biological effect	Reference
		2. MaCYP80A1 (*Mahonia aquifolium* cytochrome P450 berbamunine synthase)	combinations of domains from different plant sources 2. Construction of chimeric MaCYP80A1 containing the N-terminal integral membrane portion from BsCYP80A1	corresponding to a 10.1-fold increase over the best wild type bifunctional enzyme PbDRS-DRR 1. Almost complete reversal of the product ratio with Bs-MaCYP80A1 results in berbamunine concentrations of 25.4 mg/l and no detectable guattegaumerine	
Terpenoids					
Tetrahydrocannabinol (THC)	*S. cerevisiae*	CsTHCAS (*Cannabis sativa* Δ9-tetrahydrocannabinolic acid synthase)	Site-directed saturation mutagenesis library screened using a sensing yeast strain carrying the human cannabinoid receptor CB2	Isolation of variants producing higher CB2-mediated fluorescence response correlated with higher titers of THC	[70]
Rubudioside/rebaudiosides	*S. cerevisiae*	1. KAH 2. KO	Fusion enzymes and use of scaffolding peptide tags of target cytochrome P450 with truncated version of their reductase tCPR1	Combination of best performing metabolic channeling designs for both KAH and KO yielded an eightfold increase in precursor steviol titer over base strain reaching 40.6 mg/l	[52]
Terpenoid with inhibitory activity toward human PTP1B	*E. coli*	GHS	1. Directed evolution (epPCR) and site saturation mutagenesis of GHS associated with growth coupling genetic circuit for selection of variants producing terpenoid products inhibiting PTB1B and allowing expression of an antibiotic resistance gene	Combining two mutations increased the concentration of a minor product, a terpene alcohol that inhibits PTP1B by over 50-fold	[64]
Noncannonical C_{16} terpenes	*S. cerevisiae*	SpSodMT (*Serratia plymuthica* sodorifen methyltransferase)	Rational engineering of the substrate binding pocket based on a homology structural model	Obtention of variants producing 10 different noncanonical C_{16} terpenoid building blocks that could be converted by terpene synthases to 28 different new-to-nature terpenes	[71]
Miltiradiene	*S. cerevisiae*	Classes I and II di-TPS (diterpene synthases)	1. Combinatorial screening of types I and II di-TPS for miltiradiene production 2. Fusion of best combination of classes I and II di-TPS 3. Truncation of plastid localization tags	Best strain carrying fusion enzyme tSmKSL1-CfTPS1 produced 3.5 g/l miltiradiene in a 5-l bioreactor	[72]
Various monoterpenes (menthol, α-pinene, limonene, geraniol, sabinene, camphene, 8-hydroxygeraniol, cannabigerolic acid)	*S. cerevisiae*	1. Enzymes of the mevalonate pathway 2. Monoterpene synthases (SeCamS, SpSabS, ObGerS, MsLimS, ClLimS, PtPinS, ObGerS)	1. Relocation of the entire mevalonate pathway (producing monoterpene precursor GPP) and different monoterpene synthases to the yeast peroxisome	Overall, 15- to 125-fold increase in monoterpene titers when enzymes are targeted to the peroxisome compared with cytosolic expression	[73]
Forskolin	*S. cerevisiae*	CYP76AH15	Semirational site-directed mutagenesis approach targeting different substrate recognition sites	$CYP76AH15^{A99I}$ variant leads to 5.6-fold higher titer of 11-oxo-13R-manoyl oxide reaching 86.4 mg/l (first	[74]

Table 2 (*continued*)

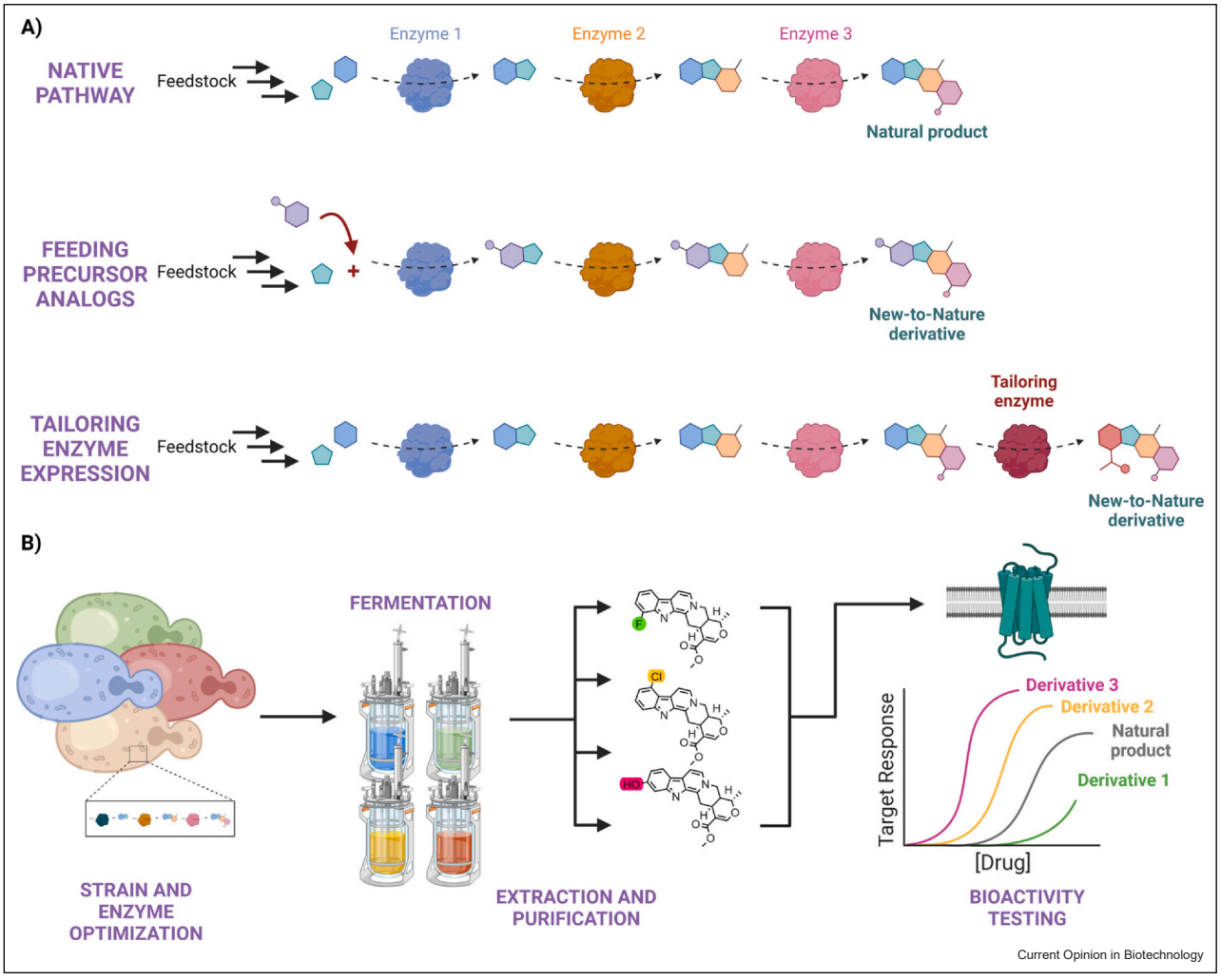
Target compound	Chassis	Enzyme(s)	Optimization method	Biological effect	Reference
				committed step of the forskolin pathway)	
Ginsenoside Rh2	*S. cerevisiae*	UGT51 (UDP-glucose sterol glucosyltransferase)	Sequential rounds of semirational engineering based on crystal structure with a colorimetric screening	Best mutant M7-1 bearing seven mutations resulted in ~1800-fold increase in *in vitro* Rh2 production activity and improved 122-fold Rh2 titer in yeast reaching 6 mg/l	[54]
Phenolics					
Resveratrol	*E. coli*	4CL1 (*Arabidopsis thaliana* 4-coumarate-CoA ligase 1)	Directed evolution (epPCR) library of 4CL screened *in vivo* with an engineered transcription factor–based biosensor	Up to 4.7-fold increase in resveratrol titer reaching > 200 mg/l	[75]
Xanthohumol	*S. cerevisiae*	HIPT1L (*Humulus lupulus* prenyltransferase)	1. Screening of homologous prenyltransferases 2. Truncation of signal peptide in best performing candidates 3. Fusion of truncated prenyltransferase with prenyl group donor IDI1 4. Increase expression level of fusion enzyme	HIPT1LΔ_{1-86} truncation increased prenylation activity, and 21-fold increase in xanthohumol precursor production achieved upon expressing fusion enzyme IDI1-HIPT1LΔ_{1-86} reaching a final *de novo* production of 140 µg/l xanthohumol	[76]

Structure-guided rational or semirational engineering of bottleneck enzymes is another common approach to optimize microbial cell factories for phytochemical production [53]. Production of anticancer ginsenoside Rh2 was improved over 900-fold in yeast by combining semirational engineering of glycosyltransferase UGT51 based on a crystal structure, with deletion of a product-degrading enzyme and increased cofactor supply [54]. While experimentally determined protein structures are not available for all enzymes, recent improvements in structure prediction, in particular, Alphafold 2 [55], are expected to have a positive impact on structure-guided engineering of key enzymes to increase therapeutic phytochemicals production in heterologous microbes. This computational approach was exemplified recently by d'Oelsnitz et al, who used an Alphafold2-generated structural model to carry out machine learning–guided semirational engineering of norbelladine 4-O-methyltransferase (Nb4OMT) involved in *Amaryllidaceae* alkaloids production. Using an engineered biosensor for screening, they identified a variant with 60% improvement in product titer, 17-fold reduced remnant substrate, and threefold lower off-product regioisomer formation in *E. coli* [56].

Alternatively, directed evolution is still widely applied to increase biocatalytic activity or alter the specificity of plant enzymes in micro-organisms. This approach encompasses a multitude of library preparation methods that are used in iterative cycles of gene mutagenesis, expression, and selection [57]. However, since gene libraries often have a very large size, and finding improved variants is difficult, the main challenge is to develop reliable high-throughput screening methods. To address this issue, promiscuity of genetically encoded biosensors based on prokaryotic allosteric transcription factors (aTFs) can be exploited to develop cheap and semiquantitative readouts to approximate accumulation of natural product titers (e.g. by increase of fluorescence). This approach was adopted by d'Oelsnitz et al. who evolved the multidrug-resistance regulator, RamR from *Salmonella typhimurium* to sense different BIAs with important therapeutic activities [58]. Using a double negative selection/positive screening approach in *E. coli*, variants with high selectivity for their cognate BIA were obtained. With one of these engineered sensors, the authors evolved the O-methyltransferase *Gf*OMT1 from *Glaucium flavum* to become more promiscuous, allowing the production of pharmaceutically important tetrahydropapaverine (THP) by methylating norcoclaurine at four different positions. Their work illustrates well how evolution-guided promiscuity engineering can yield synthetic metabolic pathways bypassing unknown or difficult-to-express enzymes.

While aTF-based biosensors are one feasible option, we argue that other approaches harnessing specific bioactivities of therapeutic phytochemicals could be considered as

Figure 3



Microbial cell factories as platforms for the derivatization of bioactive plant natural products. **(a)** Natural product producing strains bearing native plant pathways can be exploited to generate new-to-nature derivative libraries by feeding pathway precursor analogs and relying on pathway enzyme promiscuity. These strains can also be further engineered to express tailoring enzymes to decorate natural products. **(b)** The workflow to employ microbial cell factories as phytochemical derivatization platforms includes strain and enzyme optimization, fermentation, and downstream processing to yield panels of analog compounds that can be evaluated subsequently for bioactivity and improved pharmacological properties.

alternative screening methods. This is particularly relevant for compounds targeting G protein–coupled receptors [59] as many of these human receptors have been functionalized in yeast and linked to a fluorescence output [60–63]. Other types of bioactivities such as protein inhibition could also be leveraged to develop high-throughput assays. For example, the inhibition of human protein tyrosine phosphatase 1B (PTP1B) was linked to the expression of an antibiotic resistance gene in *E. coli* with a genetic circuit used to select for variants of the promiscuous terpene synthase γ-humulene synthase (GHS), producing inhibitory terpenoids from a directed evolution library [64]. Finally, certain plant secondary metabolites exhibit stress-protecting bioactivities, which can be exploited in Adaptive Laboratory Evolution (ALE) experiments to increase microbial production. ALE was applied to optimize yeast cell factories producing β-carotene and β-caryophyllene, two antioxidant terpenoids yielding threefold and fourfold respective improvements after cultivation for 20 days in oxidative conditions [65,66].

By integrating metabolic and enzyme engineering, it becomes possible to design and tailor entire biosynthetic pathways, optimize enzyme kinetics, and overcome limitations associated with natural enzymatic systems. At every stage of the optimization process, we envision that novel technological advancements will be crucial in accelerating the optimization of cell factories producing therapeutic phytochemicals. Examples include integration of

machine learning to design superior strains and enzymes, use of automated robotic platforms for large-scale strain building and fermentation optimization, as well as application of microfluidics and high-throughput screening based on LC-MS.

Bioengineering for new-to-nature natural product derivatives

Classical drug discovery pipelines usually involve finding a small-molecule 'hit' against a particular target, that is then optimized in a medical chemistry campaign, and the structural analogs screened for superior pharmacodynamic and pharmacokinetic properties [77]. Developing a library of analogs for plant therapeutics can be chemically challenging due to their complex structures, and the lack of regio- and stereo-selectivity of available synthetic chemistry processes [78]. Microbial cell factories can serve as a platform for the generation of such libraries of bioactive natural products based on biocatalysis in microbial hosts expressing highly specific enzymes (Figure 3a). One method is to feed modified precursors in microbe cultivations and rely on the promiscuity of pathway enzymes to accept these altered substrates. This approach was widely used to produce halogenated alkaloids, including noscapine [17], berberine [79], alstonine, and serpentine [80].

Metabolic engineering in microbial hosts also opens the possibility of expressing tailoring enzymes and generating scaffold modifications *de novo*. Tailoring enzymes of interest include halogenases, methyltransferases, oxidoreductases, and glycosyltransferases. For instance, bacterial flavin-dependent halogenases were recently expressed in *S. cerevisiae* cell factories allowing the introduction of chlorine and bromine atoms in bioactive plant MIA scaffolds. In addition to significantly altering the ligand's pharmacological properties on their own, such halogen modifications could also be used as chemical handles for specific chemical cross-coupling leading to a nearly infinite diversity of analogs [81]. By expressing and engineering the methyltransferase *Sp*SodMT from *Serratia plymuthica* in yeast, Ignea et al. demonstrated the production of 10 different non-canonical C_{16} prenyl diphosphate terpenoid precursors [71]. These were further converted by terpene synthases and optionally oxidized by a cytochrome P450 yielding an array of 28 different new-to-nature terpenes with 16 carbon atoms. Natural terpenes have a vast diversity of medical uses, and studies like these hold the promise of finding new hit-to-lead bioactive scaffolds.

It is worth noting that producing such derivatives in microbes entails important enzyme screening and promiscuity engineering work (Figure 3b). The analogs then must be produced through fermentation, purified, and assayed for their bioactivities. As the possibilities for enzymatic derivatization are vast, it is important to try to rationalize the types of analogs targeted, especially using Quantitative Structure Activity Relationships methods commonly employed by medicinal chemists [82].

Conclusions

In conclusion, tremendous progress has recently been made in refactoring plant biosynthetic pathways in micro-organisms. We strongly believe that microbes provide value for the bioactive plant natural product field, including as platforms for biosynthetic pathway discovery, as cell factories for manufacturing, and aiding drug discovery by creating novel, pharmacologically improved analogs of plant natural products that are challenging to produce chemically. Metabolic engineering in such microbial hosts is deeply interconnected with enzyme discovery and engineering, through the screening of enzyme homologs and mutants with increased activity, altered subcellular localization, and different promiscuity profiles either to bypass unknown enzymatic steps in a pathway or to allow the production of non-natural analogs.

Current challenges include the cumbersome and expensive means to elucidate biosynthetic pathway enzymes for a significant number of therapeutically relevant phytochemicals whose biosynthesis is still undocumented. Future research should focus on the integration of multiomics data and well-informed candidate gene selection methods. Examples of breakthrough technologies include single-cell metabolomics and transcriptomics technologies [83–85], 3D genome topology analysis using proximity-by-ligation Hi-C sequencing [83,86], or machine learning–based selection of gene candidates [87]. Another important bottleneck once prototypic microbial cell factories for the production of therapeutic phytochemicals have been built, is the long and costly optimization process for industrially relevant TRY levels. Derisking the strain engineering 'Valley of Death' [88] will require to increase in the throughput of screening of naturally diverse and engineered pathway and enzyme variants, including molecular and metabolic network modeling tools and automated strain building and phenotyping platforms. Integration of Life Cycle Analysis and Techno-Economic Analysis can prove instrumental in defining strain performance objectives and designing the fermentation process in the early project stages [9]. Once performance objectives are met at laboratory scale, up-scaling fermentation processes and developing efficient downstream processing methods are other major challenges that must be addressed to ultimately make microbial production of plant therapeutics commercially relevant.

CRediT authorship contribution statement

Holtz Maxence: Conceptualization; Funding acquisition; Data curation; Visualization; Roles/Writing – original

draft; Writing – review & editing. **Acevedo-Rocha Carlos G.:** Supervision; Writing – review & editing. **Jensen Michael K.:** Conceptualization; Funding acquisition, Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

MKJ has financial interests in Biomia ApS. MH and CGA-R declare no conflict of interest.

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