Metabolic engineering of microbes for monoterpenoid production

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Abstract

Monoterpenoids are an important class of natural products that are derived from the condensation of two five-carbon isoprene subunits. They are widely used for flavouring, fragrances, colourants, cosmetics, fuels, chemicals, and pharmaceuticals in various industries. They can also serve as precursors for the production of many industrially important products. Currently, monoterpenoids are produced predominantly through extraction from plant sources. However, the small quantity of monoterpenoids in nature renders this method of isolation non-economically viable. Similarly impractical is the chemical synthesis of these compounds as they suffer from high energy consumption and pollutant discharge. Microbial biosynthesis, however, exists as a potential solution to these hindrances, but the transformation of cells into efficient factories remains a major impediment. Here, we critically review the recent advances in engineering microbes for monoterpenoid production with an emphasis on categorized strategies and discuss the challenges and perspectives to offer guidance for future engineering.

Keywords: Natural products; Monoterpenoids; Metabolic engineering strategy; Biochemical production; Microbial cell factory

1. Introduction

Monoterpenoids are an important class of plant natural products that exhibit a broad range of biological activities. With increasing emphasis in health and environmental sustainability, some monoterpenes and related derivatives have gained attraction among customers for their unique pollution-free and environment-friendly attributes in various industrial applications (e.g., foods, medicines and cosmetics). Today, monoterpenoids are primarily acquired through plant biomass extraction method, a process that is low yielding and unable to match up to industrial demand. Moreover, this method of production may suffer from seasonal and geographical variability [1]. While chemical synthesis workflows can also be used to produce monoterpenoids, these operations may be energy-intensive and generate substantial amount of organic waste. Hence, there exists an unmet need for an alternative efficient, sustainable and eco-friendly supply route for monoterpenoids.

Recently, a promising novel approach that harnesses microbes and transforms them into cell factories for *de novo* biosynthesis of natural products has emerged. Microbes are employed as hosts in this technique due to their advantageous natural qualities, one of which being their rapid life cycles, which effectively shortens the production time from dozens of months to several days [2]. In addition, it is the core competency of a microbial-based system that strong productivity keeps robust production supply in a persistent rhythm over the course of fermentation. Furthermore, the use of abundant, renewable, cheap resources such as waste cooking oils for the manufacture of high-value natural products is more sustainable and economical as compared to customary production methods [3].

In the past decades, efforts to maximize bioproduction titers, productivities, and yields (TPYs) were amply rewarded, some even having reached commercialization in microbe platforms. These typically involved a combination of several metabolic engineering strategies rather than an isolated step alone. Moreover, improper regulation of microbes would result in stresses such as redox imbalance and toxic intermediate accumulation or even be detrimental to cell growth. Hence, the objective of this review is to offer guidance as to when, what and how each of the strategies should be adopted to build a robust monoterpenoids biosynthesis workhouse. Here, we aim to describe recent developments in molecular and process strategies of modulating the host metabolism at the level of DNA, RNA, protein, metabolite, cell and fermentation for the production of monoterpenoids (Fig. 1), explore the bottleneck issues encountered and present potential solutions.

2. The monoterpenoids biosynthesis pathway

The monoterpenoids synthesis pathway in microbes can be divided into three modules for analysis: (1) the two universal five-carbon building units, isopentenyl diphosphates (IPP), and its allylic isomer, dimethylallyl diphosphate (DMAPP), derived from two parallel routes, the mevalonate (MVA) pathway and methylerythritol 4-phosphate (MEP) pathway (Fig. 2), (2) with the catalyzation of the key enzyme geranyl diphosphate synthase (GPPS), one isopentenyl diphosphate (IPP) and one dimethylallyl diphosphate (DMAPP) are immediately condensed to generate geranyl diphosphate

(GPP), (3) GPP as the direct precursor is converted to monoterpenoids by various monoterpene synthases (MTS). Recent studies have observed that in a few plant species, neryl diphosphate (NPP), the cis isomer of GPP, also acts as the substrate for monoterpenoids formation [4] (Table 1), thereby suggesting that both MEP and MVA pathways may share common intermediates such as IPP, DMAPP and GPP. Intriguingly, the two pathways could even co-exist within some higher plants and marine alga. Metabolic reconstruction of both pathways into a single host had been demonstrated, in *Escherichia coli*, resulting in significant improvement in isoprenoids productivity and yield [5]. Nevertheless, the synthesis logic of the two pathways is completely distinct, which leads to sharp contrasts in synthesis places, synthesis substrates and the enzymes involved. The MVA pathway, mainly presenting in archaea, fungi, plant cytoplasm and other eukaryotes [6], proceeds from acetyl-CoA, referring to the key enzymes acetyl-CoA C-acetyltransferase (ERG10), hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), mevalonate kinase (ERG12), phosphomevalonate kinase (ERG8), diphosphomevalonate decarboxylase (ERG19), whereas the MEP pathway, mainly presenting in most gram-negative bacteria, green algae, and c*yanobacteria* [7], arises from the condensation of glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR), referring to the key enzymes 1-deoxy-D-xylulose 5-phosphate synthase (Dxs), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), 2-C-methyl-Derythritol-2,4-cyclodiphosphate synthase (IspF), 1-hydroxy-2-methyl-butenyl-4-diphosphate synthase (IspG), 1-hydroxy-2-methyl-butenyl-4-diphosphate reductase (IspH). In comparison with the MEP pathway, it was calculated by stoichiometry that the MVA pathway provides less yield but consumes less energy and reducing equivalents [8]. For the production of terpenoids, the heterologous MVA pathway in *E. coli* has been validated through experiments to be more excellent than the MEP [9, 10], whereas in *cyanobacteria*, the expression of the MVA pathway may be less competent than the native MEP pathway [11]. Thus, the combination of target host and MEP/MVA pathway must be carefully analyzed, as an unharmonious relationship between them could lead to suboptimal performance.

To function, the microbe platform must be designed to contain core metabolic pathways for cell growth and the production of desirable molecules. As rewired monoterpenoids pathways couple with other various pathways to constitute an intricate mesh of reactions in cellular metabolism, the undesirable interference between them arises inevitably, leading to the disruption of intracellular homeostasis. To alleviate this inherent limitation, an alternative option is to construct an artificial biosynthetic pathway that operates as an orthogonal route and is growth-independent of native metabolism with fewer cellular interactions and higher substrate utilization efficiency. For instance, constructing an isopentenol utilization pathway (IUP) has enabled the bioconversion of isopentenol, isoprenol, or prenol to IPP or DMAPP by a choline kinase from *Saccharomyces cerevisiae* and an isopentenyl phosphate kinase (IPK) from *Arabidopsis thaliana*, which is comprised of only two reaction steps with a single ATP demand [12] (Fig. 2). In another study, an alcohol-dependent hemiterpene (ADH) pathway

has been developed for the biotransformation of exogenously supplied isopentenol and dimethylallyl alcohol to isoprenoid by way of utilizing a non-specific acid phosphatase from *Shigella flexneri* and an IPK from *Thermoplasma acidophilum*, which involves two reaction steps with double equivalents of ATP demands [13]. Another such synthetic pathway is the *de novo* isoprenoid alcohol (IPA) pathway which was established as an energy efficient method to synthesizing isoprenoid precursors, and when implemented in *E. coli*, produced almost 0.6 g/L total monoterpenoids [14]. Native pathways can also be refined as seen in the improved performance of the NPP-based orthogonal pathway where NPP served as an alternative precursor to GPP in monoterpenoid biosynthesis [15]. In most of the orthogonal limonene production in *E. coli* [16], *S. cerevisiae* [17] and *Yarrowia lipolytica* [18], neryl diphosphate synthase (NPPS) performed excellently in raising the efficiency and titer of the limonene biosynthesis pathway.

3. Regulation strategies for the synthesis of monoterpenoids in microbes

3.1. Engineering of precursor supply

Despite the structural and functional diversity of monoterpenoids, all of them originate from acetyl-CoA, or PYR/G3P. Specifically, three acetyl-CoA molecules are devoted to the first two steps of the MVA pathway, while G3P and PYR assume the role of precursors for the MEP pathway. Sufficient provision of the precursors has, therefore, been the focus of metabolic engineering research, as it is a significant prerequisite for the biosynthesis for all isoprenoid products.

Both the MEP and MVA pathways are strongly coupled and tightly regulated with the central carbon metabolism, particularly in the glycolysis pathway and other several degradations of organic carbon pathways. G3P and PYR are generated in several primary central carbon pathways such as the Embden–Meyerhof–Parnas (EMP) pathway, the Entner-Doudoroff (ED) pathway and the Pentose Phosphate (PP) pathway. Among them, the EMP pathway has drawn much attention in precursor engineering due to its extensive participation in isoprenoid biosynthesis. PYR serves as the downstream glycolytic intermediate of G3P in this pathway, and as there are some competitions between PYR and other metabolites for G3P, an inequitable distribution of G3P and PYR becomes difficult to avoid. Through redirecting metabolic fluxes from PYR back to G3P, the pathway bottleneck was debugged via overexpression of *Pps* (encoding phosphoenolpyruvate synthase) and *Pck* (encoding PEP carboxykinase) or the inactivation of the *pykFA* genes (encoding pyruvate kinases) [19, 20]. To further increase the G3P and PYR amount, an attempt of enhancing the flux of EMP pathway was made by deleting the central carbon metabolism gene *zwf* (encoding glyceraldehyde 3-phosphate dehydrogenase), thereby allowing carbon access to the PP pathway and preventing the PP pathway from consuming carbon sources, which successfully improved the production of lycopene [21]. Another vital glycolysis pathway from sugar substrates is the ED pathway, but, unlike the EMP pathway, it produces equal amounts of G3P and PYR simultaneously. From investigating the ED pathway performance in precursor engineering, it was realized that the ED pathway was the only available pathway that produces both G3P and PYR through the deletion of the *pgi* (encoding phosphoglucose isomerase) and *gnd* (6-posphogluconate dehydrogenase) genes which

blocks the EMP and PP pathways, respectively. It is noteworthy to mention that the ED pathway exhibited the highest isoprene production titer and yield among all glycolysis pathways in the engineered *E. coli* [22].

The features of complicated compartmentalized synthesis and involvement in many crucial cellular processes suggest that supplying molecular building block acetyl-CoA in microorganisms is a sophisticated metabolism. For instance, acetyl-CoA is not only a pivotal precursor of monoterpenes production but also an essential intermediate of the tricarboxylic acid cycle (TCA). The disruption of the gene *gltA*, encoding cytoplasmic citrate synthetase, successfully reduced flux toward TCA and increased the intracellular abundance of acetyl-CoA in *E. coli* [23]. Comparatively, citrate in yeast is mainly produced in the mitochondrion as part of the citric acid cycle. One study demonstrated the feasibility of using mitochondrial citrate carriers in the engineered *Y. lipolytica* to divert citrate into the cytoplasm whereby it is then converted into cytoplasmic acetyl-CoA by ATP citrate lyase (ACL) [24]. Applying a strategy of increased ACL expression in combination with other approaches can potentially lead to better outcomes. For instance, isocitrate dehydrogenase (ICDH) has been identified as a key enzyme in the TCA cycle. Deletion of its encoded gene *IDH1* would channel citrate flux from the TCA cycle to ACL. Increasing ACL expression in conjunction with *IDH1* deletion reportedly had a synergistic effect on boosting flux toward the mevalonate pathway [25]. In fact, the main cytosolic acetyl-CoA was biosynthesized in yeast from acetaldehyde which derived from the pyruvate dehydrogenase (PDH) bypass pathway (or pyruvate decarboxylation pathway). To efficiently redirect the carbon flux to cytosolic acetyl-CoA, heterologous bacterial PDH was introduced to *S. cerevisiae* to take full advantage of cytoplasmic pyruvate and thus, improve the acetyl-CoA pool [26]. However, acetaldehyde can also be alternatively converted to ethanol by alcohol dehydrogenase due to the Crabtree effect in yeast, hence, in maximising the channelling of flux from ethanol to acetyl-CoA in the cytosol, the over-expression of the gene *ADH2* (encoding alcohol dehydrogenase), gene *ALD6* (encoding NADP-dependent aldehyde dehydrogenase), and a codon-optimised ACS variant (L641P) (encoding acetyl-CoA synthetase) demonstrated to be a better solution [27]. This push strategy that facilitates the drive of carbon from ethanol to cytosolic acetyl-CoA, when paired with a pull strategy that pulls acetyl-CoA towards the target products, has been proven to be a successful technique in enhancing acetyl-CoA supply. As such, in addition to the overexpression of ADH2, ALD6 and L641P, co-expression of acetoacetyl-CoA thiolase (*ERG10*) must be performed to pull the flux toward the downstream of a metabolic sink and achieve a 60% improvement in production of isoprene [28]. Overall, these examples indicate that a robust supply of precursor is indispensable for downstream metabolites.

3.2. Metabolic engineering of biosynthetic pathways

3.2.1 Overexpression of structural genes in the MEP and MVA pathway

As fine-tuning of the multiple GPP-related genes involved in the construction of the *de novo* monoterpenoids biosynthesis pathway are attain optimal efficiency in the monoterpenoids bioproduction system, pathway engineering in microorganisms has been broadly applied. Often, the overexpression of genes is employed for its efficiency.

With regard to the manipulation of the MEP/MVA pathway in genetic engineering, the most classical and effective approach would involve the enhancement of their throughput toward precursor GPP supply to the fullest extent. Towards that end, each enzyme in the MEP pathway was systematically overexpressed, which validated the potential of IspD to improve the production of terpenes in *Synechocystis PCC 6803* [29]. Similarly, overexpression of DXR was confirmed to have a significant positive effect on isoprene production in the engineered *E. coli* [30]. Furthermore, application of targeted proteomics and metabolomics analysis quantitatively revealed that Dxs dominates the flux through the MEP pathway and is regarded as a major genetic part due to its substantial flux control coefficient of 0.35 (i.e., a 1% increase in enzyme Dxs activity led to a 0.35% increase in pathway flux) in the engineered strain [31]. Besides, the concentrations of downstream intermediates 1-deoxy-D-xylulose-5-phosphate (DXP), MEP and 4-diphosphocytidyl-2-C-methylerythritol (ME-CDP) were linearly dependent on Dxs expression [31]. As the last downstream intermediate, the excess IPP would remain unconsumed and further disrupt intracellular homeostasis. One way to overcome this inherent challenge is through the overexpression of IDI which is responsible for the conversion of IPP to DMAPP. For instance, applying this approach through co-expression of *idi* (encoding isopentenyl diphosphate isomerase) and *ispS* (encoding isoprene synthase) could cue growth impairment suffered from the expression of *ispS* alone [31]. Despite overexpression of rate-limiting reaction genes being one of the easiest and most widely implemented strategies to improve metabolic flux, the impact of it was mild in some cases. Take IspD and IspE for example: they are rate-limiting enzymes in the MEP pathway, but overexpression of neither *ispD* (encoding CDP-ME synthase) [32] nor *ychB* (encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase) [33] was successful in improving metabolites production and instead, may even present a bottleneck of severe growth inhibition when co-overexpressed with other MEP enzymes [34]. Moreover, as the last two enzymes in the MEP pathway, IspG and IspH, are both $[4Fe–4S]^{2+}$ enzymes coupled with a reduction system that drives electrons from a reduced donor to an oxidized [4Fe-4S] cluster [35], simple overexpression of IspG and IspH will not be successful in enhancing the flux towards monoterpenoids [31, 36]. It is only when the redox recycling system and cofactors are operating normally that the function of IspG/H can be achieved [37]. Taken together, the use of modulating the MEP genetic route revealed the benefit and the function of the pathway debottleneck but also emphasized that each genetic part has its own natural features which should be considered carefully to generate maximum profit.

In the same regard, increased expression of genes encoding enzymes involved in rate-limiting steps is often applied in the MVA pathway as well. This is evident in the condensation reaction of acetyl-CoA to form acetoacetyl-CoA by acetyl-CoA C-acetyl transferase (ACCT) where overexpression of *ERG10*, responsible for the synthesis of ACCT, is effective in channelling carbon flux from the central metabolism towards the MVA pathway [27]. As discussed earlier that the MVA pathway can only generate IPP but not DMAPP, this therefore renders enzyme IDI an essential genetic part in monoterpenoid production, and overexpression of its encoded gene has been proven to be productive in enhancing the GPP flux. One of the more prominent examples that

have been documented would be the overexpression of *IDI1* (encoding isopentenyl diphosphate isomerase) that has achieved approximately five folds increase in cineole production [38]. When the same strategy was employed in geraniol production, a 1.45-fold titer increase was observed [39]. Another such example would involve the most frequently treated and highly regulated rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which converts HMG-CoA to MVA. Overexpression of HMGR either solely or conjunctively could be adopted to augment the accumulation pool of GPP to a considerable extent. For instance, in limonene production, overexpression of *tHMG1* (encoding 3-hydroxy-3-methylglutaryl-CoA reductase) gene alone achieved approximately 18-fold higher output than that of the control strain in *Y. lipolytica* [18] while co-overexpression of genes *ERG12* gene together with *HMG1* resulted in a 112-fold increase in yield [18]. That being said, studies have also shown that there is a limit to the increase in output of the target products through the sole overexpression of related genes. In one study, the mechanism of thiolysis activity of ACCT illustrated that overly high concentrations of acetoacetyl-CoA would be inclined to ultimately generate a futile cycle between acetyl-, malonyl- and acetoacetyl-CoA [40] instead of having enhanced production rates. As such, other approaches such as the deletion of genes have also been explored to increase production. For example, it has been studied that deleting *YPL062W* will stimulate the upregulation of several MVA pathway genes (*ERG10*, *ERG13* encoding hydroxymethylglutaryl-CoA synthase, *HMG1*, and *ERG20*), which in turn will channel the carbon flux toward monoterpenoid precursors acetyl-CoA and MVA, thereby improving the MVA and geraniol production [41].

3.2.2. Optimization of downstream module pathway

Despite the apparent advantages that the employment of MVA and MEP synthetic biology routes in metabolic engineering presents, the formation of byproducts through oxidation, isomerization, dehydrogenation and esterification [42] hinders large-scale production. In the production of (S)-perillyl alcohol by *Pseudomonas putida* KT2440, the byproducts perillyl aldehyde and perillic acid used up to 26% of the total amount of terpenes by oxidation. To impede the formation of side products and improve recovery of the target product, the outer membrane protein AlkL was expressed to enhance its hydroxylation activities, thus resulting in the formation of no perillic acid and only minor amounts of perillyl aldehyde (8% of the total products) in the two-liquid phase system. In another study of the production of geraniol using *E. coli*, the investigation of corresponding endogenous enzymes for geranoids formation recognized that *yjgB* (encoding geraniol dehydrogenase) executes the primary task of geraniol dehydrogenization isomerization into other geranoids (nerol, neral, and geranial), and thus the deletion of the gene *yjgB* led to a direct increase in geraniol production from 96.5 mg/L to 129.7 mg/L [43]. In *S. cerevisiae*, the geraniol transformation is strikingly correlated to isomerization by acid-catalysis, containing reduction to citronellol by an NADPH oxidoreductase Oye2p and esterification by an alcohol acetyltransferase Atf1p [44]. To further minimize endogenous transformation of geraniol, the gene *OYE2* or *ATF1* was solely deleted which contributed to a 1.7-fold or 1.6-fold increase of geraniol

production, respectively [45]. It is noteworthy to mention that the deletion of both genes *OYE2* and *ATF1*, however, resulted in a dramatic reduction of geraniol production along with a 35% decrease of the final biomass [45], as they both participate in the management of sterol metabolism [46]. Since simple gene deletion failed to completely eradicate geranyl acetate synthesis, this drove the discovery of overexpressing endogenous acetylesterase (AES) being the solution to transforming geranyl acetate into geraniol and it was reported to have successfully increased production of geraniol to 2.0 g/L under controlled fermentation conditions [47].

3.2.3. Dynamic control engineering

Over the long-term fermentation process, engineered strains have frequently exhibited reduced cell fitness due to internal metabolic state imbalance and external environment interference, such as metabolic burdens and physicochemical cues. Thus, dynamic control engineering via autonomously coordinating microbes' metabolic flux and adapting metabolism to suboptimal environments and stresses came into existence.

Both the two fundamental components, biosensor and regulator, constitute a dynamic control system. In this regulation paradigm, non-intuitive ligand metabolite concentration is measured and converted into a visualizable and quantifiable signal by the sensor that immediately transmits it to the coupled regulator. Subsequently, the regulator functions in detecting the corresponding signal and adjusting the metabolisms accordingly. This feedback control strategy was successfully applied for evolving strains with higher production capacity based on metabolite response. Here, through regulating the expression of two non-conditionally growth-critical genes, *folP* and *glm*, with an MVA product-responsive biosensor, the MVA-based dynamic control system was constructed to retain the high-yield MVA production across 95 generations of growth and eliminate low production cells suffering from detrimental genetic heterogeneity [48]. Stemming from the desire to maintain homeostasis, this strategy has favourably served to gain the perfect tradeoff between cell growth and synthesis of target products. Similarly, quorum sensing, a form of communication that is reliant on signal transduction triggered by the population density reaching a threshold point to initiate changes in behaviour, is an attractive approach in dynamic control to achieve this goal as well. In the production of bisabolene, LuxI/R quorum-sensing system was utilized in *E. coli* to achieve a titer of 1.1 g/L. The mechanism of this system requires the LuxR autoinducer complex to accumulate in proportion to cell density and upon reaching a threshold point in population density, will activate the transcription of the PluxI promoter to trigger bisabolene production [10]. While multiple dynamic regulation elements and gene circuits have been developed and widely employed in metabolic engineering, till date, the use of the stress-response promoter is the most extensively studied method. In one study, it was demonstrated that the stress-response promoter endows the host with the ability of dynamic gene regulation, which was exploited to regulate FPP biosynthesis in the isoprenoid biosynthetic pathway in *E. coli*, and this acquired 2-fold improvement of amorpha-4,11-diene production over either a constitutive expression system or an IPTG inducible system [49]. Similar strategies have the scope to be extended into *S. cerevisiae* as well, with one example being the side-

product regulated system that harnesses ergosterol-responsive promoters to adjust squalene synthase ERG9 expression in accordance with the required ergosterols for optimal cell growth, thereby redistributing the metabolic flux towards non-native isoprenoid production [50].

Apart from improving the performance of the platform, one of the benefits of dynamic control is that it eliminates the barrier of externally adding an inducer or a repressor, which will raise the fermentation efficiency, as well as lower production costs. Despite advances in the design of these systems, current dynamic metabolic control systems are not performing adequately and precisely, and the absence of sensor-regulators for plentiful pathway intermediates make it particularly challenging to regulate heterologous metabolic pathways.

3.3 Engineering of single elements

3.3.1 Promoter engineering

Promoter engineering is an extremely effective and accessible method for regulating enzyme expression at the transcription level. In recent years, with the improved understanding of the core elements and upstream activation sequences of promoters, promoter engineering has been applied for the fine-tuning of metabolic flux. For example, replacing the T7 promoter with the stronger constitutive FAB80 promoter increases the expression of the phosphomevalonate kinase, encoded by *ScPMK* gene, in engineered *E. coli*, hence increasing metabolic flux along the melavonate pathway [16]. Sometimes, complete knockout of certain essential genes is not a viable method. In such cases, promoter replacement strategy will be employed to down-regulate unintended secondary flux. One example is the down-regulation of ergosterol availability, an essential sterol, in yeast. Specifically, the native ERG20 promoter was replaced with an 807 bp fragment of the ERG1 promoter, containing an ergosterol-responsive element that is negatively regulated by ergosterol. This method enabled the reduction of metabolic flux toward the sterol branch without causing any cell viability issue [15]. Adjacent protein-encoding genes may play a secondary role of being a gene regulatory element. Bioinformatics analysis and serial promoter deletion assay revealed that gene *YPL062W* is also a core promoter sequence for gene *ALD6* that encodes for cytosolic aldehyde dehydrogenase. As the *ALD6* gene is negatively correlated to monoterpenoids productivity, the deletion of *YPL062W* disrupted the promoter elements to down-regulate *ALD6* expression and hence raised monoterpenoid productivity [41]. Promoter engineering also could be applied to change the diauxic growth state of microbes; the chromosomal ERG20 promoter of *S. cerevisiae* was replaced with glucose-sensing promoter PHXT1, rerouting the carbon flux from growth pathway to limonene synthetic pathway on the condition of sufficient supply of glucose [17]. Additionally, for metabolites that cause cellular stresses, using a cooperative promoter as sensors and regulators to up- or down-regulate the accumulation of the metabolites is a general design principle in dynamic control engineering. Notably, screening endogenous and heterologous promoter with higher transcriptional efficiency, stronger specificity, and more precise regulation (quantitative, timed, site-specific) are critical aspects in promoter engineering.

3.3.2. Protein engineering

Microbe performance is closely linked to cofactor balance, precursor supply, and enzyme property, among which, enzyme properties make a significant contribution. Some native rate-limiting pathway enzymes can be thought of as principally determining the yield, so extensive research efforts are made to integrate protein engineering into cellular metabolic processes. As such, protein engineering comprises of three categories: protein stability, catalytic activity and substrate specificity.

The low substrate specificity of native enzymes, since notorious for accumulating undesired side products, is yet another reason for attaining low yields for target products. Gene random mutagenesis therefore performed well in improving enzyme specificity by introducing mutagenesis to active sites and binding sites. In microbes, there are no enzymes tht are specific to GPPS alone; both GPPS and Farnesyl diphosphate synthesis (FPPS) are catalyzed by a single gene *ERG20*. Hence, overexpressing the native gene *ERG20* for increasing monoterpenoid production in *S. cerevisiae* has been attempted, but no solution seemed imminent [51]. Through the specific optimization strategies, the FPPS variant with a high functional activity of GPPS was integrated into a *Y. lipolytica* wild strain [52] to redirect the flux of GPP and FPP, which greatly promoted the GPP accumulation and linalool production compared to the control strain. Moreover, this prevented the metabolic burden of cells created by overexpression of genes. A similar strategy was applied in *E. coli* where a mutation in Ser81 to Phe (S81F) was introduced to IspA for altering its substrate specificity [53], which improved GPP availability and further boosted monoterpenoid production. Introducing mutagenesis to re-mould the binding pocket is also a good solution to improve enzyme catalytic activity, which may be useful in circumventing the bottleneck of substrate utilization and product formation. In *E. coli*, efmvaS (HMG-CoA synthase) mutation of alanine to glycine at site 110 accelerated the overall reaction rate of enzyme and streamlined the process from acetyl-CoA to mevalonate [16]. Further recombination approaches of the mutagenesis have been developed to construct and screen the mutant library for desired corresponding enzymes. Such engineering has been performed on the protein ERG20 to generate a series of mutants such as $ERG20^{F96W}$, $ERG20^{N127W}$, $ERG20^{K197G}$ in *S. cerevisiae*, and since different combinations of these mutants made up a really large variety of database, therein lied a highly efficient double mutations enzyme comprising both F96W and N127W that was expressed in mitochondria and cytosol to improve linalool production up to 2.69 mg/L, a 2-fold increase over the control strain [54].

Due to its role in the anabolic metabolism of downstream essential cellular components, direct deletion of the essential gene *ERG20* severely prevented cell growth and failed to improve monoterpenoid production when down-regulated. Hence, an effective solution is to formulate an N-degron-dependent protein degradation strategy to down-regulate FPP synthase Erg20p activity for GPP accumulation in *S. cerevisiae* [55]. As both major biosynthetic synthases from plants have an N-terminal plastid transit peptide that would be proteolyzed after targeting the plastid stroma, experiments illustrated that N-terminally truncated (targeting peptide-deleted) versions of these synthases tremendously improved their catalytic activity [56]. In practice, deleting the

plastid transit peptide of linalool synthase from *Mentha citrate* visibly enhanced its catalytic activity [53]. For further identification and modification of enzymes structure, four different active pockets (S14, L28, S43 and S52) of the N-terminal CrGES (geraniol synthase from *Catharanthus roseus*) were truncated separately by a computer application software, with the t3CrGES (S43 truncated version of CrGES) among them having shown the best secondary structural stability, resulted in a 3.46-fold increase over the control strain [57]. In another study, with the help of the Chlorop 1.1 sever, when the codon-optimized *SlNPPS* gene (NPP synthase from *Solanum lycopersicum*) was truncated at three different positions (S45, K51 and C54) in the N-terminus to improve heterologous expression, the obtained NPPS outperformed GPPS in the production of limonene [16].

Guided by protein-protein interaction (PPI) theory, another approach would require using a short peptide linker sequence to accomplish the fusion of two or more enzymes so that the obtained consecutive enzymes with multifunction not only minimize the distance between them to reduce intermediate loss but also accelerate the reaction rate. Optimal linker length and orientation of active sites are key factors to the catalytic activity of the fusion enzymes. For instance, the engineering of five CS-P450c in fusion proteins with different linker lengths by adjusting the repeat number (n) of (GSG)n linker (n = 1–5) led to these fusion proteins having exhibited obvious distinctions between production level, production rate and the overall production ratio [58]. In the production of geraniol in *S. cerevisiae*, with the analysis and distribution of t3CrGES and $Erg20^{F96W-N127W}$ instructed by surface electrostatics, co-expression of the reverse fusion of $Erg20^{F96W-N127W}$/t3CrGES and another copy of $Erg20^{F96W-N127W}$ formed a version enzyme t3CrGES-$Erg20^{F96W-N127W}$+ $Erg20^{F96W-N127W}$ that submitted the highest score in geraniol accumulation [57]. To further enhance the NPP-based orthogonal pathway performance, reconstruction of NPPS must adopt these approaches. The fusion of NPPS and Erg20p reached higher enzyme stability, and the introduction of site-directed mutagenesis in 1,8-cineole synthase elevated its catalytic efficiency and specificity toward NPP [15]. Inspired by these, future efforts could be dedicated to the research of increasing the affinity of NPPS toward the isoprenoid precursors since NPPS was less competent than GPPS in utilizing intermediates such as DMAPP and IPP as revealed by *in vitro* kinetic assays [15]. Besides, protein structural modification can indirectly regulate cell phenotypes to adapt to environmental changes as seen in one example in *S. cerevisiae* where using the truncated version of the Tcb3p (a tricalbin protein) C-terminal helped raise limonene resistance [59]. Overall, protein engineering based on computational intelligence represents a powerful tool to tailor or fuse proteins to improve protein properties, and with the deeper understanding of protein structures and functions available, novel methods such as the introduction of non-natural structural components including unnatural amino acids and non-natural cofactors were made possible for the creation of desired artificial proteins and protein complexes, Moving forward, further creating artificial enzymes with specific functions through *de novo* protein design will be the direction for the development of microbial cell factories.

3.3.3. Cofactor engineering

With the focus being on the reconstruction of the production biosynthetic pathway, the effects of cofactors on strain viability goes unnoticed in current metabolic regulations. These indispensable redox carriers are required to achieve energy transfer in cofactor dependent biosynthetic pathways, specifically, DXR, HDS, and HDR in the MEP pathway, and HMGR in the MVA pathway all use NADPH as the source of reducing power. Beyond altering the intracellular redox state, cofactors play multiple roles in governing the enzyme activity, fine-tuning energy metabolism and regulating carbon flux. Therefore, it has been experimentally demonstrated that an appropriate increment of cofactors could be beneficial to production and cell growth.

Cofactors, typically generated by microbial hosts via the central carbon pathway, have been generally categorized as metal, NADPH/$NADP^+$ (or NADH/NAD^+), ATP/ADP/AMP and vitamin. For instance, the widely studied cofactor, NADPH, is mostly derived from the PP pathway, TCA cycle, ED pathway, as well as the transhydrogenase system between NADH and NADPH [60]. As such, overexpression of genes involved in the PP pathway [61] or TCA [62] is a valuable approach to augment carbon flux from glucose toward NADPH generation. Another strategy implemented to generate excess NADPH in the PP pathway is through the interruption of glycolysis by deleting of *pgi* (encoding phosphoglucose isomerase), *pfkA* and *pfkB* (encoding phosphofructokinases I and II) [23] genes, thus rendering the glucose oxidation cyclization via the glycolytic pathway impossible and forces carbon flux through the PP and ED pathways. However, the inhibition of the cell growth from deleting *pgi* in the EMP pathway concurrently limits productivity due to the release of carbon dioxide via PP pathway [63]. To address this obstacle, the ED pathway was recruited to regenerate NADPH because of the lack of concomitant carbon loss in the process. By heterologously introducing high catalytic activity ED pathway enzymes from *Zymomonas mobilis*, an effective cofactor biosynthesis route was assembled and this attained a 25-fold higher NADPH regeneration rate than its counterpart [64]. Other than these strategies concentrating on metabolic pathways, the turnover between NADPH and NADH should also be carefully optimized through the deletion of *yjgB* (encoding NADPH-dependent aldehyde reductase) to reduce NADPH consumption [65], and overexpression of *Pos5p* (encoding NAD kinase) to facilitate NADPH supply [61]. On the other hand, a sufficient supply of ATP is also essential for the production of terpenoids compounds. The modulation of a single gene in ATP synthesis, of which the process is facilitated by ATP synthase and the electron transfer chain in *E. coli*, has led to a bigger improvement of ATP supply and production yield, as well as increasing metabolic flux towards TCA cycle [62]. Indeed, ATP and NADPH are mutually self-reinforcing, and thus, increasing the ATP content could lead to an increase in the concentration of NADPH as well [66]. Overall, natural metabolic pathway genetic parts have been the focus of current cofactor engineering, so novel techniques could be developed to adapt advanced tailor-made synthetic biology tools by in silico design such as biosensors [67].

3.3.4. Organelle engineering

As large amounts of metabolic reactions occur in the cytoplasm, yeast metabolic engineering for isoprenoids production is largely centered around the rewiring of cytoplasmic metabolic pathways, whereas the unique organelles of mitochondria, peroxisomes, endoplasmic reticulum (ER) in eukaryotes should be further lucubrated. The advantages of suitable physicochemical environments and enough precursors/enzymes make those organelles ideal orthogonal compartments for localizing partial or complete biosynthetic pathways (Fig. 3), which relieves side-pathway competition and inordinately alleviates the cytotoxicity of some hydrophobic products.

As an independent subcellular organelle, the mitochondrial acetyl-CoA pool level is approximated to be 20- to 30-fold higher than that of the cytoplasm [68], and the relatively reducing redox and ATP potential in this organelle tower over that of the cytoplasm as well [69]. To enable higher titers of the product, yeast mitochondria were recruited to translocate the biosynthesis pathway from the cytosol into this organelle. For geraniol production in *S. cerevisiae*, when the biosynthetic pathway was targeted to the mitochondria to prevent the consumption of the precursor GPP by other cytoplasmic side-pathways, a 6-fold higher geraniol production as compared to that of cytosolic producing strains was observed [70]. Similarly, in the linalool production in *S. cerevisiae*, the exploration of the capacity for biosynthesis of isoprenoid within the mitochondria and cytoplasm revealed that the titer of strain co-expressing LIS and the ERG20 mutant in mitochondria was 2.2 times higher than in the cytoplasm [54].

Peroxisome, the main organelle for β-oxidation of fatty acids in yeast, is responsible for generating a pool of acetyl-CoA necessary for the biosynthesis of heterologous natural products. In contrast with mitochondria, the peroxisome could be extensively engineered to establish an orthogonal subcellular compartment because it is not essential for cell viability, thereby suggesting that peroxisomal production could be used as a general strategy for the synthesis of monoterpenoids without inhibiting cell growth. Under semicontinuous fed-batch conditions, when a complete MVA pathway was introduced into the peroxisomes of *S. cerevisiae*, the attained results came close to the industrial and commercial production of 5.5 g/L geraniol and 2.6 g/L d-limonene, with an overall 15- to 125-fold increase over cytosolic [71]. Recently, one prominent study found that squalene overproduction in the cytoplasm of *S. cerevisiae* was distributed in inflated peroxisomes that were swollen along with the production of squalene, thus demonstrating that peroxisomes were not only subcellular compartments for squalene synthesis and but also dynamic depots for the storage of squalene, ultimately accomplishing a 138-fold improvement in squalene titer [72].

ER is a dynamic organelle that involves oxidizing conditions, progressive low PH, protein synthesis and modification [73], especially since it functions via size adjustment in response to an imbalance between the ER protein synthesis load and its folding capacity [74]. By overexpressing a key ER size regulatory factor, INO2. in *S. cerevisiae*, ER expansion supported to improve capacity to synthesize endogenous and heterologous ER-associated proteins and ultimately provided more available space to accommodate them, which in turn, increased the production of squalene and cytochrome p450-mediated protopanaxadiol by 71-fold and 8-fold [74].

Lipid bodies (LB) are organelles in oleaginous yeast that can facilitate the compartmentalization and storage of lipotoxic hydrophobic compounds, such as triacylglycerols[75]. The targeting of lipase dependent heterologous pathway to LBs may lead to more efficient conversion of TAGs [76]. Importantly, the character of size and number variation, similar to the ER, determined that LB is also a dynamic organelle [77]. By using LBs as a storage sink, oleaginous yeast *Y. lipolytica* enabled the gram-scale production of 4 g/L β-carotene [78]. As further analysis of microstructure in cells revealed the relevance between lipid-metabolism engineering and the regulation of LBs size by metabolite accumulation, it was realized that the method of regulating the TAG metabolism could be suitably and effectively applied for high-yield production of lycopene [77].

While organelle engineering in yeast can yield substantial increments over its cytoplasmic counterpart under many circumstances, the production may not always be superior compared to cytoplasmic localization. It was observed in *S. cerevisiae* that the production level of linalool by expressing *LIS* in mitochondria was lower than that of the strain with cytoplasmic expression [54]. Furthermore, there continues to be substantial obstacles that have yet to be overcome. Organelle compartmentalization targeting pathways to small spaces and resulting in flux imbalance, the localization and abundance of proteins accumulating in a particular organelle possibly resulting in growth burden even with expanded organelle numbers and volumes, and the gaps in our understanding of organelle mechanisms are just some to name.

3.4. Engineering strategies to alleviate cytotoxicity

While the production level of *de novo* biosynthesis of terpenoids in microbial cell factory stays elevated in recent years, most are still at the lab-scale. This is often attributed to the above-mentioned that microbes suffered from high toxicity of terpenoids, especially monoterpenes, in vivo bioconversion. For cytotoxic mechanism of monoterpenoids could be basically illustrated as altering cell membrane fluidity, structural or fatty acid compositions [79, 80], impairing cell walls and mitochondria membranes [81]. Moreover, accumulation of intracellular reactive oxygen species (ROS) is identified to be cause of cell death by destroying lipids, proteins, carbohydrates [82, 83] and inducing oxidative DNA damage [84].

3.4.1. Transporter engineering

Mostly, unfavorable feedback responses and growth toxicity is caused by target metabolites accumulation in cytoplasm, thus, for transporter, a membrane protein, isolation target chemicals from cells being an efficient approach to minimize intracellular concentration of metabolites (Fig. 4A). Transporter AcrAB-TolC and ATP-binding cassette (ABC) are two of the most frequently used elements in transporter engineering. The native *E. coli* multidrug efflux pump AcrAB-TolC contributed a lot in targeting multitudinous excretion of metabolites, containing monoterpenoids. According to its function, AcrAB-TolC system is departed into three basic modules: AcrB functions as the inner membrane transporter using ATP to enable export, AcrA functions as the membrane fusion protein complexing with the other proteins to

generate channels, and TolC functions as the outer membrane protein facilitating efflux outside the cell [85]. In practice, AcrR is a transcriptional repressor which was located upstream of the acrAB operon accounting for the management of this pump system, the deletion of *AcrR* could facilitate this efflux pump to transport geraniol to span the double-layer membrane into the media [86]. MarA is employed as the transcriptional activator which is a part of the marRAB operon as well as the *mar* repressor (MarR) and putative inner-membrane protein (MarB) [87]. Overexpression of *marA* successfully enhanced *E. coli* resistance to geraniol by contributing to the AcrABeTolC transporter in geraniol exportation [88]. Since no native transporters of monoterpenoids have been confirmed in *S. cerevisiae*, it is beneficial that introducing a heterologous efflux pump to develop transporting system. Applying the same principle, ABC transporters are composed of four subunits: two cytoplasmic nucleotide-binding domains that consume ATP to activate transportation and two transmembrane domains that link and transport chemical compounds in its supplied migration channel [89]. Typically, the ABC transporter GcABC-G1, using ATP hydrolysis as an energy source, functioned as efflux pumps to enhance the release of (+)-3-carene, d-limonene, and β-pinene [90].

As shown above, the transporter engineering strategy is great in terms of endowing microbes with a strong tolerance to metabolites of interest, but some limitations are still required to be overcome. Chemicals with similar structures could share the same efflux pump, yet the transporter does not work for all hosts even their functions have been identified in certain chassis cells. For instance, eight types of ScABC transporters (Pdr5p, Pdr10p, Pdr15p, Ste6p, Yor1p, Pdr18p, Pdr11p, and Aus1p), drug-responsive transcription factor (Pdr3p), and plasma membrane transporter (Tpo1p) in yeast were picked to assess the effects on transportation of intracellular d-limonene, but only the transporter Pdr5p and Pdr15p were capable of elevating the cells' tolerance capacity toward d-limonene [91]. Regarding substrate specificity, one transporter could not work for all biochemical productions, as exemplified by the failure to provide protection against 1,8-cineole by overexpression of NtPDR1 despite the transporter's ability to enhance the tolerance toward sclareol [92]. Besides, certain monoterpenes may cross the bilayer cell membrane more rapidly via diffusion than bind to transporters for efflux [93]. As a result, instead of improving strain performance, overexpression of particular transporters even has adverse side effects on cell viability [94]. Looking forward, more efforts should be devoted to exploring novel transporters with low toxicity, high transport efficiency and broad substrate specificity in transporter engineering.

3.4.2. Capturing strategies

A rather simple and efficient physical approach towards cytotoxity alleviation of monoterpenes is using an extractive solvent such as diisononyl phthalate, dibutyl phthalate, and dodecane to transport inhibitory products into the organic phase, owing to the hydrophobicity of organic compounds (Fig. 4D). The organic overlay facilitates the monoterpene production by expediting separation to relieve the cells of burden imposed by the metabolites and concurrently reducing loss of monoterpene through evaporation. In *E. coli*, a controllable aqueous-organic two-phase culture system was formed after adding isopropyl myristate to the culture medium for preventing

volatilization of geraniol [47]. Eventually, the engineered strain achieved about 2.0 g/L geraniol. A similar approach using a non-toxic organic phase has been taken in another study, which produced limonene on a gram-scale of up to 3.6 g/L in engineered *E. coli* [95]. Understandably, the organic overlay method was also employed with other microorganisms. In *Sphingobium* sp., the organic phase typically consists of hydrocarbon solvents (e.g., n-decane, n-hexadecane) and was successfully applied to biotransform d-limonene into high concentrations of α-terpineol [96]. Another efficient physical approach to specifically capture monoterpenes is the use of anion exchange resin. When the use of Amberlite resin is combined with higher-level expression of the downstream enzymes (P450 system), perilly alcohol production was increased 2.5-fold in total and about 3.5-fold in specific production [97]. In addition, as numerous monoterpenes have great application value in fragrances due to their volatility, this physical property motivated the application of headspace trapping to aid production. Headspace trapping performed significantly better than the biphasic system in isolating limonene, as its physical characteristics enable it to not mix with other metabolites in the headspace [98]. To conclude, capturing strategies based on physical properties of interested biochemicals have been developed using several distinct extraction mechanisms, and progress of both physical chemistry and material technologies will prompt the development of much more of these convenient and easy extraction tools in prospect.

3.4.3. Tolerance engineering

When the host strain faces certain stress, such as complex growth environment, specific inhibitors or precursor toxicity, the cell will prompt stress response which leads to changes in cell structure and physiological characteristics. Instead of transporting monoterpenoids out of the cell, the starting point and ultimate objective of tolerance engineering is to obtain a strain with better fitness and enhanced tolerance towards the toxic products through modulation of the cellular physiology. One approach is strain evolution, whereby the improved stress tolerance stems from random mutation and the selection pressure under a certain growth environment leads to the natural selection of desired tolerance traits (Fig. 4C). During tolerance engineering, certain traits such as an improved cell phenotype often equates to enhanced fitness of the adapted variant. This was exemplified by a 200-generation evolutionary engineering of *S. cerevisiae* that drastically improved the tolerance of the yeast toward limonene and other monoterpenes, which was conferred by a random mutation that truncated a protein ($tTcb3p^{1-989}$) that preserves cell wall integrity [59]. Most studies on monoterpenoids cytotoxicity mechanisms have basically focused on the modifications in morphology but often overlooked the lethal effect of intracellular reactive oxygen species (ROS) accumulation induced by monoterpenoids. Under oxidative stress, anti-oxidant mechanisms were triggered to generate more antioxidants and antioxidant enzymes [99], upregulate the expression of genes encoding antioxidant enzymes [100], and elevate the accumulation level of NADPH molecules [101]. Acute limonene toxicity in *E. coli* is induced by limonene hydroperoxide. During routine screening, the point mutation L177Q in the protein AhpC encoding alkyl hydroperoxidase was found to alleviate this

toxicity by reducing the hydroperoxide to a more benign compound [102]. In another study, by screening a shot-gun *E. coli* DNA library, RecA (DNA-dependent ATPase) was identified to serve as a regulatory protein to induce the SOS response, which conferred host tolerance to monoterpenoids by promoting homologous recombinational DNA repair towards DNA lesions [103]. Transcriptomics as a novel and effective approach was applied to mine for genes that alleviate limonene toxicity and 8 gene candidates were discovered to improve tolerance of *Y. lipolytica* to exogenously added limonene. Through morphological and cytoplasmic membrane integrity analysis, the underlying mechanism of limonene cytotoxicity to this yeast was elucidated. Additionally, by using short-term adaptive laboratory evolution strategy, fermentation performance of host strains and tolerance to limonene was improved [104]. Although tolerance engineering performed well to improve monoterpenoids titer, the specific cytotoxicity mechanisms of most monoterpenoids still remain enigmatic which hiders further development. For instance, there still lies controversy over whether the monoterpenoids cytotoxicity impacts on the plasma membrane [105] or cell wall [106]. Nevertheless, although conventional tolerance engineering counts on time-consuming and labour-intensive isolation and random mutagenesis of natural microbes, much progress has been made from loads of novel insights into growth kinetics and metabolism of microbes. With the ongoing rapid development of next-generation sequencing platforms, data science technologies will further mine unknown genes or loci correlated with specific phenotypes, thus great advances in tolerance engineering will be imminent with this technique.

3.4.4. Cell free biosynthesis engineering

Without the hindrance of cell viability and substrate competition, cell free biosynthesis (CFB) systems has been adopt as a novel powerful approach to chemical transformations that harbours a much larger potential to reaching monoterpenoids industrial production. In contrast to classical metabolic engineering strategies, CFB systems, which simply mixes the required enzymes, substrates and cofactors together in a reaction vessel (Fig. 4B), are easy apply for exploring a novel biosynthetic pathway incorporating with multiple enzymes derived from various organisms, and avoid dispatching energy and carbon resources to keep cell growth. Moreover, the challenge of the comparative pathway in the host is also alleviated by circumventing competing byproduct pathways. Importantly, the CFB system resolves the main limiting factor of product and precursor toxicity.

CFB has been demonstrated for monoterpene production using a stable CBF platform that comprised 27 enzymes and generated both NAD(P)H and ATP in an optimized monoterpene biosynthesis pathway. Ultimately, high yields and titers were achieved for limonene (12.5 ± 0.3 g/L) and pinene (14.9 ± 0.6 g/L), which illustrates the immense potential in further expanding the number and diversity of monoterpenes products that can be biosynthesized by CFB [107]. Although *in vitro* systems based on purified enzymes could be kept stable and active, their rate of monoterpene production still could not

satisfy the requirements of industrial production because the lifespan of the engineered enzymes is short, resulting in a high production cost. To minimize the cost of enzyme purification and cofactor supplementation, an alternative approach using a cell lysate-based system was proposed recently. This approach utilized enriched cell lysates, which comprised endogenous metabolic enzymes, to regenerate NADH and produce limonene, resulting in a productivity of 3.8 mg/L/h [108]. Furthermore, a cell-free metabolic engineering model was established recently for obtaining biosynthetic enzymes with *in vitro* prototyping and rapid optimization at high-throughput, further promoting the performance of biosynthetic pathway design and optimization [109]. To accelerate the development of CFB systems, increasing number of metabolic network models have been published recently, propelled by rapidly expanding biological databases that contain enzymatic reaction data for a continuously growing number of organisms [110]. Taken together, CFB systems will provide an indispensable driving force in the syntheses of biochemicals in the foreseeable future.

3.5. Fermentation optimization

Fermentation engineering has been explored as an avenue to improve biomass density and productivity for engineered microbes to perform optimally over the full course of fermentation. The production bioprocess is extremely complicated and easily influenced by fermentation conditions such as temperature, pH, rotation speed, oxygen and additives. The type of carbon and nitrogen sources in culture medium can also significantly affect cellular health. It is known that optimal carbon/nitrogen ratio could strikingly strengthen the viability of cells [111], and a straight substitution of glycerol for glucose was shown to bring about higher limonene formation rates, a prolonged growth phase, and enhanced stability compared to the same whole-cell biocatalyst growing on glucose [112]. More importantly, the yield of monoterpenes can be dramatically increased by adding auxiliary carbon sources. For instance, an engineered *Y. lipolytica* using glycerol as a main carbon source and citrate as auxiliary carbon source reached 165.3 mg/L limonene production during by fed-batch cultivation in a 1.5-L bioreactor [113]. Analogously, with the addition of pyruvate and mevalonolactone as auxiliary carbon sources to the medium, along with an increased cell growth, the titer of linalool was 1.8 times higher than that of the control group [54]. However, as the amount of auxiliary carbon sources rise, the titers, productivities, and yields of monoterpenoids do not increase and may even decrease sometimes, implying that excessive pathway intermediates will disrupt intracellular homeostasis. Other than auxiliary carbon sources, the addition of metal ions and vitamin was proved to deliver enhanced fermentation performance of microorganisms, as increased availability of cofactors is an essential precondition for enzymes to function well. In *Y. lipolytica*, supplementing an additional 0.2% of $MgSO_4 \cdot 7H_2O$ was proved to be beneficial for limonene production [114]. In *Candida glabrata*, thiamine improved the activity of PDH to channel the carbon flux from pyruvate to the TCA cycle, and significantly improved the cell growth of the strains [115]. Notably, from the perspective of economics and environmental protection, the approach of simultaneously degrading low-cost materials like waste cooking oils (WCO) to acquire high value-added products is highly attractive in the

context of bioremediating of the large quantity of WCO being improperly disposed [116]. By this means, the utilization of WCO as sole carbon for production of limonene was carried out in *Y. lipolytica*, resulting in approximately 11% and 16% increase in titers of d- and l-limonene, respectively, as compared to those obtained using glucose as carbon source. This work represents a major breakthrough to waste conversion and in the biochemical production industry [114].

4. Conclusion and Perspectives

The development of efficient microbial cell factories for producing natural products is a systematic project that demands careful considerations from multiple perspectives. Conventional approaches in metabolic engineering aim to increase production titers by enhancing the biosynthesis activity along an intended priori metabolic route. The lack of catalytically efficient enzymes and effective genetic engineering tools are significant hurdles that need to be overcome before cell factories can be reliably engineered. Recently, systems metabolic engineering has proven useful in circumventing the abovementioned issues. Through the integration of metabolic engineering, systems biology and synthetic biology, the molecular mechanisms underlying complex metabolic processes can be rapidly deconvoluted, thus enabling just-in-time gene expression for the fine tuning of metabolic fluxes. Potentially, fermentative processes can be monitored and evaluated real-time by process analytical tools that is coupled to machine learning algorithm to identify metabolic bottlenecks and improve production titers. Finally, advanced genetic tools like CRISPR system for synthetic modules reconstitution and novel big data-assisted cloud computing for enzymes identification will accelerate the scale-up process of commercial monoterpenoid production.

Declarations

Ethics approval and consent to participate

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors read and approved the final manuscript. All authors give consent to publish the review in Natural Product Reports.

Availability of data and material

All relevant data generated or analyzed during this study were included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The Natural Science Foundation of Tianjin, China (17JCYBJC40800), the Research Foundation of Tianjin Municipal Education Commission, China (2017ZD03), the Innovative Research Team of Tianjin Municipal Education Commission, China (TD13-5013), Tianjin Municipal Science and Technology Project (18PTSYJC00140, 19PTSYJC00060), Startup Fund for ‘Haihe Young Scholars’ of Tianjin University of Science and Technology, the Thousand Young Talents Program of Tianjin, China, the

Synthetic Biology R&D Programme (SBP-P2, SBP-P7, SBP-P9) of the National Research Foundation of Singapore, Ministry of Education, Singapore (R-MOE-A401-C001, R-MOE-E103-E001).

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Acknowledgements

This work was supported by the Natural Science Foundation of Tianjin, China (17JCYBJC40800), the Research Foundation of Tianjin Municipal Education Commission, China (2017ZD03), the Innovative Research Team of Tianjin Municipal Education Commission, China (TD13-5013), Tianjin Municipal Science and Technology Project (18PTSYJC00140, 19PTSYJC00060), Startup Fund for 'Haihe Young Scholars' of Tianjin University of Science and Technology, the Thousand Young Talents Program of Tianjin, China. JLF is supported by the Synthetic Biology R&D Programme (SBP-P2, SBP-P7, SBP-P9) of the National Research Foundation of Singapore. AW is supported by Ministry of Education, Singapore (R-MOE-A401-C001, R-MOE-E103-E001).

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Table 1

Exploration of microbes as cell factories for the production of monoterpenoids

Parental strain	Monoterpenoid	Engineering strategy	Fermentation condition	Carbon Source	Titer	Yield	Productivity	Reference
E. coli BLR (DE3)	L-limonene	*MstlLS*↑, *AgtGPPS*↑	Batch shake flask	Luria-Bertani medium (IPP and DMAPP were added as co-substrates)	5 mg/L	-	0.21 mg/L/h	117
E. coli DH1	L-limonene	*MstlLS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, *AgtGPPS*↑	Batch shake flask	Glucose	435 mg/L	43.5 mg/g	6.04 mg/L/h	97
E. coli DH1	L-limonene	*MstlLS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, $IspA^{S81F}$↑	Batch shake flask	Glucose	214 mg/L	21.4 mg/g	4.46 mg/L/h	53
E. coli BL21 (DE3)	L-limonene	*MstlLS*↑, *atoB*↑, *ScHMGS*↑, *SctHMGR*↑, *ScERG12*↑, *ScERG8*↑,	Fed-batch bioreactor	Glucose	345 mg/L	0.46 mg/g	7.67 mg/L/h	112

		ScERG19↑, *idi*↑, *AgtGPPS*↑						
E. coli BL21 (DE3)	L-limonene	*MstlLS*↑, *atoB*↑, *ScHMGS*↑, *SctHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, *AgtGPPS*↑	Fed-batch bioreactor	Glycerol	1350 mg/L	1.81 mg/g	30 mg/L/h	112
E. coli BW25113	L-limonene	*MslLS*↑, *EfmvaE*↑, *EfHMGS*↑, *MmERG12*↑, *ScERG8*↑, *ScERG19*↑, *ScIDI*↑, *SltNDPS1*↑	Fed-batch shake flask	Glucose	1290 mg/L	64.76 mg/g	15.36 mg/L/h	16
E. coli BL21 (DE3)	L-limonene	*MstlLS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, *AgtGPPS*↑	Fed-batch bioreactor	Glycerol	3630 mg/L	-	151.25 mg/L/h	95
S. cerevisiae EPY210C	D-limonene	*CltdLS*↑, *tHMGR*↑, *UPC2-1*↑	Batch shake flask	Glucose/ galactose mixture	1.48 mg/L	-	0.01 mg/L/h	118
S. cerevisiae CEN.PK2-1C	D-limonene	*CltdLS*↑, *EfmvaE*↑, *EfHMGS*↑, $HMGR^{K6R}$↑,	Batch shake flask	Glucose	76 mg/L	3.8 mg/g	0.79 mg/L/h	55

		ERG12↑, *ERG8*↑, *ERG19*↑, *IDI*↑, *ERG20*^{N127W}↑, *ERG20*↓						
S. cerevisiae yJGZ1	D-limonene	*tHMGR*↑, *IDI*↑, *ERG20*↑, *SlNDPS1*↑, *CltdLS2*↑	Fed-batch flask	Glucose/ ethanol mixture	917.7 mg/L	-	8.29 mg/L/h	17
Anabaena sp. PCC 7120	L-limonene	*PstlLS*↑, *Ecdxs*↑, IDI ↑, GPPS ↑	Batch shake flask	CO_2	0.52 mg/L	-	0.0018 mg/L/h	119
Synechococcus elongatus PCC 7942	Limonene	*MsLS*↑ *AgGPPS*↑ *BbDXS*↑	flask	CO_2	-	-	885.1 µg/L/OD/d	120
Y. lipolytica Po1f	D-limonene	*ArtdLS*↑ *SltNPPS*↑, *ERG12*↑, *HMGR*↑	Batch shake flask	Glucose/ pyruvic acid mixture	23.56 mg/L	-	0.33 mg/L/h	18
Y. lipolytica Po1f	D-limonene	*ArtdLS*↑ *SltNPPS*↑, *ERG12*↑, *HMGR*↑	Fed-batch bioreactor	Glucose/ citric acid mixture	165.3 mg/L	-	1.15 mg/L/h	113
Y. lipolytica Po1g KU70Δ	D-limonene	*CldLS*↑, *HMGR*↑	Fed-batch shake flask	Waste cooking oil	2.51 mg/L	-	0.02 mg/L/h	114
Y. lipolytica Po1g KU70Δ	L-limonene	*MslLS*↑, *HMGR*↑	Fed-batch shake flask	Waste cooking oil	2.72 mg/L	-	0.02 mg/L/h	114
Y. lipolytica ATCC 20460	Limonene	*PfLS*↑, *HMGR*↑, *ERG12*↑,	Batch shake glass tube	Glucose	35.9 mg/L	0.45 mg/g	0.50 mg/L/h	121

		IDI↑, *ERG20*$^{F88W-N119W}$↑, *ACL1*↑, *SeACS*↑, *SQS*↓						
E. coli DH1	L-linalool	*MctlLIS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, *IspA*^{S81F}↑	Batch shake flask	Glucose	505 mg/L	50.5 mg/g	10.52 mg/L/h	53
S. cerevisiae CEN.PK2-1C	Linalool	*AcNES1*↑, *EfmvaE*↑, *EfHMGS*↑, *HMGR*^{K6R}↑, *ERG12*↑, *ERG8*↑, *ERG19*↑, *IDI*↑, *ERG20*^{N127W}↑, *ERG20*↓	Batch shake flask	Glucose	18 mg/L	0.9 mg/g	0.25 mg/L/h	55
S. cerevisiae BY4742	L-linalool	*ColLIS*↑, *mColLIS*↑, *ERG20*$^{F96W-N127W}$↑, *mERG20*$^{F96W-N127W}$↑, *tHMGR*↑, *IDI*↑, *ERG20*↓, The MVA pathway genes (*ERG10*, *HMGS*, *tHMGR*, *ERG12*, *ERG8*, *ERG19* and *IDI*) were re-localized	Batch bioreactor	Glucose/mevalonolactone mixture	23.45 mg/L	-	0.33 mg/L/h	54

		from the cytoplasm to the mitochondrial compartment						
Y. lipolytica Po1f	L-linalool	*AalLIS*↑, *ERG20*$^{F88W-N119W}$↑, *HMGR*↑, *IDI*↑	Batch shake flask	Citric acid/ pyruvic acid mixture	6.96 mg/L	-	0.15 mg/L/h	52
E. coli BL21 (DE3)	Geraniol	*ObGES*↑, *AgGPPS*↑, *EfMvaE*↑, *EfMvaS*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *ScIDI*↑, *aes*↑	Fed-batch bioreactor	Glucose/geranyl acetate mixture	2.0 g/L	-	28.57 mg/L/h	47
S. cerevisiae CEN.PK2-1C	Geraniol	*CrtGES*/*ERG20*$^{F96W-N127W}$↑, *ERG20*$^{F96W-N127W}$↑, *tHMGR*↑, *IDI*↑	Fed-batch bioreactor	Glucose/ethanol mixture	1.68 g/L	-	14 mg/L/h	57
S. cerevisiae CEN.PK2-1C	Geraniol	*CltdLS*↑, *EfmvaE*↑, *EfHMGS*↑, *HMGR*^{K6R}↑, *ERG12*↑, *ERG8*↑, *ERG19*↑, *IDI*↑, *ERG20*^{N127W}↑, *ERG20*↓	Batch shake Flask	Glucose	27 mg/L	1.35 mg/g	0.28 mg/L/h	55
E. coli DH1	Perillyl alcohol	*MHahpGHI*↑, *MstlLS*↑, *AgtGPPS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑,	Batch shake flask	Glucose	105 mg/L	10.5 mg/g	2.18 mg/L/h	97

		ScERG8↑, *ScERG19*↑, *idi*↑						
E. coli DH1	1,8-Cineole	*ScCS*↑, *atoB*↑, *SaHMGS*↑, *SaHMGR*↑, *ScERG12*↑, *ScERG8*↑, *ScERG19*↑, *idi*↑, *ispA*^{S81F}↑	Batch shake flask	Glucose	653 mg/L	65.3 mg/g	13.6 mg/L/h	53
E. coli DH1	Hydroxycineole	*ScCS*↑, *CbP450cin*↑, engineering a fusion of *ScCS with CbP450cin*	Batch shake tube	Glucose	56 mg/L	5.6 mg/g	0.12 mg/L/h	58

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↑ Gene overexpression; ↓ Gene knockdown; Δ Gene knockout; / Gene fusion expression; ***MstlLS*** encodes a truncated version of *Mentha spicata* l-limonene synthase; ***AgtGPPS*** encodes a truncated version of *Abies grandis* geranyl diphosphate synthase; ***atoB*** encodes the endogenous acetoacetyl-CoA synthase; ***SaHMGS*** encodes *Staphylococcus aureus* hydroxymethylglutaryl-CoA synthase; ***SaHMGR*** encodes *S. aureus* hydroxymethylglutaryl-CoA reductase; ***ScERG12*** encodes *S. cerevisiae* mevalonate kinase; ***ScERG8*** encodes *S. cerevisiae* phosphomevalonate kinase; ***ScERG19*** encodes *S. cerevisiae* diphosphomevalonate decarboxylase; ***idi*** encodes the endogenous isopentyl diphosphate isomerase; ***IspA^{S81F}*** encodes a mutated version of *E. coli* FPP synthase (the enzyme IspA); ***ScHMGS*** encodes *S. cerevisiae* hydroxymethylglutaryl-CoA synthase; ***SctHMGR*** encodes a truncated version of *S. cerevisiae* hydroxymethylglutaryl-CoA reductase; ***MslLS*** encodes *M. spicata* l-limonene synthase; ***EfmvaE*** encodes *Enterococcus faecalis* acetoacetyl-CoA synthase/hydroxymethylglutaryl-CoA reductase; ***EfHMGS*** encodes *E. faecalis* hydroxymethylglutaryl-CoA synthase; ***MmERG12*** encodes *Methanosarcina mazei* mevalonate kinase; ***ScIDI*** encodes *S. cerevisiae* isopentyl diphosphate isomerase; ***SltNDPS1*** encodes a truncated version of *S. lycopersicum* neryl diphosphate synthase; ***CltdLS*** encodes a truncated version of *Citrus limon* d-limonene synthase; ***tHMGR*** encodes a mutated version of *S. cerevisiae* hydroxymethylglutaryl-CoA reductase; ***UPC2-1*** encodes a global transcription factor involved in upregulation of sterol biosynthesis in *S. cerevisiae*; ***HMGR^{K6R}*** encodes a mutated version of *S. cerevisiae* hydroxymethylglutaryl-CoA reductase; ***ERG12*** encodes the endogenous mevalonate kinase; ***ERG8*** encodes the endogenous phosphomevalonate kinase; ***ERG19*** encodes the endogenous diphosphomevalonate decarboxylase; ***IDI*** encodes *S. cerevisiae* isopentyl diphosphate isomerase; ***ERG20^{N127W}*** encodes a mutated version of *S. cerevisiae* geranyl/farnesyl diphosphate synthase (the enzyme Erg20p); ***ERG20*** encodes the endogenous geranyl/farnesyl diphosphate synthase (the enzyme Erg20p); ***SlNDPS1*** encodes *S. lycopersicum* neryl diphosphate synthase; ***CltdLS2*** encodes a truncated version of *C. limon* d-limonene synthase; ***PstlLS*** encodes a truncated version of *Picea sitchensis* l-limonene synthase; ***Ecdxs*** encodes gene from *E. coli*; ***IDI*** encodes gene from *Haematococcus pluvialis*; ***GPPS*** encodes gene from *Mycoplasma tuberculosis*; ***MsLS*** encodes gene from *spearmint (M. spicata)*; ***AgGPPS*** encodes gene from a *fir (A. grandis)*; ***Bb*DXS** encodes gene from *Botryococcus braunii*; ***ArtdLS*** encodes a truncated version of *Agastache rugosa* d-limonene synthase; ***HMGR*** encodes the endogenous hydroxymethylglutaryl-CoA reductase; ***CldLS*** encodes *C. limon* d-limonene synthase; ***MslLS*** encodes *M. spicata* l-limonene synthase; ***PfLS*** encodes *Perilla frutescens* limonene synthase; ***IDI*** encodes the endogenous isopentyl diphosphate isomerase; ***ERG20$^{F88W\text{-}N119W}$*** encodes a mutated version of *Y. lipolytica* geranyl/farnesyl diphosphate synthase (the enzyme Erg20p); ***ACL1*** encodes the endogenous ATP citrate lyase; ***SeACS*** encodes *Salmonella enterica* acetyl-CoA synthetase; ***SQS*** encodes the endogenous squalene synthase; ***MctlLIS*** encodes a truncated version of *M. citrate* l-linalool synthase; ***AcNES1*** encodes *Actinidia chinensis* trans-nerolidol synthase; ***ColLIS*** encodes *Cinnamomum osmophloeum* l-linalool synthase; ***mColLIS*** encodes mitochondrial-targeted *C. osmophloeum* l-linalool

synthase; ***ERG20$^{F96W-N127W}$*** encodes a mutated version of *S. cerevisiae* geranyl/farnesyl diphosphate synthase (the enzyme Erg20p); ***mERG20$^{F96W-N127W}$*** encodes mitochondrial-targeted and mutated version of *S. cerevisiae* geranyl/farnesyl diphosphate synthase (the enzyme Erg20p); ***ERG20*** encodes the endogenous geranyl/farnesyl diphosphate synthase; ***ERG10*** encodes the endogenous acetoacetyl-CoA synthase; ***HMGS*** encodes the endogenous hydroxymethylglutaryl-CoA synthase; ***ERG8*** encodes the endogenous phosphomevalonate kinase; ***ERG19*** encodes the endogenous diphosphomevalonate decarboxylase; ***AalLIS*** encodes *Actinidia arguta* l-linalool synthase; ***ObGES*** encodes *Ocimum basilicum* geraniol synthase; ***AgGPPS*** encodes *A. grandis* geranyl diphosphate synthase; ***EfMvaE*** encodes *E. faecalis* acetoacetyl-CoA synthase/hydroxymethylglutaryl-CoA reductase; ***EfMvaS*** encodes *E. faecalis* hydroxymethylglutaryl-CoA synthase; ***ScIDI*** encodes *S. cerevisiae* isopentyl diphosphate isomerase; ***aes*** encodes the endogenous acetylesterase; ***CrtGES*** encodes a truncated version of *C. roseus* geraniol synthase; ***MHahpGHI*** encodes *Mycobacterium* HXN-1500 P450 enzyme system; ***ScCS*** encodes *Streptomyces clavuligerus* 1,8-cineole synthase; ***CbP450cin*** encodes *Citrobacter braakii* P450 enzyme.

Figure captions

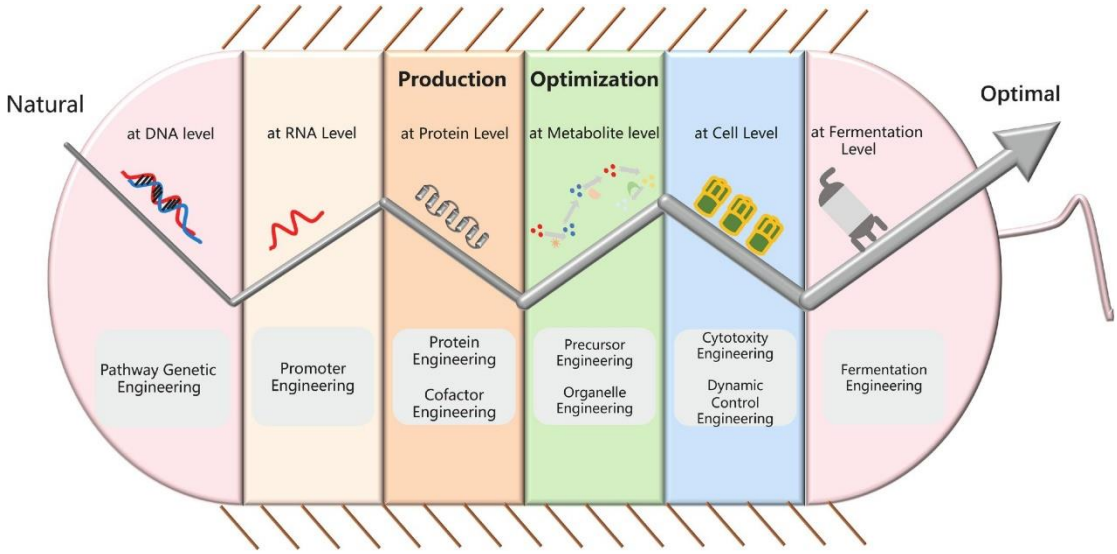

Figure 1. Overview of metabolic engineering strategies for monoterpenoid production. Microbial factories have been systematically engineered at DNA level, RNA level, protein level, metabolite level, cell level, fermentation level to efficiently synthesize high-value-added monoterpenoids by powerful metabolic engineering tools that were summarized and classified as pathway genetic engineering, promoter engineering, protein engineering, cofactor engineering, precursor engineering, organelle engineering, cytotoxity engineering, dynamic control engineering, fermentation engineering based on methods and techniques.

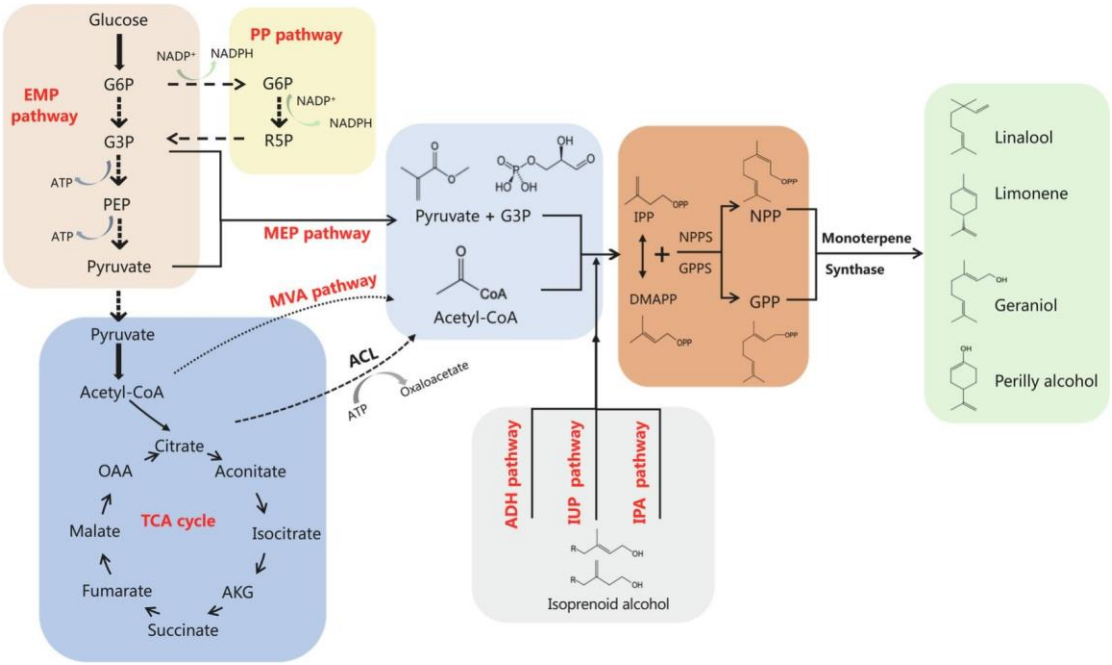

Figure 2. General biosynthesis pathways of plant natural monoterpenoid products. These include central carbon metabolism pathways, MVA/MEP pathways and artificial isoprenoid pathways. Embden–Meyerhof–Parnas pathway (EMP), Pentose Phosphate pathway (PP), mevalonate pathway (MVA), 1-deoxy-Dxylulose-5-phosphate pathway (MEP), tricarboxylic acid cycle (TCA). Alcohol-dependent hemiterpene pathway (ADH), isopentenol utilization pathway (IUP) and isoprenoid alcohol pathway (IPA) are artificially designed. G6P, glyceraldehyde-6-phosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; R5P, Ribulose-5-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl disphosphate; NPP, neryl diphosphate; ACL, ATP citrate lyase; NPPS, neryl diphosphate synthase; GPPS, geranyl diphosphate synthase.

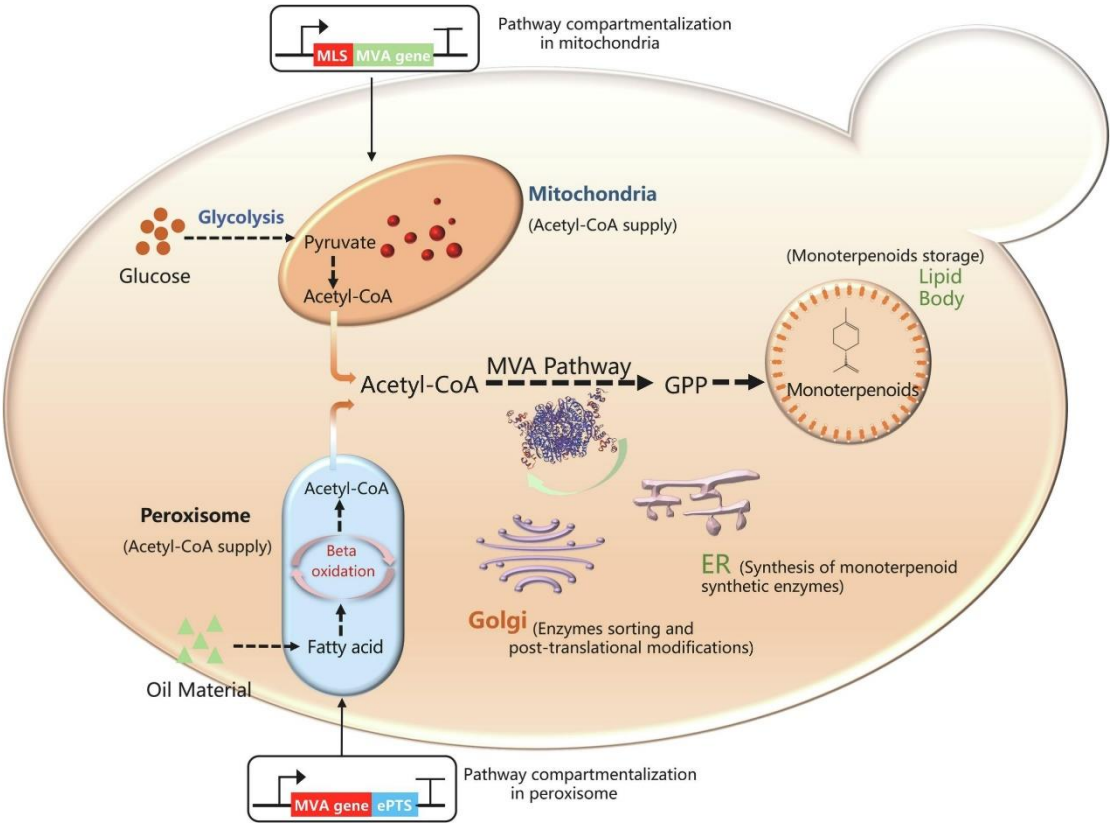

Figure 3. Overview of yeast organell engineering. Organell engineering is a direct approach to modify organelles for producing targeted chemicals in eukaryotic cells, because the unique microenvironment of subcellular organelle provides favorable conditions for various metabolic reactions, which can reduce intermediates waste, improve the efficiency of the biosynthetic pathway enzymes, moreover relieve the toxicity from monoterpenoids.

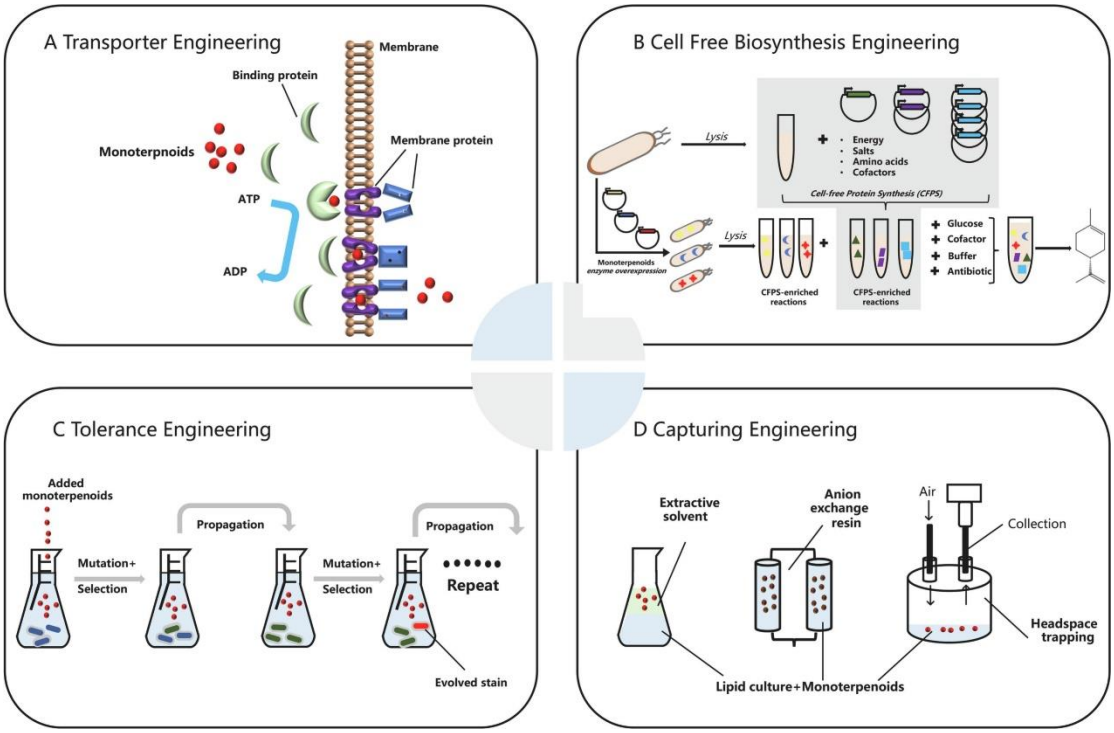

Figure 4. Overview of cytotoxicity engineering. (A) Transporter engineering is used for targeting chemicals excretion. (B) Cell free biosynthesis engineering is utilized for constructing cell free system to alleviate the toxicity of monoterpenoids toward host strains. (C) Tolerance engineering is adopted for improving resistance to monoterpenoids and fermentation performance of host strains. (D) Capturing strategies are applied for using physical approach to sequester target monoterpenoids.