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Review

Metabolic engineering and *in vitro* biosynthesis of phytochemicals and non-natural analogues

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ABSTRACT

Over the years, natural products from plants and their non-natural derivatives have shown to be active against different types of chronic diseases. However, isolation of such natural products can be limited due to their low bioavailability, and environmental restrictions. To address these issues, *in vivo* and *in vitro* reconstruction of plant metabolic pathways and the metabolic engineering of microbes and plants have been used to generate libraries of compounds. Significant advances have been made through metabolic engineering of microbes and plant cells to generate a variety of compounds (*e.g.* isoprenoids, flavonoids, or stilbenes) using a diverse array of methods to optimize these processes (*e.g.* host selection, operational variables, precursor selection, gene modifications). These approaches have been used also to generate non-natural analogues with different bioactivities. *In vitro* biosynthesis allows the synthesis of intermediates as well as final products avoiding post-translational limitations. Moreover, this strategy allows the use of substrates and the production of metabolites that could be toxic for cells, or expand the biosynthesis into non-conventional media (*e.g.* organic solvents, supercritical fluids). A perspective is also provided on the challenges for generating novel chemical structures and the potential of combining metabolic engineering and *in vitro* biocatalysis to produce metabolites with more potent biological activities.

Contents

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1. Introduction

Over many decades, a number of natural compounds extracted from plants have been associated with health benefits. Despite the controversy regarding whether or not there is a true benefit to humans, many *in vitro* studies suggest that natural products have had a positive impact on the treatment of cancer, Alzheimer's disease and cardiovascular diseases [1]. The major advantages of using natural products as pharmaceuticals are the low toxicity many of them display and their better success rate entering into a Phase I testing (25% *versus* 6% of conventional chemicals) [2]. However, extraction and isolation of natural compounds from plants can be difficult because the availability of plant material is often limited and extraction yield is generally low. Moreover, due to their complex structures, the chemical synthesis of natural products is also difficult because multiple steps are required for synthesis and purification, and reaction products are commonly mixtures of isomers and epimers that compromise the biological activity of the products; the latter has led into a decrease of interest in natural products-based drugs by pharmaceutical companies [3]. Some of the most interesting plant-derived pharmaceuticals, such as taxol and arteminisin, have been synthesized by conventional synthesis and in both cases the synthesis requires a large number of steps and results in poor yields [4].

To address these issues, *in vivo* and *in vitro* biosyntheses of plant natural products have been optimized, and both strategies have been expanded to generate libraries of non-natural products [5–7]. The biosynthesis of natural products and non-natural analogs has several advantages over conventional synthesis. Biotransformations are performed under mild conditions, are highly specific and few side products are generated facilitating product recovery and purification. Metabolic engineering of microbes requires the development of strains capable of achieving high titers and high yields. Several important factors need to be considered in the design of a microbial catalyst such as host selection and the manner in which natural product synthesis interacts with a microbe's central metabolism. Product degradation and byproduct formation also pose challenges for natural product preparation in whole-cell systems.

The *in vitro* reconstruction of plant natural product biosynthesis is attractive because it allows the isolation of metabolic pathway intermediates, and the use of non-conventional substrates to generate natural product analogs. Such analogs can show biological activities and improved physicochemical properties (*i.e.* solubility). However, one of the main challenges of *in vitro* biosynthesis is the need to produce and purify individual recombinant enzymes having high stability and activity [8]. Moreover, a complementary challenge is to optimize the use of cofactors which are expensive in many cases (*i.e.* NADPH).

In this review, we discuss the latest advances in the production of natural plant products using three different strategies: (1) metabolic engineering in microbes, (2) metabolic engineering in plant cells, and (3) *in vitro* reconstruction of natural products. We also review the diversification of natural products into non-natural analogs using both *in vivo* and *in vitro* approaches. Finally, the combination of metabolic engineering and *in vitro* biocatalysis is examined for the generation of libraries of novel compounds with therapeutic potential.

2. Metabolic engineering in microbes

2.1. Optimization of heterologous expression

Traditional metabolic engineering consists of optimizing regulatory processes within cells, improving the carbon flux, ultimately leading to increased yields of a specific compound, the production of which can be further scaled up. The use of recombinant expression systems to reconstruct natural product pathways has improved significantly due to advances in metabolic engineering and synthetic biology. *Escherichia coli* and *Saccharomyces cerevisiae* have generally been used to avoid the more difficult technical issues associated with the metabolic engineering and growth of plant cell cultures. *Escherichia coli* has the advantage of its relatively simple metabolism characterized by minimal central metabolic pathways and robust, yet centralized regulatory systems. However, it is not always the ideal host due to relatively low stress tolerance, a lack of mechanisms for post-translational modifications, difficulty in expressing complex enzymes, and lack of subcellular compartments. In contrast, yeasts often possess these ideal characteristics and also have favorable bioprocessing characters such as a larger cell size, a lower growth temperature, and higher tolerance against pH and side products. Moreover, yeast mating allows for improved cellular engineering and can lead to diploids with robust growth and increased adaptation. In metabolic engineering, complete biosynthetic pathways are often moved from native hosts into heterologous organisms with the aim to improve product yields. Therefore, gene expression needs to be balanced, promoter strength needs to be tuned and the endogenous regulatory network needs to be modified. As a consequence, conventional multi-step, sequential-cloning methods that consist of primer design, PCR amplification, restriction digestion, *in vitro* ligation and transformation, have been typically used and multiple plasmids are frequently necessary. Such approaches are time consuming and inefficient [9]. The development of methods such as the DNA assembler [10,11] have contributed to the discovery, characterization and engineering of natural products by the design and rapid construction of biochemical pathways in a one-step fashion. DNA assembler allows the design and rapid construction of biochemical pathways in a one-step fashion by exploitation of the *in vivo* homologous recombination mechanism in *S. cerevisiae*. In addition, synthetic biology platforms, like ePathBrick, allow the development of vectors compatible with BioBrick standards for the precise tuning of gene expression and support the modular assembly of pathways [12]. The ePathBrick vectors feature in four isocaudamer pairs (AvrII, XbaI, SpeI, and NheI) and support the modular assembly of several molecular components (*e.g.* promoters, operators, ribosome binding sites, and terminators) and multigene pathways. Furthermore, ePathBrick offers a platform for optimizing pathway configurations and efficient generation of pathway diversities. This platform addresses several of the main challenges in metabolic engineering

and synthetic biologist such as lack of efficient tools for fine-tuning gene expression and construction of pathways with multiple gene components.

Two main approaches are usually considered during the optimization of the synthesis of targeted products. The first one is process analysis, which targets the optimization of the operational conditions (*e.g.* aeration, temperature, pH, nutrients). During the process analysis, statistical experimental design methods are necessary to reduce the number of experiments required to understand the biocatalyst physiology. The second parameter, biocatalyst analysis, involves the organization and reconstruction of data related to intracellular mechanisms. This leads to the development of theoretical methods that help identify genetic targets for deletion or over-expression of genes [13,14]. For example, Fowler et al. [15] developed a cipher for evolutionary design (CiED) to identify genetic perturbations, such as gene deletions and other network modifications that resulted in optimal phenotypes for the production of end products. This approach demonstrates the utility of computational methods for predicting improved *E. coli* genotypes that more effectively channel carbon flux toward malonyl coenzyme A (CoA) and other cofactors in an effort to generate recombinant strains with enhanced flavonoid production capability. For example, engineered *E. coli* strains were constructed first by the targeted deletion of native genes predicted by CiED and then by incorporating selected overexpressions resulting in enhanced flavonoid production (>600% increase of naringenin, and >400% increase of eriodictyol). In addition, computational modeling has been used to understand how transcription factors interact with a cell's transcriptional machinery. Other computational algorithms have been created to predict over-expression targets to improve taxadiene production [16]. Such methods identify targets and genes both within and outside the isoprenoid pathway that are then experimentally verified in microorganisms such as *E. coli*. Experimental results match computational predictions on the synergy between the isoprenoid precursor pathway and targets outside this pathway. The use of such computational models can be expanded to other host systems and other desired natural products.

2.2. Reconstruction in vivo of natural and non-natural compounds

2.2.1. Isoprenoids

Terpenoids are a class of isoprenoids with a large number of diverse structures exhibiting many biological activities (*i.e.* anti-inflammatory, anti-infectious, and anti-cancer properties). However, not all terpenoids have beneficial properties; some can be poisonous to insects and mammals, such as gossypol. Terpenoids can be sub-classified by their structure as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenes, where the chain length specificity dictates the final terpenoid structure generated by the terpene cyclase [17,18]. The two main precursors are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [19,20]. There are two pathways to generate isoprenoids: the mevalonic acid pathway (MVA; for some bacteria, plants, and higher eukaryotes) [21,22] and the 2-C-methyl-D-erythritol4-phospate/1-deoxy-D-xylulose5-phospahte pathway (DXP, for plants and most of the bacterial strains) [23]. The end products of both pathways are the precursors of all terpenoids, some with pharmaceutical relevance such as taxol, artemisinin, and lycopene.

Taxol (paclitaxel) was first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*, was approved by FDA, and has been widely used as an anticancer drug (*e.g.* against ovarian, breast and lung cancer). Taxol can be produced by chemical conversion of precursor molecules derived from needles of the more prevalent European yew, *T. baccata* or from cultured plant cells. Both methods are difficult, and the price of taxol remains high [24]. Over the past few years significant advances have been made in the generation of taxol, taxol precursors or taxol analogs by metabolically engineered microbial hosts [20]. In a study, the metabolic pathway of taxadiene was partitioned into two modules [24] (Fig. 1). The first module is the native upstream methylerythritol-phosphate (MEP) pathway that generates IPP. The second module is a heterologous downstream pathway responsible for generating the terpenoid. The two modules were balanced to maximize the production of taxadiene and minimize the amount of the pathway inhibitor indole. Taxol biosynthesis was then engineered through a P450-mediated 5α-oxidation of taxadiene to taxadien-5α-ol.

In another study, computer modeling was used to optimize the biosynthesis of taxadiene in *E. coli* comparing the maximum theoretical IPP yields and the thermodynamic properties of the DXP and MVA pathways using different hosts and carbon sources, and demonstrated that genetic manipulation of the DXP pathway and chromosomal engineering were powerful tools for heterologous biosynthesis of taxadiene [19]. Computational modeling establishes that better yields of IPP can be obtained through DXP pathway leading to the optimization of taxadiene biosynthesis. The combination of chromosomal engineering and codon usage redesign through the DXP pathway gave yields of ~870 mg/L of taxadiene which is the highest value reported in a heterologous host. In another report [25], the production of taxadiene was studied and compared between K- and B-derived *E. coli* strains (two of the most used *E. coli* strains in laboratories as a tool and model organism) through the recombinant parameters of MEP pathway. In this study, different promoters (T7, Trc, and T5) and cellular backgrounds were varied during the taxadiene biosynthesis. Results showed that a K-derivative *E. coli* strain produced 2.5-fold higher amounts of taxadiene. While operational parameters such as temperature did not affect yield significantly, the inhibitory effect of indole was greater on the K-derivative *E. coli*. In addition, significant differences in the pyruvate metabolism were observed between the K and B strains. *E. coli* clearly provides an excellent recombinant host for the heterologous biosynthesis of taxol. The yields of taxadiene were greater using T7 promoter because numerous genes (*dxs, idi, ispD, and ispF*) within the MEP pathway were overexpressed in both *E. coli* strains. However, significant efforts were required to rewire cellular metabolism to obtain high yields of taxol [26,27].

Artemisinin was first isolated, during early 70s, from the *Artemisia annua*, and is the most effective anti-malarial drug known [28]. The current production of artemisinin requires several steps that increase the final cost of the drug. These steps include: first, growing the source plant, *A. annua*; second, the extraction of the active moiety; third, the creation of the desired artemisinin derivative; and fourth, the co-formulation with the companion drug. Despite significant efforts to optimize the yield of artemisinin in plant cell cultures [29], the yield remains low, and a significant improvement of our knowledge of the biosynthetic pathway is required to engineer it into recombinant microorganisms. Several biosynthetic enzymes have been characterized, and introduced in heterologous systems [30,31]. For example, a gene encoding an amorpha-4,11-diene synthase from *A. annua* was introduced into yeast to produce 600 μg/L of the artemisinin precursor amorpha-4,11-diene [32]. Another study reports the engineered heterologous expression of the yeast mevalonate pathway, achieving yields of 27 g/L of amorpha-4,11-diene in recombinant *E. coli* [33]. In a recent study, it was reported the overexpression of every enzyme of the mevalonate pathway in *S. cerevisiae* CEN.PK2 leading to production of >40 g/L of amorpha-4,11-diene [34]. Conversion of amorpha-4,11-diene to dihydroartemisinic acid was then achieved

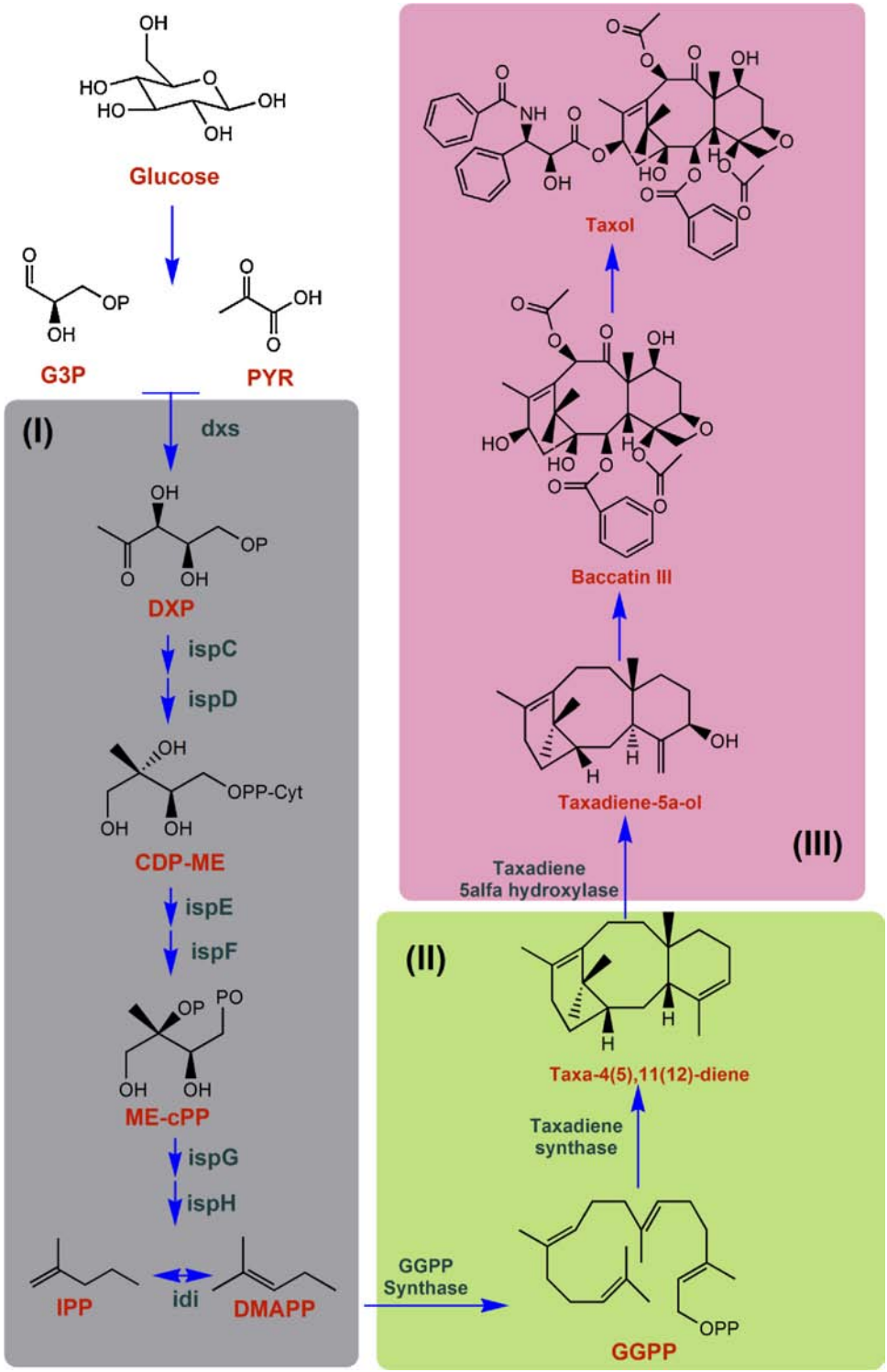

Fig. 1. Isoprenoid pathway optimization for taxol biosynthesis. (I) Native upstream methylerythritol-phosphate (MEP) module that generates IPP and DMAPP. Enzymatic bottlenecks have been targeted (*dxs*, *idi*, *ispD*, and *ispF*). MEP isoprenoid pathway is initiated by the condensation of the precursors glyceraldehyde-3 phosphate (G3P) and pyruvate (PYR) from glycolysis. (II) For taxol biosynthesis, a synthetic operon of downstream genes GGPP synthase and taxadiene synthase was constructed. The taxol pathway bifurcation starts from the isoprenoid precursors IPP and DMAPP to form GGPP, and then taxadiene. (III) Taxadiene undergoes stereospecific oxidations, acylations, and benzoylation to form the late intermediate Baccatin III and, after assembling the side chain, taxol.

through a chemical process, whose product could be subsequently be converted to artemisinin.

2.2.2. Flavonoids

Flavonoids are plant secondary metabolites derived from a group of precursors named chalcones. Chalcones are derived from malonyl-CoA and *p*-coumaroyl-CoA [35] by the action of the chalcones synthase (CHS) [36]. Diversification can be achieved using a series of enzymatic modifications (Fig. 2) that yield a large number of structures (*e.g.* flavanones, dihydrofavonols, anthocyanins, flavonols, flavan-3-ols, tannins, and other polyphenolic compounds) [37–40]. Optimization of flavonoid production has

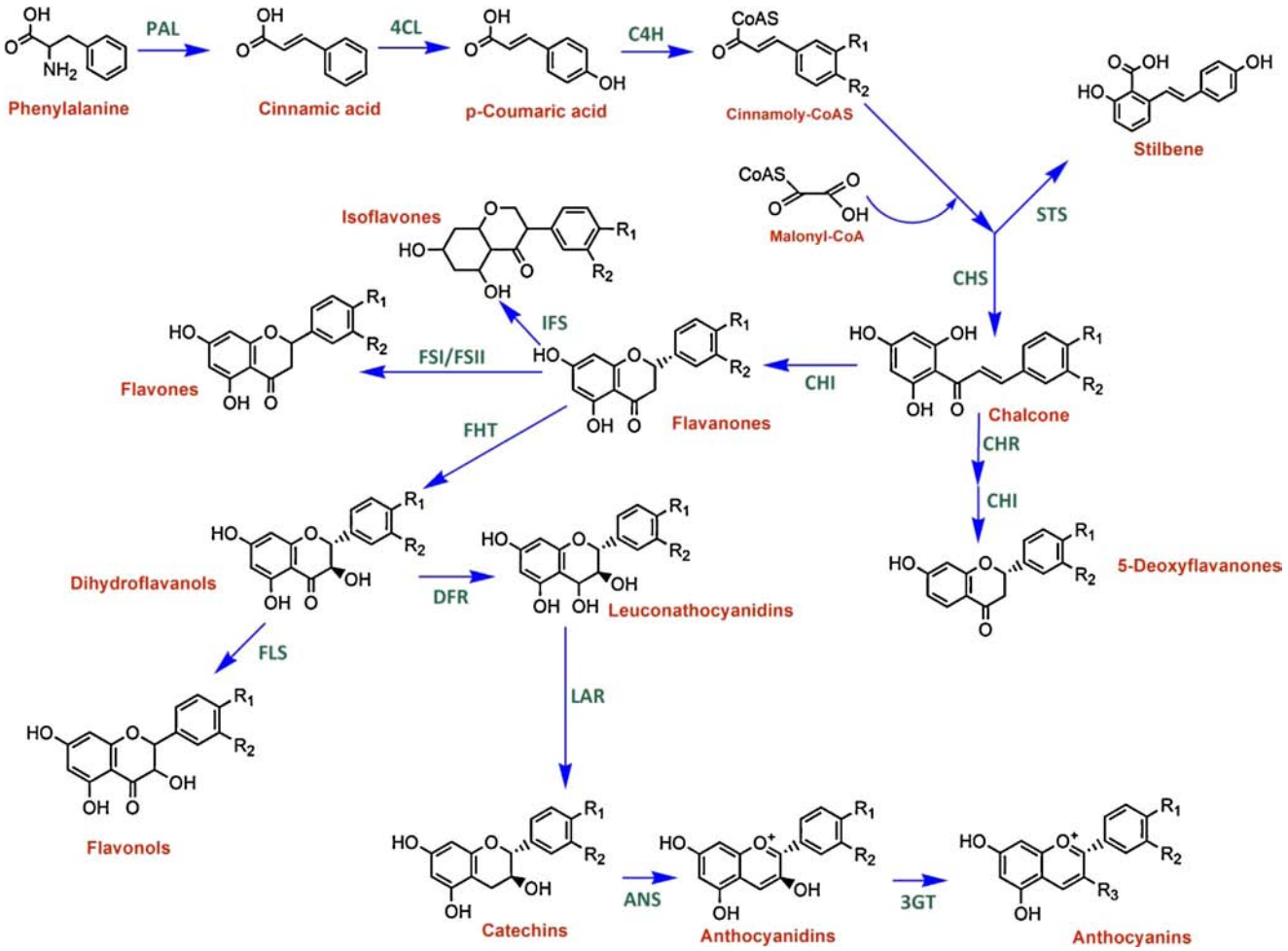

Fig. 2. Phenylpropanoid pathway. The R-groups denote the hydroxylation patterns for the natural flavonoid compounds although unnatural substitutions can be introduced at these positions. Abbreviations within the figure: Phenylalanine Ammonia Lyase (PAL), 4-coumaroyl-CoA Ligase (4CL), coumarate-4-hydroxylase (C4H), stilbene synthase (STS), chalcone synthase (CHS), chalcone reductase (CHR), chalcones isomerase (CHI), isoflavone synthase (IFS), flavonol 3β-hydroxylase (FHT), dihydroflavonol reductase (DFR), leucocyanidin reductase (LAR), anthocyanin synthase (ANS), anthocyanidin 3-O-glucosyltransferase (3GT).

been carried out in heterologous hosts such as *E. coli* [41–43] and *S. cerevisiae* [44]. For example, an *E. coli* cell factory was developed to generate the anti-cancer and anti-inflammatory 7-*O*-methyl aromadendrin. Moreover, flavonoids have recently attracted attention for their potential use as drugs for treating Type II diabetes. One of the main treatments of Type II diabetes relies on digestive enzyme inhibition. Flavonoids (and other natural products) have demonstrated inhibitory activity against digestive enzymes with reduced side effects [45]. In addition, natural flavonoids (catechins and afzelechin) have shown to trigger secretion of insulin in pancreatic β-cells at micromolar and nanomolar scale respectively [42].

In addition to producing natural flavonoids, metabolic engineering can be used also to generate non-natural flavonoid derivatives by feeding with various precursors. For example non-natural flavanones were synthesized including novel halogenated-flavanones that were then tested as anti-microbials and anti-fungals [46]. From the compounds tested, 4-chloro-flavanone was the most potent antimicrobial compound with a minimum inhibitory concentration (MIC) value of 70 μg/mL in *E. coli* when combined with the efflux pump inhibitor Phe-Arg-β-naphthylamide (PABN) and a MIC of 20 μg/mL against *B. subtilis* (with and without the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine, NMP). In addition, the same compound, when used alone, showed antifungal activity with MICs of 30 μg/mL against *S. cerevesiae* and 30 μg/mL against *C. neoformans*. Diversification of flavanones into their corresponding isoflavones was achieved in a three-enzyme system in *S. cerevesiae* by screening a library of synthetic flavanone precursors that produced 4 natural isoflavones and 14 unnatural analogs. Several end-products showed significant binding affinities to human estrogen receptors, particularly the β isoform [47].

2.2.3. Stilbenes

Stilbenes are produced by the aldol condensation of the tetraketide intermediate formed by the addition of three acetyl groups to *p*-coumaroyl-CoA by stilbene synthase (STS). Among this class of compounds, resveratrol has attracted the most attention. Resveratrol is a stress metabolite produced by *Vitis vinifera* grapevines and helps to defend against pathogens [48]. Resveratrol has been associated with the "French paradox" (the observation that French people suffer from relatively low rates of heart diseases, despite their diet rich in saturated fats). Over more than a decade, resveratrol has also been shown to have cancer chemopreventive activity, to reduce the risk of coronary heart disease, and shows biological activity against Alzheimer's disease [49–52]. Due to its promising pharmaceutical properties, there have been many attempts to produce resveratrol in heterologous hosts, such as bacteria and yeast. Different methods have been employed to improve the biosynthesis of resveratrol in *S. cerevisiae*. First, the enzyme tyrosine ammonia lyase (TAL) was mutated, codon-optimized and re-synthesized, in order to improve the production of *p*-coumaric acid and resveratrol (~2.5-fold higher

resveratrol than control cells). Using this engineered yeast, resveratrol biosynthesis was detected in sucrose and grape juice medium that allowed the production of white wines with comparable amounts of resveratrol as found in red wines [53]. In a previous study, another attempt to make white wines with engineered yeast that generates resveratrol introducing the phenylpropanoid pathway in *S. cerevisiae* to produce *p*-coumaroyl-CoA [54]. To this end, the genes coding for 4-coumaroyl-CoA ligase (*4CL216*) and the grapevine resveratrol synthase gene (*vst1*) were co-expressed in *S. cerevisiae*. Resveratrol biosynthesis could also be improved using a synthetic scaffold strategy [55] (based on engineered synthetic protein scaffolds) that interact with the enzymes involved in the biosynthesis pathway of the resveratrol through small peptide ligands in a programmable manner [56]. Using this approach, a 2–6 fold improvement in resveratrol yields was observed. Mathematical algorithms, such as OptForce, have been used to predict genetic interventions for redirecting the malonyl-CoA flux towards the optimization of natural products. In a recent report, an improved titers of resveratrol (∼60%) was achieved using such strategy [57].

3. Metabolic engineering in plant cells

Plant secondary metabolites are frequently involved in plant environmental adaptation as protection against biotic factors such as herbivores, predation and competition or non-biotic factors such as UV light [58]. Due to the complexity of incorporating biosynthetic pathways into heterologous hosts, an alternative approach takes advantage of recent advances in tissue culture technology combined with improvement in genetic engineering, making these processes economically feasible. Plant cell cultures can be propagated, genetically manipulated, and can be grown under controlled conditions. End-product yields can be increased through optimization of the culture medium or through the addition of specific elicitors [59]. The use of bioreactors provides more precise control of environmental parameters (agitation, pH, illumination, temperature) that are critical to the production of different secondary metabolites, allowing the optimization of the yields of the end-products [60]. Below we describe the approaches and strategies developed to improve the yields of different metabolites from their native plant host. In addition, we discuss new compounds produced through *in vitro* modification or through mutasynthesis to improve their pharmacological activity.

3.1. Terpenoids

The anti-cancer drug taxol is currently produced through both a semi-synthetic process and a process relying on plant cell culture. Diversification of taxol analogs has been achieved by combining both strategies [61,62]. For example, the biosynthesis of two taxol analogues, carbatizel and larotaxel, was carried out by isolating the 10-deacetylbaccatin III from *Taxus baccata* cell lines, which is used to synthesize the taxol analog, docetaxel, and more recently water soluble-derivatives carbazitaxel and larotaxel [62].

Phyton Biotech Inc. and Samyang Genex Corp., produce taxol using large scale fermentation of *Taxus* spp. cell lines that can produce large amounts of taxol and taxol analogs [61,63]. The primary difficulties associated with establishing a plant cell culture-based bioprocess are the low and variable yields in secondary metabolite accumulation. Taxol accumulation is significantly enhanced upon methyl jasmonate addition, an integral component of the signal transduction process that regulates the inducible defense systems of plants [64]. The extent of taxol accumulation upon methyl jasmonate induction has been improved in different cultures [63,65] and combining media and process optimization strategies have led to taxol yields of 900 mg/L at industrial scale [66].

Direct and indirect molecular biology techniques (*i.e.* qRT-PCR) have been useful tools to acquire knowledge of regulation, transport, and degradation, helping the optimization of taxol yields, either by the overexpression of genes controlling limiting steps or by suppressing the undesired byproducts by employing antisense technology [63,67,68].

Similar to the work done in microbes, many engineering attempts in plant cells are focused on the MVA or MEP pathways responsible for the generation of precursors for terpenoid biosynthesis. For example, in *Salvia miltiorrhiza* the enzyme labdadienyl/copalyl diphosphate synthase (SmCPS) and a kaurene synthase-like enzyme (SmKSL) are responsible for the transformation of geranylgeranyl pyrophosphate (GGPP) into miltiradiene, a key intermediate of tanshinone, an important pharmacological set of compounds used in treatment of cardiovascular diseases [22]. The introduction of gene constructs containing cDNAs of SmHMGR and/or SmGGPPS as well as SmDXS under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter into *S. miltiorrhiza* cells caused a significant enhancement in the production of tanshinone [69].

Production of terpenoids can be optimized by performing deep sequencing on the transcriptome of the plant. For example, a set of genes and markers from *A. annua* were identified for fast-track propagation, and a genetic map was built for accounting a significant amount of the variation in key traits controlling artemisinin yield; enrichment of such genetic maps in parents of new high-yielding hybrids leads to the conversion of *A. annua* into a robust crop for artemisinin production [70].

Other kinds of terpenoids with potential pharmacological activity are the saponins that have demonstrated high anti-bacterial, anti-viral, anti-inflammatory, and anti-leishmanial activities. Despite limited knowledge of the saponin biosynthetic pathway, it is believed that incorporating enzymes from other plant cell lines can significantly increase the production of saponins [59]. Finally, there are still no reports on new structures of triterpene saponins prepared by combinatorial biosynthesis in plants. However, some hypothetical examples have been proposed, including the introduction of dammarenediol synthase from *P. ginseng* (PgDDS) into *Medicago truncatula*, to produce a hybrid-type saponin [59].

3.2. Alkaloids

Alkaloids are produced by bacteria, fungi, plants and animals, and have several pharmacological applications. Their biosynthesis requires a large number of steps and their biosynthetic pathways remain largely unknown. Metabolic engineering has been used as the strategy to optimize their production in plant cell and tissue cultures (*e.g. Catharanthus roseus* and *Rauvolfia serpentine* have been used extensively). However, rather than focusing only on the accumulation of the final products, the analysis of secondary metabolites has significant advantage since it allows the examination of the distribution of fluxes around key branch-points. In the early 2000s, it was developed a model that organizes the flux analysis by grouping metabolites of similar biosynthetic origin and quantified temporal profiles of metabolites from several branches of the indole alkaloid pathway in *C. roseus* hairy root cultures [71]. *C. roseus* produces more than 100 different alkaloids most of which have potent anti-cancer properties (*e.g.* vinblastine and vincristin are two well-known alkaloids with clinical use as antitumor drugs) [72,73]. In a later study, it was showed that the *in situ* photoactivation of the catharanthine (an indole alkaloid extracted from *C. roseus*) results in better yields of vinblastine, avoiding the side reactions observed during the photoactivation in cells [72].

In a recent study, the use of C10 catharanthine and C12 indole substituents on the biomimetic Fe(III)-mediated coupling

with vindoline led to the discovery of two new derivatives, 10′-fluorovinblastine and 10′-fluorovincristine. The fluorine substitution at C10′ substantially enhances the cell-based activity (8-fold) against tumor cell lines (IC_{50} = 800 pM for vinblastine-sensitive HCT116, and IC_{50} = 80 nM, for vinblastine-resistant HCT166/VM46) [74].

New technologies such as natural product genomics have been applied in *C. roseus*. The principal natural product genomics strategy combines activation tagging mutagenesis and cytotoxic selection, using repeated rounds of mutations to "direct" evolution toward specific phenotype. In *C. roseus*, cytotoxin 4-methyl tryptophan was studied for increasing the production of alkaloids. Natural product genomics can also lead to the generation of new molecules resulting from activation tagging mutagenesis process [75]. Another case of natural product diversification was reported in a previous study, where it is reported the introduction of the chlorination biosynthetic machinery from soil bacteria into *C. roseus* [76]. These prokaryotic halogenases function within the context of the plant cell to generate chlorinated tryptophan, which is then shuttled into monoterpene indole alkaloid metabolism to yield chlorinated alkaloids (Fig. 3). Overexpression of genes in plants has been also used to increase the yields of alkaloids. Overexpression of codeinone reductase (the final enzyme in morphine biosynthesis) in transgenic *Papaver somniferum* results in higher yields of ~30% [77]. Moreover, overexpression of cytochrome P450 monoxygenase (*S*)-N-methylcoclaurine 3′-hydrolase (CYP80B3) resulted in an increase of 450% of total morphine alkaloids [78]. Diversification of non-narcotic alkaloids in opium poppy can be mediated through RNAi-mediated replacement of morphine to produce reticuline and methylated derivatives of it [79].

Plant tissue and cell cultures are prospective scalable alkaloid production platforms. However, one major disadvantage of plant tissues and cell lines is the inability to produce certain alkaloids due to the lack of specialized cell types. Metabolic reconstruction is consequently required for increasing the efficacy of plant cell lines and tissues for industrial scale, and this requires the development of robust genetic tools for plant transformation. However, the existence of multiple alkaloid synthetic pathways and regulatory control mechanisms increase the degree of unpredictability for metabolic engineering strategies. A potential way to address the complexity of plant cellular systems is to develop mathematical models of the plant metabolism and methods capable of effecting simultaneous changes in multiple metabolic points, such as the use of transcription factors [80]. Finally, strategies like *de novo* combinatorial biosynthesis in plant systems can be a useful approach to diversify natural into non-natural products and requires constituent enzymes that have been reengineered for broad specificity [81].

3.3. Flavonoids

Structural gene overexpression or silencing, transcriptional regulation, flux control, and transporter overexpression have been applied in the manipulation of the flavonoid pathway in plants and plant cell cultures [82]. Chalcone isomerase, a key enzyme in the flavonoid biosynthetic pathway, has been studied extensively. For example, overexpression of chalcone isomerase gene from *Scutellaria baicalensis*, *Glycyrrhiza uralensis* and *Arachis hypogaea* led to increased production of flavonoids in hairy roots [83,84]. In addition, the overexpression of maize ZmMYB31 greatly reduces the synthesis of lignin and represses the accumulation of sinapoylmalate, redirecting the metabolic flux toward flavonoid biosynthesis [85]. Overexpression of the bacterial TAL gene in *Arabidopsis* led to higher accumulation of anthocyanins, enhancing the metabolic flux into the phenylpropanoid pathway, and finally resulting in an increased accumulation of flavonoids and phenylpropanoids [86]. The enzyme activities of the anthocyanin biosynthetic pathway can be regulated partially by UV and by the phytochrome-activating wavelengths of 700–800 nm, resulting in better yields of flavonoids [87].

Diversification of polyphenols can be achieved by overexpressing the prenyltransferases of both bacteria (*Streptomyces* sp.) and plants (*Sophora flavescens*) in *Lotus japonicus* for the production of prenylated polyphenols 6-dimethylallylnaringenin, 8-dimethylallylnaringenin, 6-dimethylallylgenistein, and 7-O-geranylgenistein. These prenylated polyphenols are not naturally generated by *Lotus japonicus* and have pharmaceutical value as antioxidant, antibacterial, and anticancer agents [88].

Resveratrol biosynthesis is restricted to only a few plant species commonly used for human consumption (*e.g.* pine, peanuts, grapes, bilberry, and mulberry) [89]. Stilbene synthase genes, encoding for the first dedicated enzyme in resveratrol biosynthesis, have been transferred to a number of crops, either to improve the resistance of plant to stresses, such as fungal pathogens and UV radiation, or to improve their nutritional value [90].

Some investigators have used chimeric genes or a combination of two stilbene synthase encoding genes to increase the levels of stilbene production [91]. Several suspension plant cell cultures employing elicitors (*e.g.* methyl jasmonate, or other stress inducers including cyclodextrins or chitosan) have been used for the production of *trans*-resveratrol under *in vitro* conditions [92–94]. Also, other plant cells (*e.g.* cotton cells) in suspension can produce *trans*-resveratrol. However the most commonly used are grape cell cultures with the addition of β-cyclodextrins acting as elicitors, which help to greatly improve resveratrol yields (600–4000 mg/L) as well as stabilize the final product [95,96]. Tailoring plants for resveratrol synthesis is another simple approach to enhance its yields because stilbene synthase is a key enzyme in resveratrol synthesis utilizing as substrates precursor molecules that are present throughout the plant kingdom. The introduction of a single gene is thus sufficient to synthesize resveratrol in heterologous plant species [97]. Finally, a recent study has proposed that DNA methylation may be involved in the control of resveratrol biosynthesis through the regulation of stilbene synthase gene expression. Also, the *rolB* oncogenes have been shown to induce alterations in the DNA methylation patterns, causing high levels of resveratrol and lower hyper-methylation levels [98].

4. *In vitro* diversification of natural and non-natural compounds

In vitro reconstruction of plant natural products and their non-natural analogs is an attractive alternative to generate pure biosynthetic intermediates as well as final products. In addition, *in vitro* reconstruction reduces side reactions and cell metabolites that frequently occur *in vivo*; for example, some of them could be potentially toxic for microbes [99]. Finally, *in vitro* reconstruction avoids poorly controlled post-translational limitations (*i.e.* protein–protein interactions) [100,101] but can be limited because of the lack of factors that may help with metabolite channeling into the pathway.

4.1. Terpenoid synthases

Despite the ~50,000 terpenoids known, the *in vitro* diversification of terpenoids is particularly limited because of the difficulties with the isolation of active terpenoid synthases. However, diversity is possible through three different reactions: chain elongation, branching, and cyclization. For example, chimeric proteins were generated by fusing catalytic fragments of FFPase and chrysanthemyl pyrophosphatase (CPPase) allowing the proteins to show an

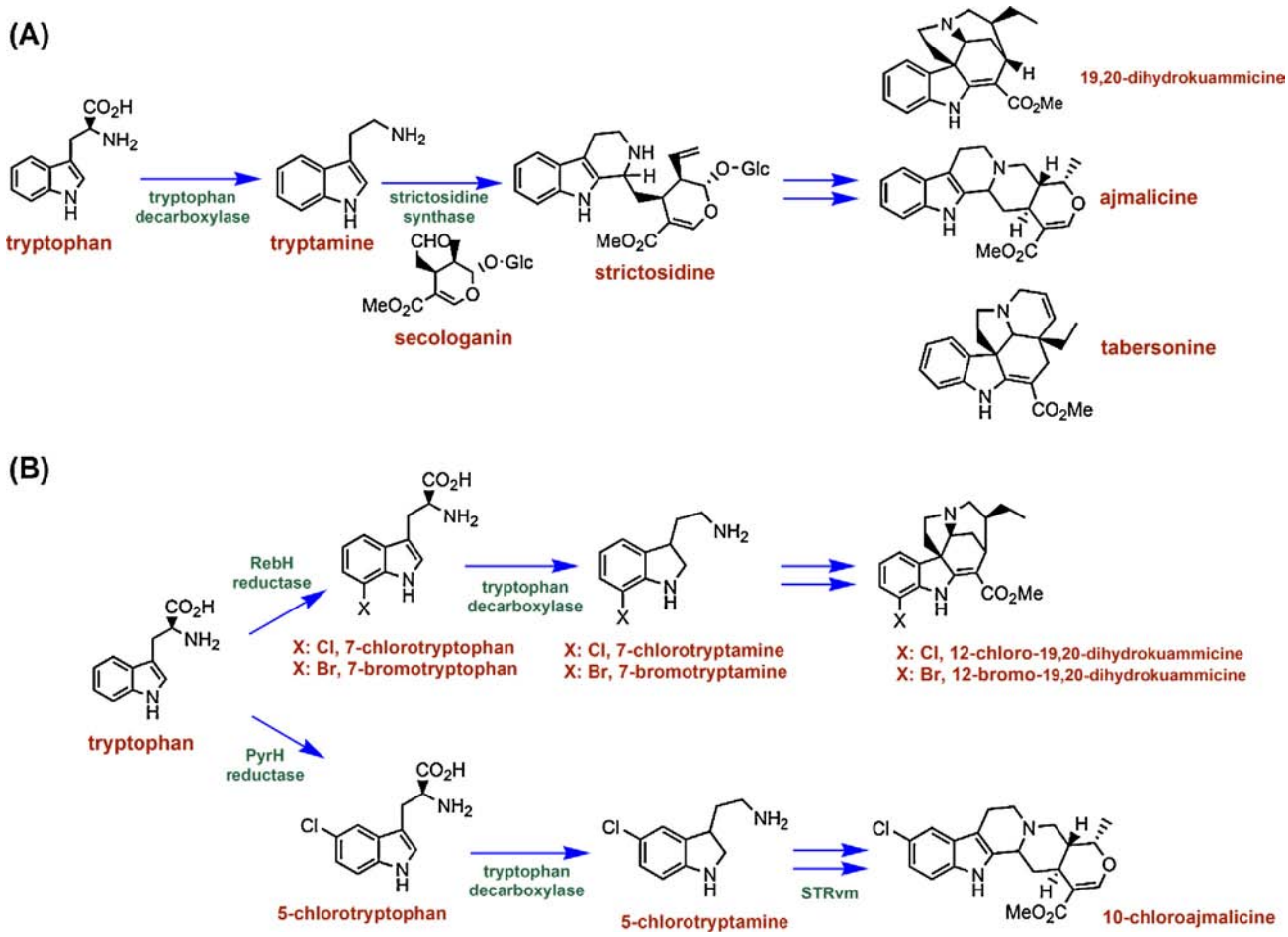

Fig. 3. Monoterpene indole alkaloid biosynthesis in plant cells. (A) Biosynthesis of a series of alkaloids with potential pharmacological activities from tryptophan. (B) Diversification of monoterpene indole-halogenated-alkaloids. RebH and PyR catalyze the halogenation of tryptophan [76].

enzymatic transition from elongation to branching, up to cyclopropanation [102]. The latter is relevant because the enzymes involved in construction of monoterpenoids seem to have a common ancestor [103], and generation of chimeras would be a useful tool to engineer enzymes involved in the production of regular and irregular patterns of isoprenoids; even patterns where no enzymes have yet been characterized [100,102,104] (Fig. 4A).

Monoterpenoids are among the simplest terpenoids and are used in flavors and fragrances. Chlorinated analogs of isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) were synthesized and evaluated as substrates of farnesyl pyrophosphatase (FPPase) (Fig. 4B) demonstrating substrate flexibility. For example, 3-chloro-geranyl pyrophosphate (3-ClGPP) and IPP were incubated to generate 7-chloro-farnesyl pyrophosphate (7-ClFPP). Moreover, the combination of FPPase with geranylgeranyl pyrophosphatase (GGPPase) provides diversification of chlorinated GGPP analogs at positions 3, 7, 11, and 15 [105].

Few attempts have been made for the *in vitro* diversification of diterpenoids. In particular, taxadiene synthase has gained significant attention because of its role as the terpenoid cyclase involved in forming the taxol ring structure. This enzyme catalyzes the cyclization of the C-20 GGPP [106,107], however, very little has been done in terms of assessing whether the enzyme can accept a wide range of GGPP analogs. Once the core structure is generated, however, a series of tailoring reactions take place *in vivo*, thereby resulting in selectively placed hydroxyl, methyl, and glycosyl residues among other functional groups. Biocatalytic modification of the mature paclitaxel has been performed using thermolysin (a bacterial protease) in organic solvents to selectively acylate the 2′-OH of the paclitaxel side chain, giving rise to a library of more water-soluble analogs [108]. Perhaps the most significant biocatalytic transformation on paclitaxel would be the selective addition of the phenylisoserine side chain onto the C-13 position of the baccatin III ring. To date, no definitive enzyme has been identified that catalyzes this highly specific transformation. However, the exploitation of such an enzyme would dramatically reduce the process of paclitaxel semi-synthesis from the baccatin III diterpenoid scaffold.

Triterpenoids derive from squalene and include significant structural diversity. Diversification of triterpenoids was achieved by isolating baruol synthase (BARS1) from *Arabidopsis thaliana* to produce more than 20 oxidosqualene analogs (Fig. 4C) [109]. This diversity was due to the large number of deprotonation sites within oxidosqualene. Triterpenoid diversity was also achieved through the construction of cyclase mutants. A set of lanosterol synthase mutants (from *S. cerevisiae*), were prepared for the synthesis of lanosterol from oxidosqualene (the expected product) but also afforded a novel triterpenoid, isomalabaricatrienol, a potential precursor of isolambricane [109] (Fig. 4C). This finding suggests that several triterpenoid cyclases have a common ancestor. The same concept was demonstrated for diterpene and sesquiterpene cyclases [18,110]. A series of sesquiterpene synthases from *Coprinus cinereus*, Cop1–Cop6, were used to generate different patterns of cyclization of FPP stereoisomers [111]. The control of cyclization patterns is influenced by the reaction conditions (*i.e.* pH), but also by specific chemistry and structure (*i.e.* protein loops) at the active

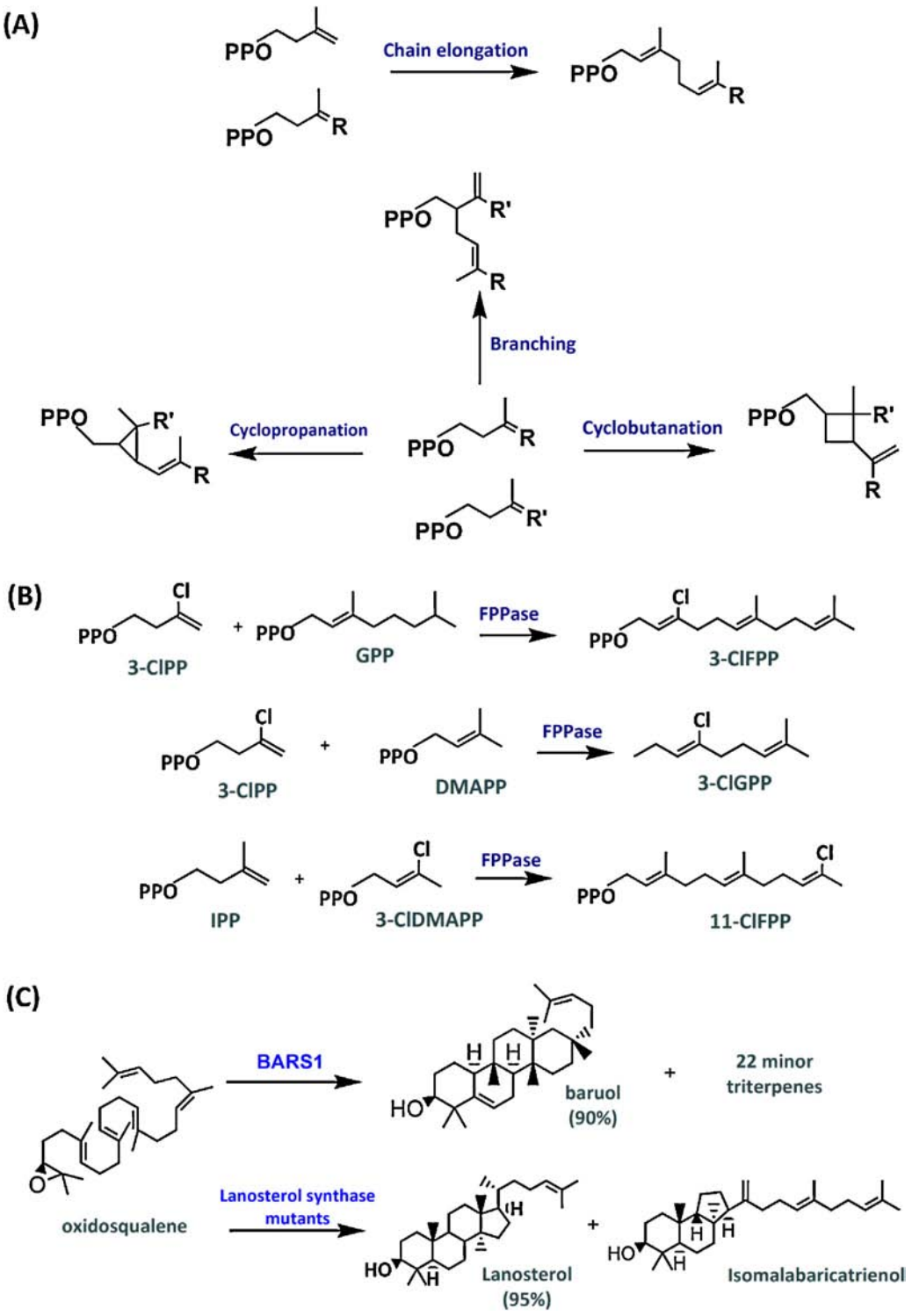

Fig. 4. *In vitro* biosynthesis of terpenoids. (A) Fundamental reactions for production of terpenoids: chain elongation, branching, and cyclization (cyclopropanation and cyclobutanation) [102,104]. (B) Chlorinated isoprenoid synthesis by FPPase [105]. (C) *In vitro* synthesis of triterpenoids by using BARS1 and lanosterol synthase mutants [109].

sites of the enzymes, depending on the fidelity or promiscuity of the enzymes. For example, Cop6 maintains its fidelity, and no significant changes in product profiles were observed after changes in its loop structure, while amino acid mutations in the H-α1 loop region have a marked effect on the product diversity of Cop3 and Cop4 enzymes [110,111]. Indeed, active site loops of sesquiterpene synthases are now considered targets to increase the product diversity of sesquiterpenoids. In another study, Yoshikuni and coworkers [112] explored the plasticity residues of the active site of the promiscuous sesquiterpene synthase γ-humulene synthase to construct a large novel terpene synthase using an approach for systematic recombination, each catalyzing the synthesis of one or a few different sesquiterpenes from a predominant reaction pathway while largely maintaining the specific activity of the original enzyme. Such observations suggest that plasticity residues could significantly drive molecular evolution affecting protein functions.

Moreover, γ-humulene synthase can bind substrates in two different conformations resulting in a different set of products [113]. Finally, point mutations of amino acids in the vicinities of the active site of γ-humulene (a pair of DDxxD motifs) have shown to play a critical role in substrate binding and could promote multiple orientations of the substrate alkyl chain from which multiple families of cyclic olefins could be generated [114].

4.2. Tailoring enzymes

4.2.1. Peroxidases

Peroxidases are a class of enzymes that are considered as tailoring enzymes for natural compounds [8]. Because phenols and hydrogen peroxide (that serves as an electron donor) are the main substrates, several phenols extracted from plants can be derivatized to generate novel compounds with better biological activity. A clear example of this is the apocynin derivatives. Since the last decade, apocynin has been identified as an inhibitor of human NADPH oxidase, a complex enzyme that consists of four cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac) and two membrane subunits $p22^{phox}$ and catalytic $gp91^{phox}$, in neutrophils. However, apocynin by itself is a poor inhibitor of the enzyme but it is converted into active metabolites by myeloperoxidases (MPO); several researchers have identified an apocynin dimer as an effective inhibitor of NADPH oxidase [115]. Peroxidases are capable of generating a complex mixture of oligomers that may contain several active inhibitors. *In vitro* oxidation products of apocynin have been prepared using soybean peroxidase (SBP) [6,7,116] resulting in a complex mixture of oligomers in their demethylated, hydroxylated, or quinone forms. From this complex mixture, a trimer hydroxylated quinone (IIIHyQ) was isolated and proved to be an effective inhibitor of human vascular NADPH oxidase (which has 100% homology with neutrophils) preventing the interaction of $p22^{phox}$-$p47^{phox}$ subunits. This inhibition is believed to take place through a Michael addition between the IIIHyQ and cysteine residues of $p47^{phox}$ (Fig. 5A) [6,7]. The $p47^{phox}$–$p22^{phox}$ interaction is essential for the activation of the Nox2 isoform of NADPH oxidase. Nox2 is upregulated approximately 8-fold and its production of $O_2{\cdot}^-$ increases 2- to 3-fold under induced oxidative stress.

A later study reported the testing of a resveratrol-*trans*-dihydrodimer isolated from the oxidation of resveratrol mediated by SBP [5]. Resveratrol has been widely studied because of its relation with cancer, cardiovascular diseases and Alzheimer's disease. However, more attention has been paid to its analogs and oligomers because this offers the possibility of making compounds with higher biological activity [117]. The resveratrol dimer was tested *in vitro* for the disaggregation of toxic β-amyloids fibers that are related with Alzheimer's disease. The resveratrol dimer was capable of disrupting the fiber structure to generate non-toxic high molecular weight disordered aggregates at concentrations as low as 2 μM, an order of magnitude better than monomeric resveratrol (Fig. 5B). However, the enzymatic oligomerization of phenolic compounds mediated by peroxidases does not necessarily improve the biological activities of new metabolites; for example, the dimerization of the anti-cancer vinblastine mediated by horseradish peroxidase produced a metabolite named catharinine which showed a reduction on its anti-cancer efficacy (~77 fold) against the human T-cell leukemic cell line [118].

4.2.2. Glycosyltransferases

Glycosyltransferases (GTs) are a group of enzymes that transfer sugar residues (from sugar donors) to acceptor molecules named aglycones, and are attractive biocatalysts for generation of glycosides, that may have improved biological activity and/or pharmacokinetic properties. Glycosides are widespread in plant metabolism (coupled to hormones, xenobiotics). Thus, there is an interest in performing enzymatic glycosylation reactions taking advantage of the region and enantioselectivity of GTs that allow the synthesis of glycosides in a single step.

Glycosides of naringenin can be generated *in vitro* using a GT (DicGT4) from the yeast *Dianthus caryophyllus* giving higher yields of naringenin-7-O-glucoside compared to a second glycoside, naringenin-4′-O-glucoside [119] (such compound was identified as a potential therapeutic agent for cardiomyopathy) [120]. The same approach has been applied to other flavonoids. For example, the *in vitro* glycosylation of 28 flavonoids with a recombinant uridine diphosphate glycosyltransferase, UGT78D1, was performed. Using UDP-glucose as donor nucleoside, only five acceptors (quercetin, myricetin, kaempferol, fisetin and isorhamnetin) showed glycosylation products, with selectivity for the 3-position [121].

Resveratrol glycosylation with sucrose to generate piceid can be performed in aqueous buffer using whole cells of *Streptococcus mutans* that contain the glycosyltransferase [122]. The yields were relatively low ~20% due the low solubility of resveratrol. However, further glycosylation reactions of piceid can take place at higher yields. The glycosylation of piceid using glucose, maltose, sucrose, maltotriose and α-cyclodextrin as donor substrates and obtained high yields with α-cyclodextrin (79%) and maltodextrin (72.1%) [123]. Piceid and its glycosides are valuable compounds for the treatment of cardiac ailments such as atherosclerosis and inflammation. Piceid has been shown to inhibit platelet aggregation [124] and lower serum cholesterol levels [125]. Although *trans*-piceid has been shown to reduce elevated lipid levels, it is less active than its aglycone *trans*-resveratrol [126]. In addition, it was found that polyphenolic glycosides (*e.g.* piceid, naringin, rutin, apigenin 7-glucoside) are capable of disaggregating β-amyloid fibers, showing a different pathway for remodeling β-amyloids [5].

4.2.3. Lipases

Lipases have been widely used in enzymatic catalysis in non-aqueous media because of their stability and their application with non-polar substrates (*e.g.* fatty acids, long chain esters) for esterification, transesterification and hydrolysis reactions. Some extracts from plants have been modified enzymatically by lipases in solvents such as hexane or even supercritical CO_2 (SC-CO_2). For example, lutein is a C_{40} carotenoid that acts as a filter for high-energy blue light in tanning lotions. Lutein is also a good quencher and scavenger of photoinduced reactive oxygen species in food preparations and pharmaceutical formulations. Along with its stereoisomer zeaxanthin, lutein is naturally deposited in the lens and macula of the human eye preventing two important age-related diseases; macular degeneration (AMD) and cataracts [127]. Lutein is extracted from Marigold flowers [128] (*Tagetes erecta*) as a complex mixture of at least six lutein diesters (lutein is esterified with C_{12}–C_{16} fatty acids) (Fig. 6). Lutein is commonly liberated from the diester moieties by saponification and to improve its absorption in the digestive track; however, such a process causes degradation of the final product. Lipases are capable of performing the hydrolysis of the lutein diesters in non-aqueous solvents. Lutein diesters hydrolysis can be catalyzed in a two-step reaction (Fig. 6A) by two commercial lipases from *Candida antarctica* (Novozym 435) and *Mucor miehei* (Lipozyme RM 1M) in hexane, isooctane and SC-CO_2 [129]. Fig. 6B shows HPLC-DAD chromatograms of the mixture of six lutein diesters that are converted into a mixture of lutein monoesters and free-lutein. In contrast to most of the hydrolytic reactions, the hydrolysis of lutein diesters reaches its highest rates at very low water activities (a_w = 0.1). The nature of the solvent plays a significant role in this reaction. In solvents with high log P values, like isooctane, the result is higher lutein diesters conversions (~85%). Moreover, the reaction rate is significantly higher in SC-CO_2, and the separation of the product from the solvent just requires that CO_2 be removed from supercritical conditions to

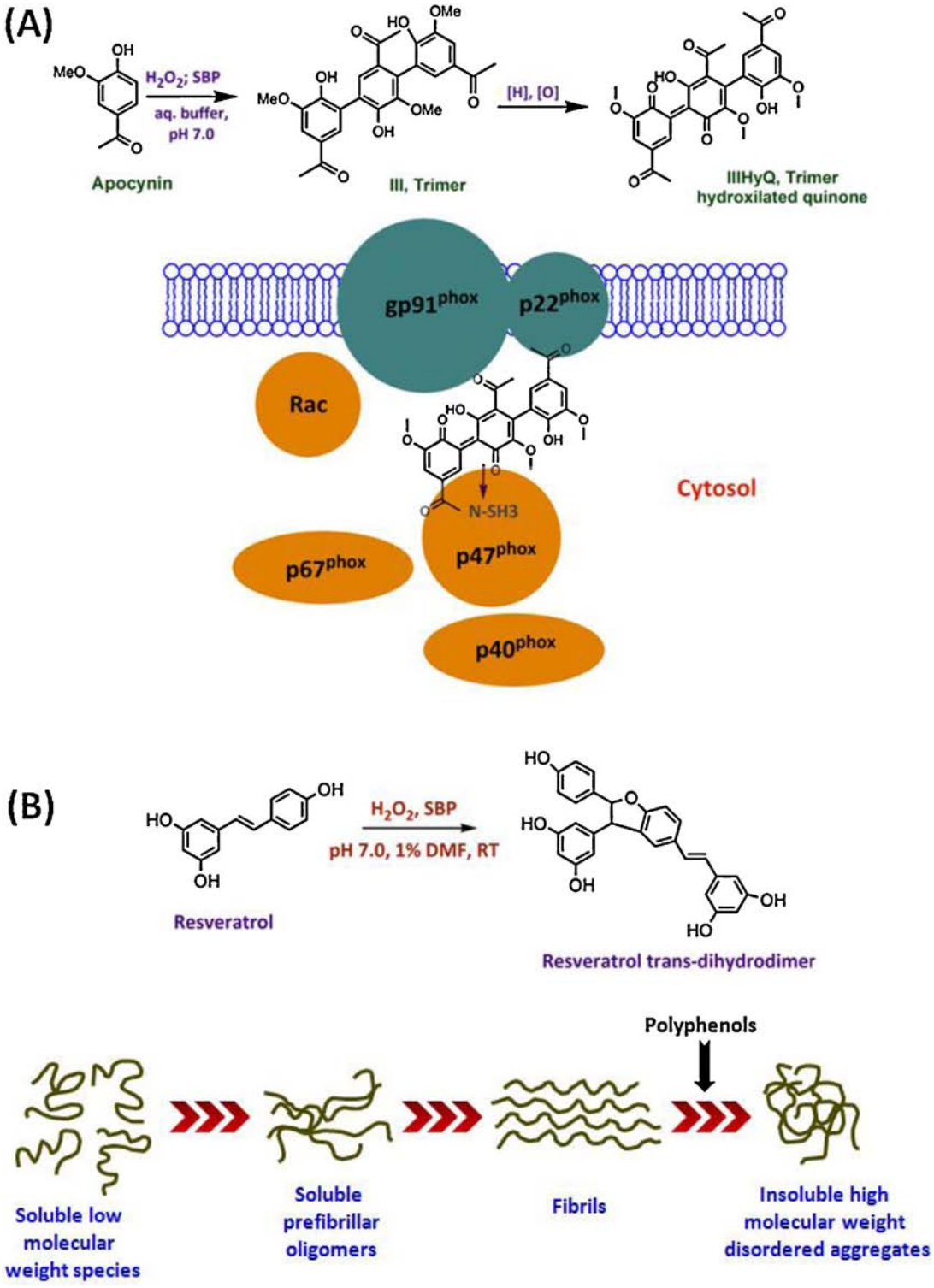

Fig. 5. Oligomerization of natural polyphenols mediated by soybean peroxidase in aqueous buffer. (A) Enzymatic oxidation of apocynin resulted in the IIIHyQ, a strong inhibitor of the human vascular NADPH oxidase [6,7]. IIIHyQ prevents the activation of the enzyme blocking the interaction of cytosolic $p47^{phox}$ subunit with membrane $p22^{phox}$. (B) Enzymatic oxidation of resveratrol generates the resveratrol trans-dihydrodimer which is capable of disrupting the fiber structure to generate non-toxic high molecular weight disordered aggregates [5,52].

recover the product. The commercial enzymes can then be reused without significant loss of activity.

Lipases have been also used to synthesize analogs of capsaicin, a molecule responsible for pungent principle in *Capsicum* plants. Despite its biological activity, the use of capsaicin as a drug or food supplement is limited because of its strong pungency. To avoid this issue, analogs of capsaicin can be synthesized through enzymatic catalysis mediated by lipases. By replacing the acyl residue of the capsaicin into another acyl group (*e.g.* natural oils, fatty acids and methyl esters) eliminating its pungency and nociceptive activity can be eliminated. Product diversity is achieved using different sources of acyl groups (*e.g.* olive oil, Safflower oil, Perilla

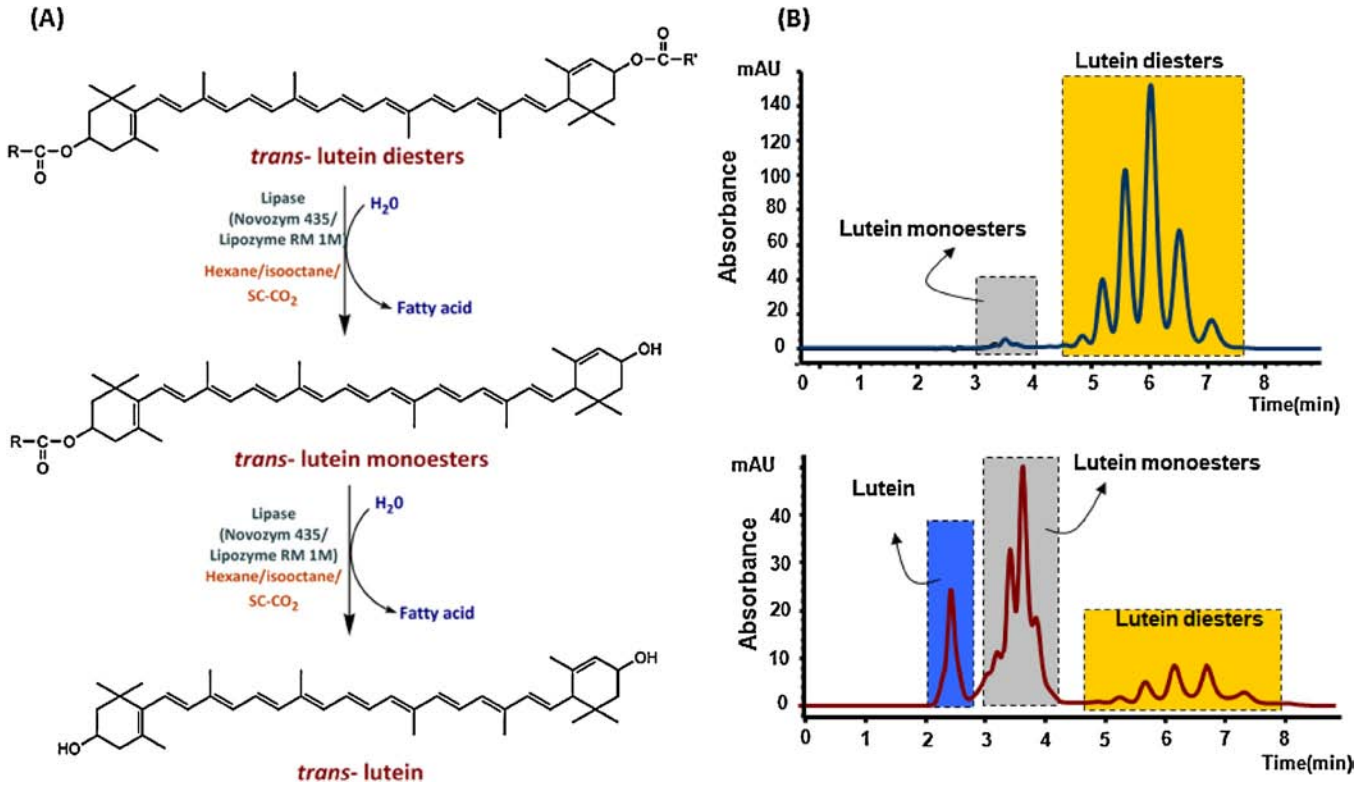

Fig. 6. Enzymatic hydrolysis of lutein diesters extracted from Marigold flowers [129]. (A) The reaction is performed in organic solvents (hexane, isooctane) or under SC-CO_2 mediated by two commercial lipases: lipase B from *Candida antarctica* (Novozym 435) and from *Mucor mihei* (Lipozyme RM 1M). The hydrolysis proceeds in two steps giving the lutein monoesters as intermediates. (B) HPLC-DAD analysis of the lutein diesters (reaction substrate) and their conversion into monoesters and free lutein (unpublished chromatograms from [129]).

oil) in hexane with different lipase preparations, resulting in a mixture of capsaicin analogs like olvanil, linoleoyl vanillylamide, and myristoyl vanillylamide [130]. More recently, Gram-negative antibacterial N-vanillylnonanamide, another analog of capsaicin, was generated in SC-CO_2 mediated by Lipozyme RM 1M. The addition of two divalent salts, $CuCl_2$ and $ZnCl_2$ (2 mM), enhanced lipase activity, reaching yields over 40% with Lipozyme RM 1M retaining more than 50% of its activity [131].

Enzymatic reactions in non-aqueous media catalyzed by lipases have been widely studied. The optimization of reactions mediated by lipases depends strongly on the level of hydration of the system because this determines in which direction the reaction can proceed. This is probably the most important parameter to control in reactions catalyzed by lipases. The best way to control the level of hydration of both, solvent and biocatalyst, is through the control of water activity (a_w) using over-saturated salt solutions, resulting in a specific relative humidity, and therefore, a_w [132].

5. Perspective: diversification of non-natural products combining strategies

Till today, numerous examples of metabolic engineering strategies to produce phytochemicals in recombinant microorganisms have been presented in the literature. However, only a handful of these studies have resulted in commercially viable processes, mainly due to issues related to production titers and overall yields. It is pretty evident from the previous literature review that significant improvements need to be made before microbial production platforms can become complementary and even competitive alternatives to currently used production methods. To that effect, new tools that allow the more efficient rewiring of the cell's native metabolism, efficient expression of plant enzymes and alleviation of the toxicity displayed by many of the target compounds have emerged and continue to be described in recent literature.

Several studies have also presented the use of both *in vivo* and *in vitro* approaches for the diversification of plant natural products to generate libraries of compounds with potential pharmacological applications, demonstrating that such diversification efforts should not be constrained to a single strategy. Combination between metabolic engineering and *in vitro* biocatalysis can also facilitate the future generation of non-natural analogs of plant natural products. For example, non-natural analogs of flavonoids, produced through metabolic engineering using precursors with specific functional groups (*i.e.* halogens [46]), can further be diversified using tailoring enzymes. There is strong evidence that oligomerization, mediated by peroxidases of polyphenols, results in the improvement of their biological activity. Moreover, combination of more than one tailoring enzyme can also improve their physicochemical properties (*i.e.* using GTs) as well as their biological activity. Micro array platforms could be ideally used to combine multiple tailoring enzymes and generate diversified libraries of new molecules. Microarrays are also amenable to high throughput screening to identify active molecules before scaling-up the reactions and isolate the individual molecules for characterization [133,134]. This approach would significantly increase the variety of chemical structures that could be tested as pharmaceutical candidates among a broad number of diseases.

Individually, several challenges are ahead for the *in vivo* and *in vitro* production of plant natural and non-natural products. From the metabolic engineering perspective, a number of variables have to be optimized at the same time (*i.e.* feed stocks, fermentation operational variables, precursor selection, and identification of the ideal host organisms) [135]. This optimization still

poses challenges in the development of mathematical models, the use of novel cloning techniques, and general synthetic biology approaches. In addition, the full expression of a pathway into host organisms for the efficient production of natural and non-natural products remains very difficult as the reconstruction of a pathway involves a large number of permutations and combinations. Moreover, diversification of natural and non-natural products can result stressful for cells resulting in altered patterns of gene expression and metabolic enzyme inhibition. *In vitro* pathway reconstruction first requires the characterization of biosynthetic pathways. This increases our understanding of the array of enzymes required for synthesis and diversification of natural and non-natural compounds. The resulting structural diversity is not accessible by traditional chemical synthesis and offers enormous potential in pharmaceutical applications. However, the main issue is the identification, production and isolation of active enzymes, as well as the identification and viability of cofactors needed for their catalytic activity. Moreover, generation of pathway intermediates and final products by *in vitro* biocatalysis should provide a better insight on the synergy between the constituent enzymes that permit the generation of natural products and analogs.

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