Adv Biochem Eng Biotechnol
DOI: 10.1007/10_2014_284

Enabling Technologies to Advance Microbial Isoprenoid Production

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Abstract Microbial production of isoprenoids provides an attractive alternative to biomass extraction and chemical synthesis. Although widespread research aims for isoprenoid biosynthesis, it is still in its infancy in terms of delivering commercial products. Large barriers remain in realizing a cost-competitive process, for example, developing an optimal microbial cell factory. Here, we summarize the many tools and methods that have been developed in the metabolic engineering of isoprenoid production, with the advent of systems biology and synthetic biology, and discuss how these technologies advance to accelerate the design–build–test engineering cycle to obtain optimum microbial systems. It is anticipated that innovative combinations of new and existing technologies will continue to emerge, which will enable further development of microbial cell factories for commercial isoprenoid production.

Keywords Terpenoids · Sesquiterpenes · Metabolic engineering · Enabling technologies · *E. coli* · *S. cerevisiae*

Contents

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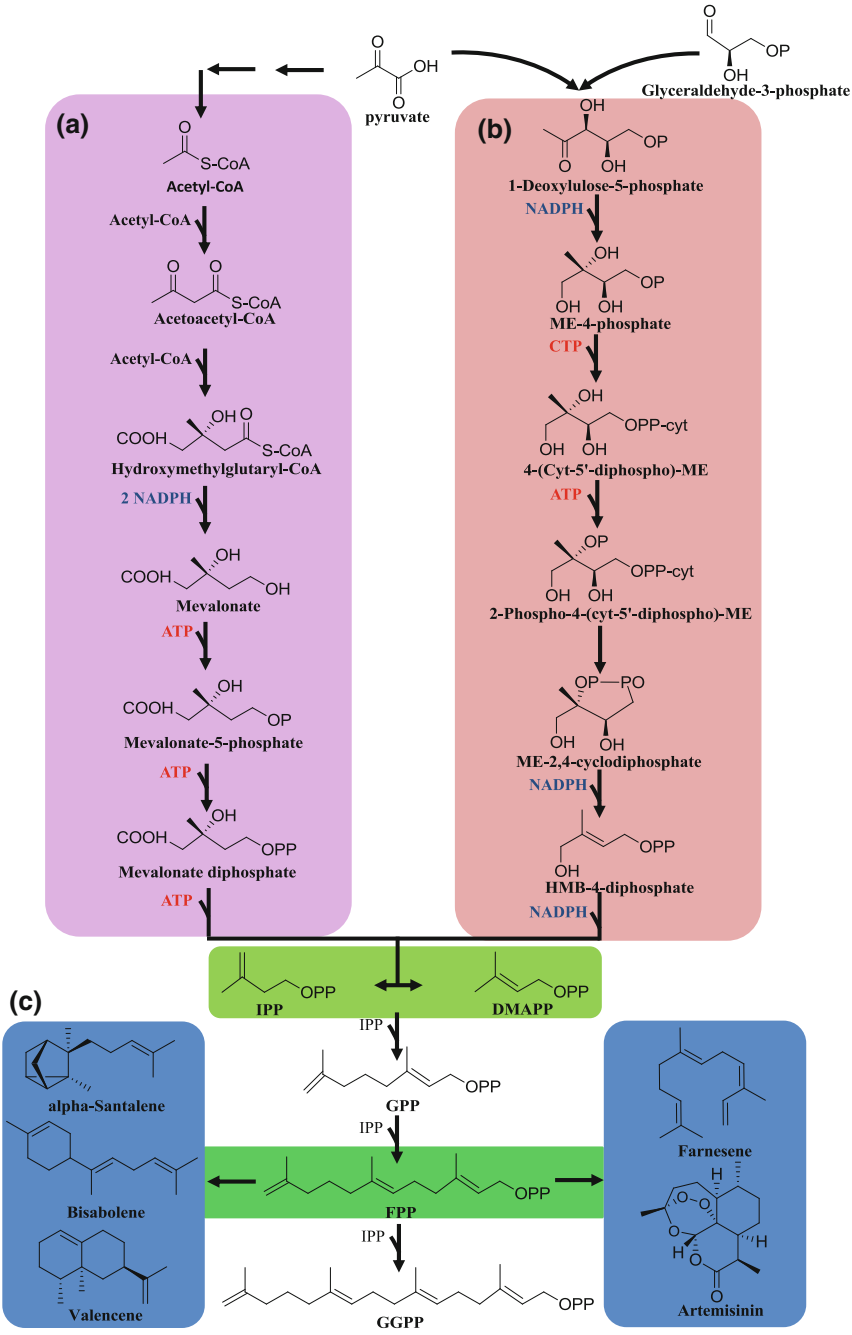
1 Introduction

Isoprenoids constitute a large and diverse group of natural products, encompassing more than 40,000 structures known thus far [1]. This extremely diverse array of chemical structures is probably a reflection of their many varied biological functions, which have great attraction for traditional and modern human exploitation, for instance, as pharmaceuticals, flavors, fragrances, dietary supplements, food ingredients, biomaterials, and biofuels [2, 3]. From environmental and economic concerns, microbial production of isoprenoids has gained more and more attention as most isoprenoids are present only in low quantities in their natural plant resources and are structurally complex, making it difficult to produce them by chemical synthesis.

All isoprenoids are derived from the universal C_5 monomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP; Fig. 1). Two distinct biosynthetic pathways, the mevalonate (MVA) pathway (Fig. 1a) and the methylerythritol phosphate (MEP) pathway (Fig. 1b) have evolved for the biosynthesis of these two vital building blocks [2, 4]. Condensations of DMAPP with one or more molecules of IPP result in the linear prenyl diphosphate precursors geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), as well as larger units. These skeletons normally pass through a series of reactions, such as cyclization, methylation, acetylation, phenylation, and rearrangements to form a diversity of isoprenoid families.

Owing to the excellent progress in metabolic engineering with the advent of synthetic biology and systems biology, much success has been seen in the field of microbial production of isoprenoids. The production of artemisinic acid, a chemical precursor for the production of the important antimalarial drug artemisinin, represents a milestone achievement in this area [5]. However, microbial production of most other isoprenoids is still far behind and will require further development before they reach the commercial stage. One of the key factors is that construction and improvements of microbial cell factories are time consuming and need massive endeavors. Herein, we attempt to cover the recent developments in metabolic engineering of bacterial and yeast microbial platform strains for isoprenoid production with a focus on applied technologies.


(a)
(b)
(c)
pyruvate
Glyceraldehyde-3-phosphate
Acetyl-CoA
Acetoacetyl-CoA
Hydroxymethylglutaryl-CoA
2 NADPH
Mevalonate
ATP
Mevalonate-5-phosphate
Mevalonate diphosphate
1-Deoxylulose-5-phosphate
NADPH
ME-4-phosphate
CTP
4-(Cyt-5'-diphospho)-ME
2-Phospho-4-(cyt-5'-diphospho)-ME
ME-2,4-cyclodiphosphate
HMB-4-diphosphate
IPP
DMAPP
GPP
FPP
GGPP
alpha-Santalene
Bisabolene
Valencene
Farnesene
Artemisinin

◀**Fig. 1** Biosynthesis of isoprenoids. Complementary routes: **a** the mevalonate (MVA) pathway and **b** the methylerythritol phosphate (MEP) pathway are responsible for the biosynthesis of two vital precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Condensations of DMAPP with one or more molecules of IPP result in the immediate precursor geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), from which various functional isoprenoids are formed. **c** The concept of cell factory platform is simplified as illustrated. A strain with efficient provision of FPP precursor can be easily adapted to produce a range of different sesquiterpenes

2 Component Discovery

Gene discovery and mechanism elucidation in isoprenoid biosynthesis not only facilitates functional studies in the plant, but also represents the prerequisite for engineering a microbial cell factory for production of the compound. Though more and more active compounds are discovered and their biological functions are well-characterized, determination of their biosynthetic pathways lags far behind. For example, the efficient anticancer terpenoid Taxol was first isolated from the bark of the Pacific yew, *Taxus brevifolia* Nutt in 1971. However, to date its biosynthetic pathway has not been completely elucidated [6]. Cloning of genes differentially expressed under production compared to control conditions is a common strategy for identifying the biosynthesis genes in natural production hosts [7, 8]. Evaluation of sequence similarity can further assist in identification of terpene synthases as the first step of diversified isoprenoid biosynthesis [9]. However, the high substrate specificity and low sequence homology of downstream modifying enzymes such as cytochrome P450s makes their identification and characterization extremely challenging. Fortunately, recent rapid progress in functional genomics sequencing [10] as well as differential transcriptome sequencing [11] has enabled faster identification of related candidates. Of course, functional determination strongly relies on in vitro and/or biochemical analysis [11, 12], but functional genomics has enabled significant progress and been helpful in narrowing down the candidate genes and accelerating the procedure of gene identification.

Sometimes, knowledge gaps in target compound biosynthesis will hinder the reconstruction of the entire heterologous biosynthetic pathway. Fortunately, directed evolution provides a feasible strategy to modify an enzyme towards a specific function [13]. For instance, evolution of a methyl ketone-preferring aminotransferase towards acceptance of prositagliptin ketone as substrate enabled establishing a bioprocess for sitagliptin production by increasing the substrate binding pocket [14]. Recently, exciting studies showed that the cytochrome P450 P450BM3 from *Bacillus megaterium* could be evolved towards catalyzing a carbene transfer reaction for olefin cyclopropanation, rather than its natural monooxygenation [15, 16]. Directed evolution not only produces enzymes with novel specific activities, but may also increase catalytic efficiency. For example, directed evolution of a sesquiterpene cadinene synthase led to increased terpene–

synthase–cyclization activity [17]. Here, an efficient high-throughput screening strategy is essential for rapid enzyme selection during the evolution procedure.

Exploiting enzyme catalytic promiscuity, such as condition promiscuity, substrate promiscuity, and catalytic promiscuity, provides another possibility of gap repairing for constructing artificial synthesis pathways that are currently not available [18]. For example, a substrate-promiscuous P450BM3 from *B. megaterium* was engineered and incorporated into the semibiosynthetic route for artemisinin production in *Escherichia coli*, which enabled efficient conversion of its precursor amorphadiene to artemisinic-11S,12-epoxide, at titers of 250 mg/L [19]. Similarly, a D-lactate dehydrogenase (encoded by *d-ldh* from *Lactobacillus pentosus*) and a hydroxylase complex (encoded by *hpaBC* from *E. coli*) were shown to have enough promiscuity to catalyze the consecutive reactions for salvianic acid A production from 4-hydroxyphenylpyruvate in *E. coli* [20].

3 Cell Factory Construction

3.1 Host Selection

Saccharomyces cerevisiae and *E. coli* are commonly used hosts for isoprenoid production due to the well-characterized genetic background and excellent accessibility of molecular techniques. *E. coli* showed great performance in terpene production due to its fast growth [21, 22]. However, the biosynthesis of many functional terpenoids, especially phytoterpenoids, involves several modification steps catalyzed by complex enzymes including membrane-bound cytochrome P450 enzymes, which can be difficult to establish in bacteria including *E. coli* due to differences in translation and membrane structure. Though engineering of these enzymes can be helpful for functional expression in *E. coli*, their catalytic efficiency usually remains low. Actually, CYP71AV1 expressing *E. coli* just produced artemisinic acid at a titer of 105 mg/L even though CYP71AV1 expression was systematically optimized including codon-optimization, N-terminal modification, and reductase balancing [23], whereas the corresponding engineered *S. cerevisiae* produced artemisinic acid at up to 25 g/L [5]. Alternative prokaryotic hosts such as *Corynebacterium glutamicum* [24] and *Bacillus subtilis* [25] have also been engineered for isoprenoid production, but with relatively low efficiency. To date, the *S. cerevisiae* has been showing good performance in producing a number of functional isoprenoids including artemisinic acid [5], tanshinone precursor [11] and ginsenosides [26]. Nevertheless, its endogenous MVA pathway awaits further improvement for high-level production. The red yeast *Rhodosporidium toruloides*, which has efficient lipid biosynthesis, is well suited for isoprenoid biosynthesis, and its genome, transcriptome, and proteome have been systematically analyzed [27], which makes it a potential efficient host for functional isoprenoid production after establishing a genetic manipulation platform.

3.2 Genetic Implementation

Rapid and efficient DNA assembly and genome engineering are the prerequisites for fast pathway construction and optimization. Yeast homologous recombination-based DNA assembler [28] and scarless DNA assembly via ligase cycling reaction (LCR) [29] both enabled assembly of up to 12 DNA parts with 60–100 % of individual clones being correct, and provided fast and reliable workflow for assembly of DNA constructs from PCR amplification or DNA synthesis. In yeast efficient seamless gene disruption [30] enables rapid blocking of by-product formation and one-step multigene genomic integration [31] is helpful for constructing genetic stable strains. Similarly, in *E. coli* ePathBrick enables rapid assembly of up to 7 genes (around 9 kB in total) [32] and chromosomal gene disruption using PCR primers as the homologue to the targeted gene is well established [33]. However, genetic engineering of unconventional microorganisms such as *R. toruloides* is still difficult. Any endeavor, on establishing a genetic engineering platform for this, is helpful for constructing alternative microbial isoprenoid cell factories [34].

4 Rational Design and Optimization

4.1 Precursor Supply

High-level production of isoprenoids using either *E. coli* or *S. cerevisiae* requires engineering a strain for efficient provision of the universal C_5 monomer IPP or its isomer DMAPP, as all isoprenoids are assembled from these monomers. Two pathways exist for the biosynthesis of these precursors (Fig. 1). One approach to increase the precursor flux is to employ a heterologous pathway to supplement the native pathway, which may bypass endogenous regulation and alternative dissipative pathways. Importing the MVA pathway from *S. cerevisiae* into *E. coli* substantially increased the availability of isoprenoid precursors [35]. However, it should be noted that this procedure may also cause undesired effects such as the toxicity of pathway intermediates to the host. In this case, the accumulation of hydroxymethylglutaryl-CoA (HMG-CoA) imposed through heterologous expression of the MVA pathway was shown to be toxic for *E. coli* [36]. In addition, the host may lack the appropriate machinery to functionalize the heterologous enzyme. In a study, it was found that the difficulty functionally to express iron sulfur cluster proteins in yeast, which are essential for the last two reactions in the MEP pathway, and this led to failure in establishing a functional MEP pathway in *S. cerevisiae* [37, 38].

Another approach to increase the precursor pool is to manipulate the metabolic flux and regulation of the native pathway. Extensive endeavors have focused on metabolic engineering of the MVA pathway for enhanced supply of IPP/DMAPP, and further FPP, which is the universal precursor for sesquiterpenes. Except for the well-elucidated targets for engineering the MVA pathway in yeast such as

overexpression of a truncated Hmg1, transcriptional factor mutant Upc2-1, and FPP synthase Erg20 combined with down-regulation of squalene synthase gene *ERG9* [5, 12, 39–41] additional strategies have been developed to increase the flux to FPP. These include, for example, overexpression of all genes in the MVA pathway either by integration of an additional copy [42] or replacing the promoters of all relevant MVA pathway genes [43], or integration of one more copy of *tHMG1* with strong promoter (P_{TDH3}) into the chromosome [42]. Owing to all efforts made, a thoroughly optimized strain was developed and combined with the discovery and implementation of plant dehydrogenase and cytochromes; this enabled an efficient biosynthetic route in *S. cerevisiae* to artemisinic acid with fermentation titers of 25 g/L [5].

Taking advantage of a developed platform strain that has a high flux towards FPP, a commercial process was developed for the production of farnesene [44], another sesquiterpene derived from FPP. In addition, the flexibility of the FPP overproducing platform allowed a rapid switch from the overproduction of amorphadiene to that of bisabolene, the immediate precursor to bisabolane, which is a novel biosynthetic alternative to D2 diesel fuel [45]. These examples well elucidate the concept of a cell factory platform (Fig. 1c), where the cellular metabolism has been optimized towards formation of universal precursors, which can then be used by "plug-and-play" for the production of different types of related products.

Similar to this concept, a supply of precursor acetyl-CoA is essential for high-level production of isoprenoids via the MVA pathway and many other commercially interesting compounds as well [46]. However, acetyl-CoA metabolism in yeast is quite complex and this metabolite is present in three different compartments and is not directly interchangeable among these compartments. Therefore, the metabolism of acetyl-CoA in *S. cerevisiae* has been thoroughly investigated [47]. Based on this, a combined pull–push–block strategy has been recently developed to increase the availability of acetyl-CoA in the cytoplasm [46]. The strategy involved overexpression of endogenous alcohol dehydrogenase Adh2, aldehyde dehydrogenase Ald6, and expression of acetyl-CoA synthetase variant from *Salmonella enterica*. The use of this variant had been previously demonstrated to redirect flux from acetaldehyde to acetyl-CoA successfully and to lead to improved production of the sesquiterpene amorphadiene [48]. Furthermore, through combining these with deleting acetyl-CoA consuming reactions, such as peroxisomal citrate synthase Cit2 or cytosolic malate synthase Mls1, the developed acetyl-CoA platform improved the production of the sesquiterpene santalene fourfold [46]. The platform host was also shown to have a substantially improved production of 1-butanol by eightfold [49], and polyhydroxybutyrate by 18-fold [50]. However, these strategies exclusively depend on the native pyruvate dehydrogenase (PDH) bypass pathway, which is highly ATP-consuming for provision of acetyl-CoA making this route unfavorable. Alternative pathways for cytosolic acetyl-CoA synthesis such as replacement of native acetyl-CoA synthetases (ACSs) by acetylating acetaldehyde dehydrogenase or pyruvate formate lyase [51], may overcome the constraints on the maximum yields and increase the production of acetyl-CoA–derived isoprenoids and other compounds.

4.2 Pathway Balance

It is important to increase the flux to important precursors and the final products, however, it is also essential that the relative levels of the enzymes are coordinated in such a way that no intermediates are accumulated, as these metabolites may be toxic to the host and/or affect further product formation. In order to balance the multigene pathways, an excellent example by using an approach named "multivariate modular pathway engineering" was demonstrated to improve production of the diterpene taxadiene successfully [52]. This technique redefines the metabolic network as a collection of distinct modules. Four key enzymes (encoded by *dxs*, *ispD*, *ispF*, and *idi*) in the MEP pathway were grouped into one module, and two enzymes catalyzing the synthesis of GGPP and taxadiene were grouped into another module. The expression of the different modules in *E. coli* was sequentially and systematically tuned by, for example, varying plasmid copy numbers and altering promoter strengths. Consequently, the highest producer among these strains reached production levels of 1 g/L taxadiene, a nearly 15,000-fold improvement compared to the control strain [52]. This framework allows for the assessment and elimination of regulatory and pathway bottlenecks, offers the opportunity to evaluate a complicated system in a more tractable way, and is particularly useful for experimentation lacking high-throughput screens. The module-balancing strategy has also been proven to be useful for the production of different types of products, such as the diterpene miltiradiene [53], fatty acids [54], and the flavonoid pinocembrin [55].

4.3 Spatial Optimization of Metabolism

To obtain the desired cell factories, it is crucial to coordinate the performances of many different enzymes and multiple cellular pathways. In addition to modular optimization, spatial coupling of complex metabolic reactions at multiple scales has become an attractive strategy to improve pathway function. These optimization approaches function at many scales and are responsive to external signals, ensuring that the cell does not waste metabolic resources by producing unnecessary enzymes and channeling flux through appropriate metabolic routes.

One strategy is organizing pathway enzymes as macromolecular complexes by generation of unnatural fusion proteins. Direct fusion of FPP synthase with a heterologous sesquiterpene synthase, patchoulol synthase in *S. cerevisiae* was found successful in diverting the flux towards formation of the desired product and resulted in increased patchoulol production by about twofold [56]. Physical fusion of diterpene synthases SmCPS (labdadienyl/copalyl diphosphate synthase, from *Salvia miltiorrhiza*) and SmKSL (kaurene synthase-like, from *S. miltiorrhiza*) as well as the fusion of GGPP and FPP synthase led to significantly improved diterpene miltiradiene production and reduced by-product formation in *S. cerevisiae* [53]. Although a fusion of two normally monomeric enzymes is straightforward,

fusion of two normally multimeric or more than two enzymes may be problematic due to misfolding or proteolysis of the fusion proteins.

As an alternative to artificial protein fusions, synthetic scaffolds with the assistance of proteins, DNA, or RNA have been recently developed to colocalize multiple enzymes in a designable manner. Dueber et al. [57] expressed scaffolds from the interaction domains of metazoan signaling proteins to assemble three mevalonate biosynthetic enzymes in optimal stoichiometry, and achieved a 77-fold improvement in mevalonate production with lower enzyme expression levels and reduced metabolic load in *E. coli*. Similar to protein scaffolds, RNA aptamer-based scaffolds are recruited to control the spatial organization of two metabolic enzymes involved in biological hydrogen production expressed in *E. coli*, resulting in about 50-fold enhancements in hydrogen production [58]. Unlike protein-based approaches, the use of RNA scaffolds permits the assembly of complex multidimensional architecture with nanometer precision. However, these techniques have not been exploited in application of isoprenoid production. Along similar lines, DNA-based scaffolds were used in combination with chimeras between target biosynthetic enzymes and zinc fingers (ZFs) to increase mevalonate production up to threefold with optimal scaffold designs in *E. coli* [59].

In addition to the linear design and channeling of metabolic pathways, intracellular spatial organization of cellular functions to membrane-surrounded organelles offers great benefits such as concentrating reactions and pathways that need special conditions (e.g., pH) or may be toxic to other organelles. Mitochondrial targeting of a heterologous FPP synthase and a sesquiterpene synthase improved the production of sesquitrepene valencene and amorphadiene by eight- and 20-fold, respectively, in yeast coengineered with a truncated *HMG1* [60]. Targeting the Ehrlich pathway into yeast mitochondria increased isobutanol production more than twofold, whereas overexpression of the same pathway in the cytoplasm only improved yields by 10 % [61]. These successes are not only due to higher concentrations of enzymes, substrates, and cofactors, but might also result from removing the need to transport intermediates between organelles and from reducing the loss of intermediates to competing pathways.

4.4 Cofactor Engineering

Cofactors such as NAD(P)H, ATP, and CoA are among the most highly connected metabolites and influence a broad range of cellular functions. Manipulation of cofactor availability not only allows overcoming bottlenecks in cofactor-dependent biosynthesis pathways, but might also have a widespread impact on cellular metabolism.

Pathways for synthesis of isoprenoids often involve oxidation-reduction reactions catalyzed by enzymes using cofactors NADH and NADPH that are as critical for catalysis as the enzymes themselves. In native cell metabolism NADH is the major redox product of catabolism, and NADPH is predominantly required in

anabolism where it serves as a reducing agent for amino acid, lipid, and nucleotide biosynthesis. Thus, pathway enzymes such as, for instance, HMG-CoA reductase, which is a key player in the MVA pathway and requires NADPH as a reducing agent, will increase the demand of NADPH aimed for isoprenoid production and often have to compete with the requirements of other anabolic pathways for this essential cofactor.

One approach is altering cofactor requirements by enzyme substitution. Ma et al. [62] characterized various HMG-CoA reductases to substitute the corresponding component in the yeast MVA pathway that was heterologously expressed in *E. coli*. A NADH-dependent HMG-CoA reductase from *Delftia acidovorans* improved production of the sesquiterpene amorphadiene by 50 % over the yeast enzyme. A further enhanced performance was observed by increasing intracellular NADH availability using a NAD^+-dependent formate dehydrogenase from *Candida boidinii* along with formate supplementation.

Another approach is targeting native host pathways that can bring about cofactor recycling. In order to restore the NADH/NADPH balance impaired by installing a sesquiterpene santalene biosynthesis pathway in *S. cerevisiae*, the ammonium assimilation pathway was modified to increase the availability of the reductive cofactor NADPH. This was achieved by deleting the NADPH-consuming reaction of glutamate dehydrogenase encoded by *GDH*1, and simultaneously overexpressing the NAD^+-dependent glutamate dehydrogenase encoded by *GDH*2. This strategy was found to improve the production of the sesquiterpene cubebol by 85 % [63], and together with other strategies the production of the sesquiterpene santalene could be increased fourfold [64].

Altering cofactor specificity via protein engineering is another approach as demonstrated in the enhanced synthesis of butanediol [65], in which the coenzyme specificity of butanediol dehydrogenase was altered from NAD(H) to NADP(H).

5 Computational and Omics-Aided Engineering and Optimization

5.1 Computational Tool-Driven Optimization

The identification of gene targets for strain improvement outside the actual biosynthetic pathway is a difficult task as it needs comprehensive knowledge about all pathways and genes functioning in the cell and their interactions with each other. Increasing availability of stoichiometric models for a range of organisms with developed metabolic optimization algorithms enables researchers to identify such gene targets in silico, and may thereby accelerate the process of metabolic engineering for strain improvement towards the enhanced production of desired products.

A gene deletion strategy using minimization of metabolic adjustments (MOMA) allowed the successful identification of genes to be deleted among a large number of gene candidates, and improved the biosynthesis of lycopene in the engineered *E. coli* strain by nearly 40 % [66]. This was further proved to be useful by using OptGene [67] as a modeling framework and MOMA as an objective function to identify gene knockouts in *S. cerevisiae* and led to enhanced production of the sesquiterpene cubebol by 85 % [63].

On the other hand, it has been difficult to identify gene overexpression targets using similar techniques. One strategy called flux scanning based on enforced objective flux (FSEOF) has been successfully used to select genomewide gene amplification targets in *E. coli*, and improved lycopene production [68]. These successes point out examples where genome-scale metabolic models can be used effectively. However, there are areas where models that incorporate regulatory constraints and signaling should be used in combination with additional tools, for example, constraint-based reconstruction and analysis (COBRA) [69], which would allow for more precise target identification.

5.2 Omics-Based Technique Aided Optimization

The advancements of omics techniques have enabled a rapid acceleration in the ability to obtain system-level information for the cell, capturing cell physiology in a holistic manner from several different levels. These high-throughput omics data, integrated with computational approaches now serve biomolecular network analysis and model-based prediction in the field of systems biology [70, 71].

Transcription analysis, as the most widely implemented omics technology in metabolic engineering, has enabled quantitative measurements of the dynamic expression of mRNAs between different states at the genome scale. This allows integration of genome-scale metabolic models using different approaches to, for instance, map the transcriptional and metabolic regulation in metabolic networks [72, 73]. This valuable information enabled uncovering global regulatory mechanisms and complex metabolic networks. By exploiting whole-genome transcription analysis, a dynamic stress–response promoter strategy was developed to control accumulation of toxic intermediates in *E. coli* and improve the final titers of the sesquiterpene amorphadiene twofold over those from inducible or constitutive promoters [74].

Because mRNA levels do not necessarily correlate with protein levels, there is also much interest in the use of proteomics in metabolic engineering, to quantify protein levels or characterize states of proteins. A targeted proteomics approach via selected-reaction monitoring (SRM) mass spectrometry was employed to measure protein levels of the MVA pathway heterologously expressed in *E. coli* to produce the sesquiterpene amorphadiene [75]. The analysis revealed two proteins, mevalonate kinase (MK) and phosphomevalonate kinase (PMK), as potential bottlenecks. Manipulating the expression of these proteins led to a threefold improved

final titer of amorphadiene. Nowadays, advancements in mass spectrometry combined with liquid chromatography have expanded the number of proteins that can be quantified in a cell lysate by several orders of magnitude. Also, other methods, such as stable isotope labeling-based isotope coded affinity tags (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), or label-free comparative quantitative proteomics have enabled more accurate proteome measurements. However, these methods need to be employed to monitor abundance of proteins in metabolic engineering.

Similar to proteomics, the advent of mass spectrometry also permits measurements of intra-and extracellular metabolites for high-throughput metabolome studies [76]. However, some challenges still lie in sample preparation, as it is difficult to obtain quantitative information at the metabolite levels whereas relative levels can be measured [77]. Instead of looking at all metabolites simultaneously, target profiling compares the spectrum of interest to a library of reference spectra of pure compounds. In one study, targeted metabolite profiling coupled with transcriptome analysis was employed to identify the cytotoxic effects of accumulated HMG-CoA in an engineered *E. coli* strain expressing the MVA pathway [78]. It was revealed that HMG-CoA accumulation inhibits the biosynthesis of fatty acids. Based on these results a new fermentation strategy was suggested including supplementation of palmitic acid or oleic acid, which markedly improved the growth of the host strain.

As high-throughput omics analyses contribute to a better understanding of the biological system, this kind of analysis is expected to be more frequently employed for isoprenoid production to identify metabolic engineering targets.

6 Novel Fermentation Process Design and Optimization

A fundamental challenge to the microbial production of isoprenoids is product (and/or intermediate) tolerance; for instance, monoterpenes are generally highly toxic to microorganisms [79]. In addition to cellular engineering approaches such as transporter engineering [80] and modifying the components of the global transcription machinery [81] that can be used to overcome toxicity limitations, an alternative approach is novel fermentation process design. By adding an immiscible organic layer onto the cultivation medium, the product can be removed in situ, where the product is toxic or inhibits further product formation. A biphasic extractive system was demonstrated to reduce significantly toxicity of the monoterpene limonene to the host *S. cerevisiae*, with growth rates far beyond the ones being reached in the solvent-free system or after attempted adaptation in chemostat cultivation [79]. For example, using dibutyl phthalate as solvent, the minimum inhibitory concentration to limonene improved by about 700-fold compared to a solvent-free system.

The biphasic approach is also a suitable tool for recovery of the product. In an attempt to eliminate product loss through air-stripping, a two-phase partitioning fermentation process with a dodecane organic phase was used to trap the volatile sesquiterpene amorphadiene [82]. In another study, a gas-phase bioprocess was employed for isoprene production by using an extensively metabolically engineered *E. coli* strain reaching a high titer of over 60 g/L, a volumetric productivity of 2 g/L, and a yield of 11 % isoprene from glucose [22]. Numerous in situ product recovery techniques such as pervaporation, perstraction, and gas striping have been reported for the recovery of different kinds of compounds [83]. It can be expected that fermentation process design will gain more attention for microbial isoprenoid production in the future.

Optimization of the fermentation process is also a key factor for the viability of the overall bioprocess. Inasmuch as metabolic engineering of microorganisms is typically implemented in the process of using test tube or shake flask cultivation, a superior strain developed in this process does not necessarily perform well in a large-scale fermentation used in industrial production. Furthermore, an optimized fermentation process will further increase the performance of the metabolically engineered strains. Optimization of nitrogen delivery in the fermentation process using an engineered *E. coli* strain further enhanced the titers of the sesquiterpene amorphadiene, and up to 27 g/L amorphadiene were reached by using a dual restriction of carbon and nitrogen supply in the fed-batch process [21]. In another study, development of a fermentation process with ethanol feeding for the re-engineered yeast CEN.PK2 strain further enhanced amorphadiene production and led to the production of more than 40 g/L [43]. Moreover, during the fermentation optimization or process scale-up it might be necessary to use integrative analysis based on omics data to further identify metabolic engineering targets.

7 Outlook

Encouraged by recent advances, the production of isoprenoids by bacterial or yeast host platforms holds great promise. There are also an increasing number of research efforts on the molecular biochemistry and genetics of isoprenoid biosynthesis, and, to some extent, on their biological functions. This not only expands the resources for further exploitation, but also provides increasing opportunities for microbial production of isoprenoids. However, most of the catalytic diversity of plant enzymes is unexplored, because previous efforts have focused on a relatively small number of species. One limiting factor lies therefore in the elucidation of the biosynthetic pathways and identifying the appropriate genes. Of special note, terpene-modifying enzymes typically belonging to the P450s family pose great challenges in terms of both gene discovery and microbial expression. Advances and decreasing costs in sequencing technology will accelerate the rate of exploring new resources. Creating new enzymes by powerful protein engineering and directed evolution is another direction, as reported for an engineered cytochrome P450 that

catalyzes a reaction that is fundamentally different from the one catalyzed by the natural enzyme [15]. This is expected to expand the range of isoprenoid products that can be synthesized by microbial cell factories.

The past few years have witnessed several successful examples of industrial production of isoprenoids in large-scale fermentation processes, such as isoprene, valencene, amorphadiene, and farnesene, by either *E. coli* or *S. cerevisiae* host platforms. However, expansion to many other isoprenoid products to become industrially relevant will depend on the continued development of technologies. Continuous efforts in systems biology to elucidate the complex regulatory and metabolic networks with novel algorithms emerging will advance the predictive potential of mathematical models, and thereby enhance the process for optimal microbial factory development. Moreover, the progress in design and construction of biological components in the field of synthetic biology will facilitate faster construction and reliable and desirable control of metabolic pathways. It can be predicted that new technologies and innovative combinations of new and existing technologies will continue emerging, which will enable further development of microbial factories for commercial isoprenoid production.

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