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Biochemical strategies for enhancing the *in vivo* production of natural products with pharmaceutical potential

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Natural products have been associated with significant health benefits in preventing and treating various chronic human diseases such as cancer, cardiovascular diseases, diabetes, Alzheimer's disease, and pathogenic infections. However, the isolation, characterization and evaluation of natural products remain a challenge, mainly due to their limited bioavailability. Metabolic engineering and fermentation technology have emerged as alternative approaches for generating natural products under controlled conditions that can be optimized to maximize yields. Optimization of these processes includes the evaluation of factors such as host selection, product biosynthesis interaction with the cell's central metabolism, product degradation, and byproduct formation. This review summarizes the most recent biochemical strategies and advances in expanding and diversifying natural compounds as well as maximizing their production in microbial and plants cells.

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Introduction

Over the last century, a number of natural compounds extracted from plants and animals have been associated with health benefits in the prevention and treatment of chronic diseases [1]. However, there is a diminished interest in developing natural product-based drugs because of the limited availability of source materials from which they are extracted and the complexity in producing natural products through conventional chemical synthesis. To address these issues, *in vivo* biosynthesis has emerged, which relies on bacterial, yeast or plant cells for the large-scale production of natural products under controlled conditions [2•]. Cell-based biotransformations are highly specific, and the recovery of the resulting products is considerably easier than natural product extraction or conventional chemical synthesis, as fewer side products and less waste are generated. The optimization of *in vivo* processes requires the development of strains capable of affording high titers and high yields, as well as finding the best operational conditions for process scale up (e.g. pH, aeration, and agitation). Recent advances in metabolic engineering have contributed significantly in the expression of entire metabolic pathways, allowing the tuning of the biosynthesis of high value end products [3]. Significant work has been done to generate a broad number of natural products, their analogs, and different classes of useful intermediates (e.g. isoprenoids, flavonoids, stilbenes, polysaccharides and glycoproteins, alcohols) having potential applications as pharmaceuticals, fine chemicals and biofuels. Here, we present an overview regarding the latest advances in the *in vivo* production of natural products with an emphasis on compounds with potential applications as pharmaceutical compounds. Finally, we provide a perspective on the challenges that have to be addressed for scaling up the *in vivo* processes to prepare such natural products.

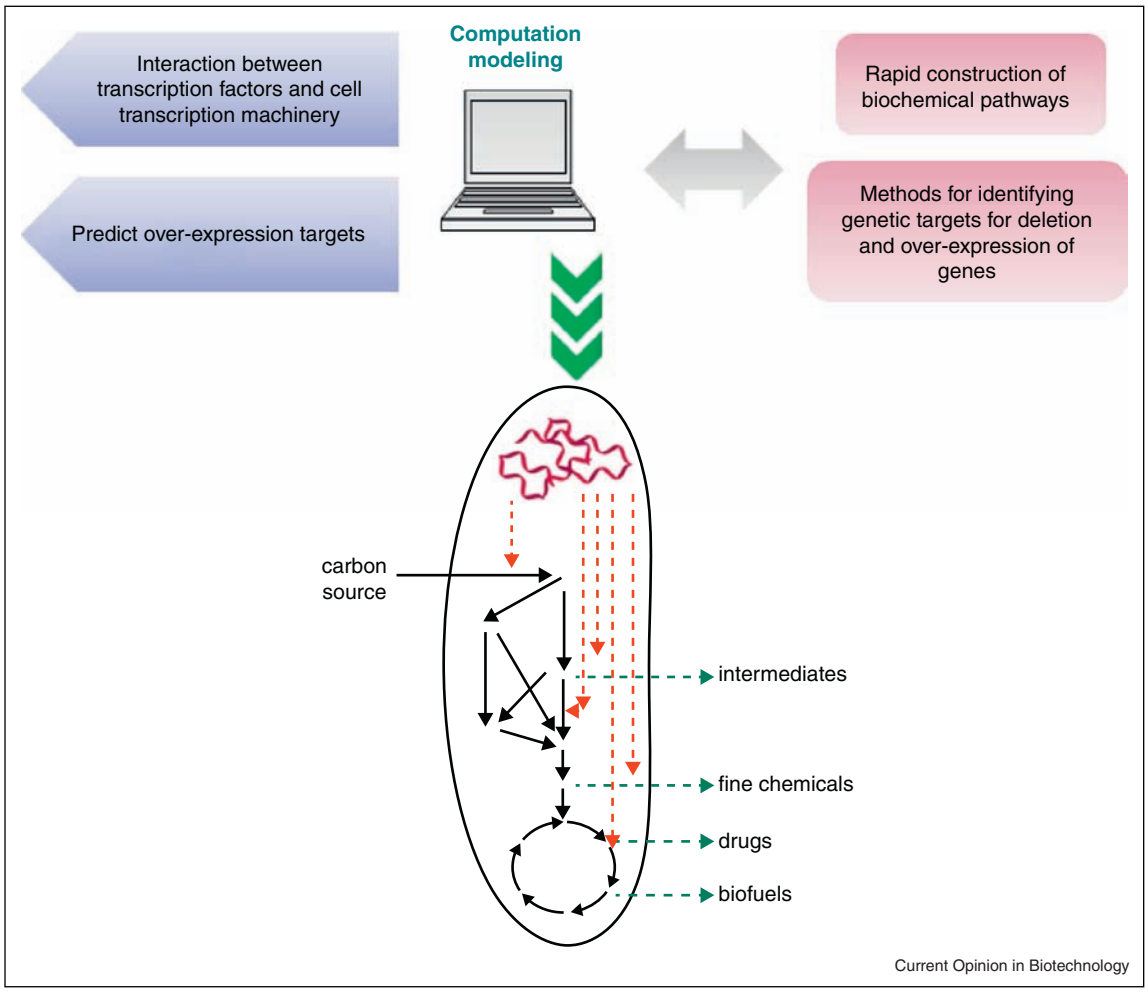
Strategies for optimizing the heterologous expression

In metabolic engineering, complete biosynthetic pathways are typically transferred from native hosts into heterologous organisms with the intention of improving product yields. Consequently, gene expression needs to be balanced, promoter strength needs to be tuned, and the endogenous regulatory network needs to be adapted. Metabolic engineering requires the optimization of regulatory processes within cells, and improving the carbon flux. The use of recombinant expression systems to reconstruct natural product pathways has improved significantly due to advances in metabolic engineering and

synthetic biology. Among the different heterologous systems, *Escherichia coli* and *Saccharomyces cerevisiae* have been extensively used because of their genetic tractability and rapid growth. *E. coli* possess a relatively simple metabolism. However, it has low stress tolerance, lacks mechanisms for post-translational modifications, has difficulty in expressing complex enzymes, and lacks subcellular compartments. Yeast has additional bioprocessing characteristics (e.g. larger cell size, lower growth temperature, and higher tolerance against low pH). As a consequence, inserting metabolic pathways into a selected host requires sequential cloning methods that can be time consuming and are often ineffective [4]. Methods for the rapid construction of biochemical pathways in a one-step fashion (i.e. DNA assembler), can facilitate the biosynthesis of natural products through the manipulation of the *in vivo* homologous recombination mechanism in *S. cerevesiae* [5,6]. Moreover, synthetic platforms, such as ePathBrick, can assist in developing vectors for the precise tuning of multigene pathways, and support the modular assembly of molecular components (e.g. promoters, operators, ribosome binding sites, and terminators) [7•]. In addition, ePathBrick is an efficient platform for the efficient generation of pathway diversity (Figure 1).

Theoretical methods have been developed for identifying genetic targets for deletion and over-expression of genes [8]. For example, by coupling a cipher for evolutionary design to an evolutionary search identified gene deletions and other network modifications, optimal phenotypes can be generated for the production of plant flavanones. Predictions of improved *E. coli* genotypes, which more effectively channel carbon flux, have resulted in an engineered *E. coli* strain capable of improving the production of naringenin and eriodictyol by >600% and >400%, respectively [9]. Computation modeling has also

Figure 1



Metabolic engineering design for the conversion of sugars into intermediates, fine chemicals, drugs, and biofuels. The scheme is a modification of one presented in Ref. [2•].

been used to understand the interaction between transcription factors and cell transcription machinery and to predict over-expression targets to improve yields [10].

Metabolic engineering in bacteria

The production of isoprenoids in microorganisms has been one of the major achievements of metabolic engineering over the past two decades. Isoprenoids can be generated through two pathways: (1) the 2-C-methyl-D-erythritol-4-phospate/1-deoxy-D-xylulose5-phospahte pathway (DXP); and (2) the mevalonic acid (MVA) pathway. The end products of both pathways are the precursors of all terpenoids, including some with pharmaceutical relevance such as taxol, and artemisinin [11••]. Ajikumar and coworkers [12••] applied a 'multivariate-modular pathway engineering' approach for producing taxadiene, by partitioning the overall pathway into two modules. The first module contained an eight-gene, upstream, native methylerythritol-phosphate pathway (MEP), and the second module comprised a two-gene, downstream, heterologous pathway to taxadiene. Such an approach allows the sampling of parameters affecting pathway flux and demonstrated the role of indole as inhibitor of the isoprenoid pathway (Figure 2a). In addition, this multivariate approach showed a maximum yield of taxadiene of ~1.2 g/L (a 15,000-fold increase over the control) in fed batch fermentations [12••]. Examination of different promoters (T7, Trc, and T5) for the overexpression of genes within the MEP (*dxs*, *idi*, *ispD*, *and ispF*) showed that the greatest yields were obtained when T7 promoters was used in *E. coli* K derivative [13]. Simultaneous *in silico* and *in vivo* studies were also used to maximize the biosynthesis of taxadiene by optimizing the yields of isopentyl diphosphate, improving the thermodynamic properties of DXP, and exploring different carbon sources and hosts. Chromosomal engineering and codon usage optimization of the DXP pathway genes resulted in yields of >850 mg/L of taxadiene, corresponding to the highest production reported in a heterologous host [14].

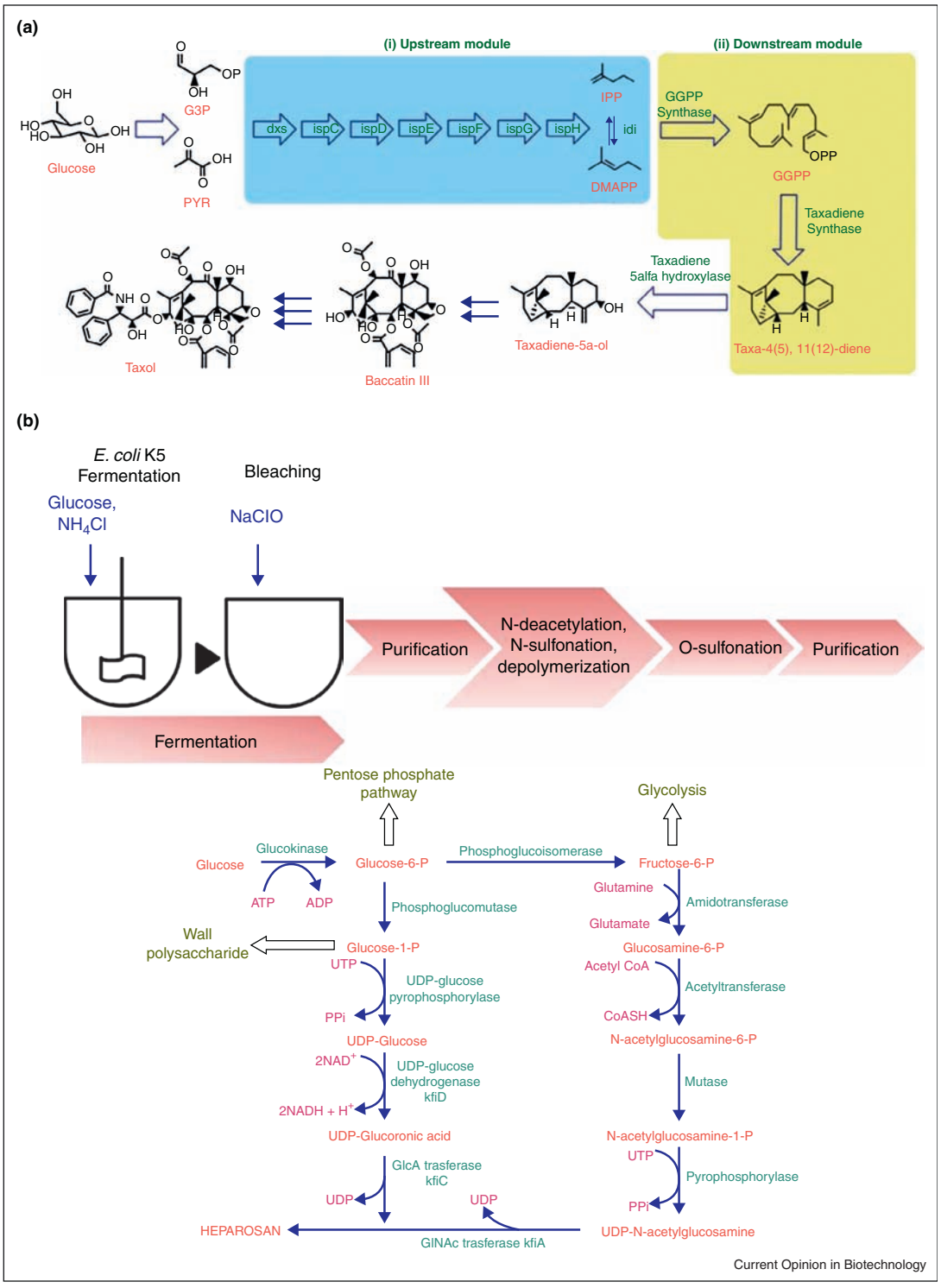
The preparation of diversified natural compounds, of more potent biological activity, is another important application of metabolic engineering. For example, flavonoid derivatives have been produced through precursor feeding of recombinant microorganisms. However, such precursors are often prohibitively expensive. Katsuyama and coworkers [15] developed an artificial biosynthetic pathway for the production of unnatural flavonoids and stilbenes in *E. coli* batch culture. This study included a substrate synthesis step for CoA esters, a polyketide synthesis step for conversion of the CoA esters into flavanones and stilbenes, and a modification step for modification of the flavanones. This precursor-directed biosynthesis produced a large number of flavanones, flavonols, flavones and stilbenes [15]. Recently, an eight-step pathway was developed to bypass the issue of feeding phenylpropanoid precursors [16]. Mathematical algorithms (i.e. OptForce) were used to predict genetic interventions for redirecting more carbon flux towards malonyl-CoA. Bhan and coworkers [17] improved titers of resveratrol (~60%) using such approach.

There has recently been intense interest in the production of microbial polysaccharides for their potential biotechnological applications. In particular, glycosaminoglycans (GAGs) have several pharmaceutical applications. Although most commercial GAGs are extracted from animals, their structural similarity to bacterial polysaccharides, make bacteria an ideal source for these GAGs. Heparin (currently extracted from porcine intestines) is one of the oldest drugs in use today for clinical prevention of blood coagulation. In 2008, an oversulfated chondroitin sulfate contaminant found in certain lots of heparin caused several deaths across the globe, including as many as 100 deaths in the US. As a consequence, significant effort has been made to produce heparosan (a precursor of heparin) from bacterial fermentations. *E. coli* K5 strain has been used as a host for producing high yields (15 g/L) of heparosan (average MW = 58 kDa) in a defined medium using fed-batch fermentation (Figure 2b) [18•]. In the heparosan biosynthetic pathway, the oxidation of UDP-glucose to UDP-GlcA is catalyzed by UDP-glucose dehydrogenase and is thought to be the limiting step. The activity of K5 lyase seems to play a key role on the amount of heparosan released in the medium as well as in the structure and molecular weight of heparosan produced (a crucial property related with its anticoagulant activity) [19]. Capsular polysaccharides with close structural similarity to chondroitin (another polysaccharide used both as a nutraceutical and pharmaceutical) can be also produced by fermentation of *E. coli* K4 strain in fed-batch fermenter. Carbon source plays a crucial role on the yield of capsular polysaccharide with glycerol being the best carbon source to obtain the highest yields.

Metabolic engineering in yeast

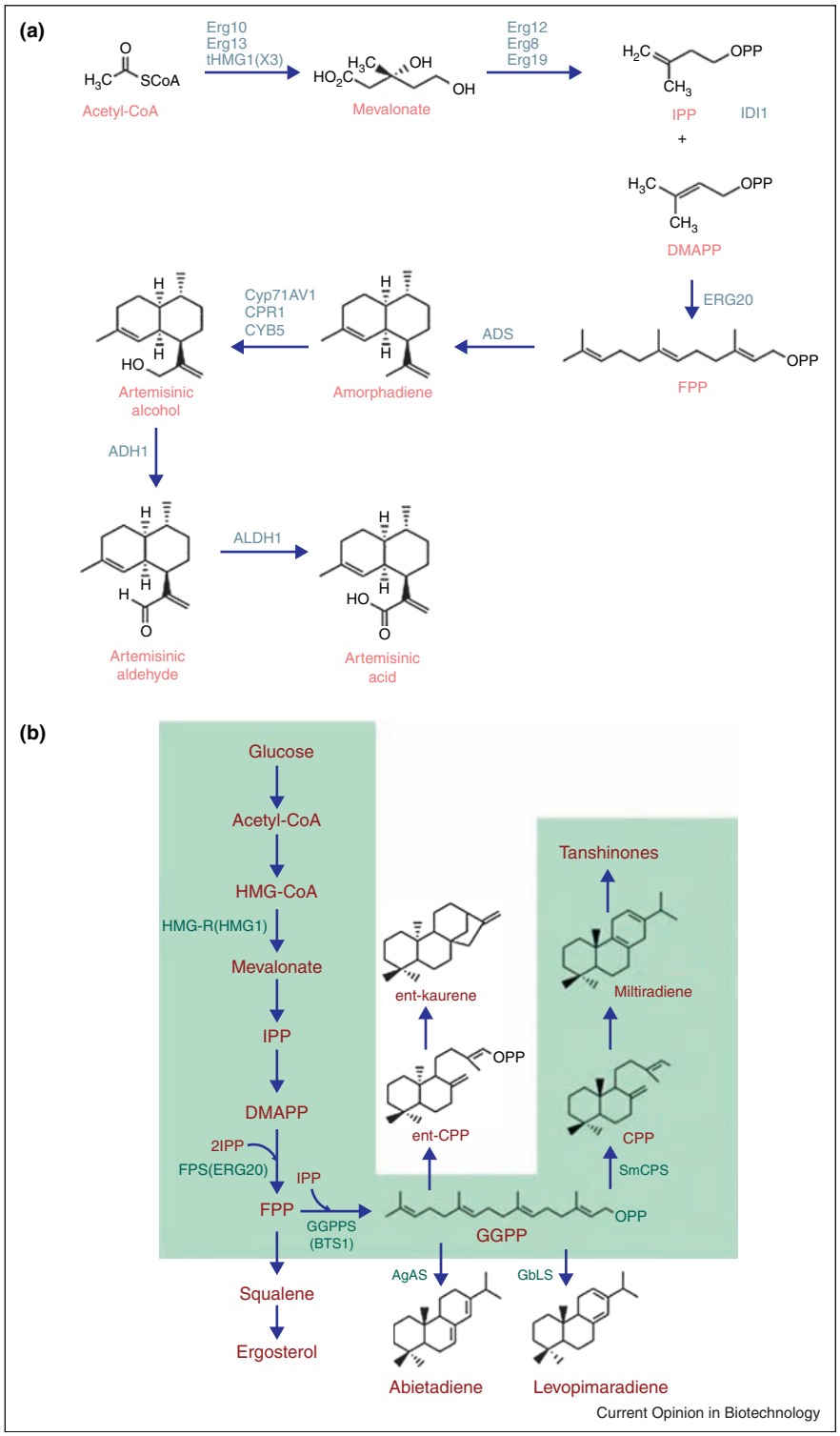
The extensive use of *S. cerevisiae* in fermentations producing alcohol has resulted in a deep knowledge of *Saccharomyces* genetics, physiology, biochemistry, genetic engineering and fermentation technologies. Significant advances have been achieved on the production of the anti-malaria sesquiterpene lactone, artemisinin, by combining metabolic engineering with chemical synthesis. Westfall and coworkers [20•] produced amorpha-4,11-diene (an artemisinin intermediate) through fermentation by overexpressing every enzyme of the mevalonate pathway. Amorpha-4,11-diene can be transformed by conventional chemical synthesis to artemisinic acid and then to artemisinin [20•]. Similarly, Paddon and coworkers [21••] incorporated a plant dehydrogenase and a second cytochrome that provided an efficient biosynthetic route to artemisinic acid (25 g/L) (Figure 3a). Furthermore, fermentation can be coupled to a scalable chemical process

Figure 2



Recombinant metabolic pathways in bacteria for the production of taxol and heparosan. **(a)** Isoprenoid pathway optimization for taxol biosynthesis. (I) Native upstream methylerythritol-phosphate (MEP) module that generates IPP and DMAPP. Enzymatic bottlenecks have been targeted (*dxs*, *idi*, *ispD*, and *ispF*). MEP isoprenoid pathway (blue) is initiated by the condensation of glyceraldehyde-3 phosphate (G3P) and pyruvate (PYR) from glycolysis. (II) A synthetic operon of downstream genes GGPP synthase and taxadiene synthase was constructed for taxol biosynthesis (yellow). The taxol pathway bifurcation begins from the isoprenoid precursors IPP and DMAPP to form GGPP, and finally taxadiene. (III) Taxadiene goes through acylations, and benzoylation to produce the intermediate Baccatin III and assembling the side chain taxol [12••]. **(b)** Fermentation of *E. coli* K5 strain for the production of heparosan. Heparosan building blocks GlcA and GlcNAc are used in the form of UDP-GlcA and UDP-GlcNAc [19].

Figure 3



Metabolic pathways in yeast for the production of artemisinic acid (a precursor for artemisinin), and miltiradiene (a precursor for tanshinones). **(a)** The oxidation of amorphadiene to artemisinic acid from *A. annua* expressed in *S. cerevisiae*. CYP71AV1, CPR1 and CYB5 oxidize amorphadiene to artemisinic alcohol; ADH1 oxidizes artemisinic alcohol to artemisinic aldehyde; ALDH1 oxidizes artemisinic aldehyde to artemisinic acid [21••]. **(b)** Schematic representation of the mevalonic acid (MVA) pathway and several diterpenoids biosynthetic pathways for the production of miltiradiene (green).

to transform artemisinic acid to artemisinin. Modular pathway engineering has been also implemented to generate intermediates of natural terpenoids (i.e. miltiradiene a precursor of tanshinone) (Figure 3b). Such approaches require the prediction and analysis of molecular interactions between terpene synthases to engineer their active sites for enhanced metabolic flux channeling to the end product biosynthesis [11••]. The use of translation fusion proteins in yeast has been another strategy to improve yields of metabolites. For example, resveratrol yields can be improved by ~15 fold in yeast using an unnatural engineered fusion protein of *Arabidopsis thaliana* 4-coumaroyl-CoA ligase (At4CL1) and *Vitis vinifera* (grape) stilbene synthase (VvSTS) [22]. Crystallographic and biochemical analysis of the At4CL::VvvSTS fusion protein demonstrates that this fusion protein improves catalytic efficiency by ~3-fold. Structural and kinetic analysis suggests that co-localization of the two enzyme active sites within a 70 Å distance of one another affords the enhanced biosynthesis of resveratrol [23•]. Biosynthesis of stilbenes can also be improved using a synthetic scaffold strategy (based on engineered synthetic protein scaffolds) with the enzymes involved in the stilbene pathway interacting through small peptide ligands in a programmable manner [24,25]. *S. cerevisiae* has proven to be an excellent platform for the engineering of several branches of flavonoid metabolism [26]. This yeast can be engineered to generate glycosylated flavonoids which have gained attention as drug candidates because of their better water-solubility and their variety of bioactivities compared to the aglycone product [27]. Flavone-C-glycosides are generated through a polyprotein comprising a flavanone 2-hydroxylase (F2H), co-expressed with a C-glucosyltransferase (CGT) [28].

Plant cell cultures

Plant cell cultures have been used extensively for the production of plant metabolites with pharmacological activities [29–31]. For example, Python Biotech, the largest producer of the anticancer drug, paclitaxel, uses plant tissue culture, and employs a large-scale fermenter (~75,000 L) [32].

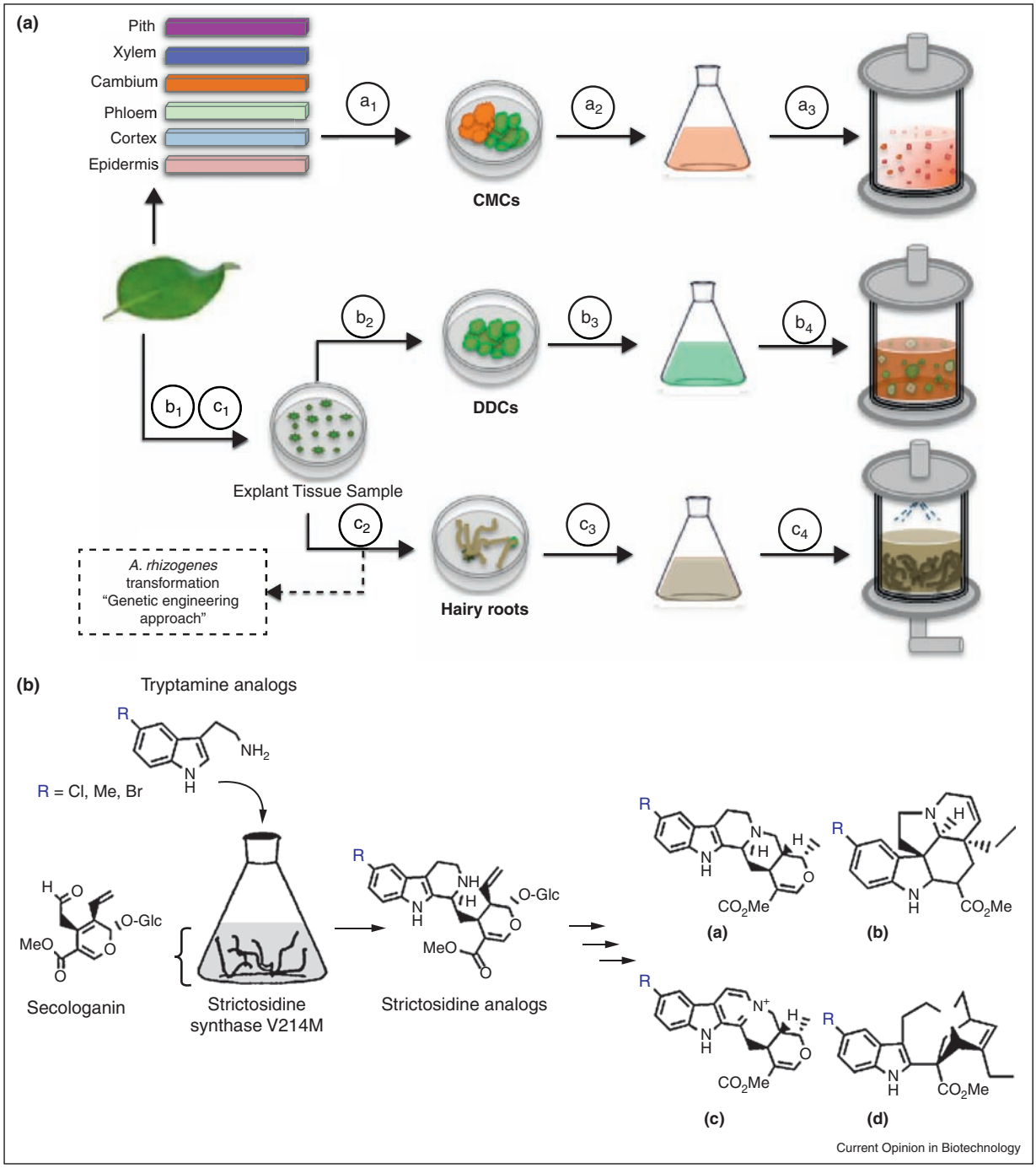
Plant cell cultures are usually induced from established callus cultures, also known as dedifferentiated cells (DDCs), by transferring callus into an appropriate liquid medium favoring suspension culture growth. A recent alternative for DDCs cultures is using multipotent plant cambial meristematic cells (CMCs). Instead of culturing heterogeneous mixtures of dedifferentiated cells, isolated cells derived from vascular cambium are propagated in solution and exposed to the appropriate growth regulators to form callus cultures that can be transferred to liquid media and disaggregated into single cells [33••,34] (Figure 4a).

Also, *in vitro* hairy root cultures, generated by infecting tissue with the soil bacterium *Agrobacterium rhizogenes*, have shown the potential to improve yields of end products [35]. The Swiss company ROOTec is the only company that uses hairy root cultures at industrial scale. By contrast to cell cultures, hairy roots are genetically stable and preserve the entire biochemical potential of the original plant. In addition, hairy roots can potentially be genetically engineered. For example, the cDNAs of genes encoding 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and geranylgeranyl diphosphate synthase (GGPPS) were cloned in *S. miltiorrhiza* hairy root cultures, improving the yield of tanshinone [36]. *Nicotiana tabacum* hairy root with reduced expression levels of a nicotine uptake permease (NUP1) showed increased nicotine (a potential anti-inflammatory agent) levels in culture media [37••,38]. Along the same line, genetically engineered *Papaver bracteatum* hairy roots, expressing codeinone reductase gene (CodR), improved the yields of morphinans, an important group of potential pharmaceuticals [39]. Finally, hairy root cultures can be used for the production of foreign proteins [40].

Similarly to microbes, optimization of culture conditions, selection of cell lines, optimization of growth and production media, induction of secondary metabolites and the use of two-phase culture system, are variables to be considered. For example, cultures of *C. roseus* under stress conditions (e.g. UV exposure or abiotic stress by protein kinases) along with the elicitor methyl jasmonate (MJ) treatment showed an enhanced accumulation of alkaloids (serpentine, vindoline, vincristine and catharanthine) [41]. The use of elicitors, like β-cyclodextrins in *V. vinifera* cultures, has proved to improve the yields of resveratrol (600–4000 mg/L) [42]. Moreover, a screening of different elicitors (MJ, fungal mycelia, proline and hydroxyproline) used in *Teucrium chamaedrys* cultures was capable of enhancing the yields of anticancer agent teucrioside; interestingly, proline and hydroxyproline potentiated the yields of teucrioside when they were combined together (>50 mg/g fresh weight) [43]. Recently, a transcriptomic analysis on *Taxus cuspidata* P991 cell line identified the up-regulation of 12 paclitaxel biosynthetic genes after incubation with MJ that lead to paclitaxel accumulation [44•]. The control of DNA methylation levels allows the reuse of plant cells avoiding a gradual decrease of paclitaxel yields [45].

Another approach involves the combinatorial biosynthetic pathway of genes from different organisms for producing libraries with novel metabolites. For example, overexpression of prenyltransferases of both bacteria (*Streptomyces* sp.) and plants (*Sophora flavescens*) in *Lotus japonicus* produced a set of prenylated polyphenols [46]. Human cytochrome P450 monooxygenase 3A4 has also been cloned into tobacco cells and used to bioconvert antihistaminic drug loratadine to desloratadiene [47]. Moreover, hybrids of triterpene saponins can be produced

Figure 4



(a) Different strategies for plant cell culture. For CMCs culture, (a_1) Remove xylem and pith for culture solid medium, (a_2) Single cell culture suspension of cambium cells, (a_3) Scale-up optimization. For DDCs culture, (b_1) Culture entire explant comprising all tissue-types on solid medium, (b_2) Reprogramming for callus induction and proliferation in solid medium, (b_3) Single cell culture suspension of a mixture of dedifferentiated cells derived from multiple tissue types, (b_4) Scale up optimization. For hairy roots culture, (c_1) culture entire explant comprising on solid medium, (c_2) *Agrobacterium rhizogenes* transformation and hairy root culture on solid medium, (c_3) Liquid culture of hairy root culture, (c_4) scale up optimization. Liquid and suspension culture are approached for optimize the production of specific metabolites, applying different strategies such as, selection of high production lines, ambient and nutrients optimization, elicitation, and genetic engineering [34]. **(b)** Reengineered strictosidine synthase V214M (enzyme that catalyzes formation of strictosidine) into *C. roseus* hairy root tissue, has expanded substrate specificity enabling turnover of tryptamine analogs to produce chlorinated, brominated and methylated 'unnatural' products: (a) ajmalicine analog, (b) tabersonine analog, (c) serpentine analog, (d) catharanthine analog [49••].

by introducing the dammarenediol synthase from *Panax ginseng* (PgDDS) into *Medicago truncatula* [48]. Diversification of metabolites can be achieved by reprogramming metabolic pathways, using unnatural precursors and combined with either RNA-meditated gene silencing or transformation with biosynthetic enzymes with altered substrate specificity [49••] (Figure 4b). Novel mutagenic-screening technologies (i.e. Natural Products Genomics) are designed to leverage the capacity of the plants' own genome for biosynthesis of complex metabolites [50]. This supports the notion that plants can be manipulated either biochemically or genomically to produce novel compounds.

Perspective

A number of examples regarding metabolic engineering strategies for the laboratory-scale preparation of natural products in recombinant microorganisms and plant cells have been presented. The transition to commercial-scale processes is often difficult due to the issues related with production titers and overall yields. In particular, substantial knowledge of the biochemistry of the metabolic steps in a pathway is required for rewiring and balancing cellular metabolism. The complexity of such systems needs the efficient (but not necessarily high-level) expression of enzymes and the understanding of the effect of intermediates, end products, and elicitors on gene regulation. The development of these platforms can be combined with other strategies (e.g. conventional synthesis or *in vitro* biocatalysis) to obtain the desired end products or to develop new platforms for diversifying natural products and their intermediates into novel metabolites with unexplored bioactivity.

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