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# *In vitro* reconstitution guide for targeted synthetic metabolism of chemicals, nutraceuticals and drug precursors

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With the developments in metabolic engineering and the emergence of synthetic biology, many breakthroughs in medicinal, biological and chemical products as well as biofuels have been achieved in recent decades. As an important barrier to traditional metabolic engineering, however, the identification of rate-limiting step(s) for the improvement of specific cellular functions is often difficult. Meanwhile, in the case of synthetic biology, more and more BioBricks could be constructed for targeted purposes, but the optimized assembly or engineering of these components for high-efficiency cell factories is still a challenge. Owing to the lack of steady-state kinetic data for overall flux, balancing many multistep biosynthetic pathways is time-consuming and needs vast resources of labor and materials. A strategy called targeted engineering is proposed in an effort to solve this problem. Briefly, a targeted biosynthetic pathway is to be reconstituted *in vitro* and then the contribution of cofactors, substrates and each enzyme will be analyzed systematically. Next is *in vivo* engineering or *de novo* pathway assembly with the guidance of information gained from *in vitro* assays. To demonstrate its practical application, biosynthesis pathways for the production of important products, e.g. chemicals, nutraceuticals and drug precursors, have been engineered in *Escherichia coli* and *Saccharomyces cerevisiae*. These cases can be regarded as concept proofs indicating targeted engineering might help to create high-efficiency cell factories based upon constructed biological components.

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## 1. Introduction

The challenges posed by the energy crisis, environmental degeneration, disease or food shortage and the concerns of achieving sustainable development have prompted great interest in the development of new biological processes and organisms designed for specific purposes.[1–3] Thanks to developments in metabolic engineering and synthetic biology in recent decades,[4] the great potential of microbes as solutions to these dilemmas has entered public knowledge.[5] Metabolic engineering aims to endow cells with improved properties and performance. Synthetic biology could create new biological parts, modules, devices and systems, in addition to re-engineering cellular components and machinery that nature has provided.[6] Through the integration of metabolic engineering and synthetic biology, more efficient microbial cell factories can be constructed to produce biofuels,[7,8] biomaterials[9] and drug precursors[10,11] from renewable biomass. In the World Economic Forum 2012 (WEF2012), synthetic biology and metabolic engineering were included in the Top 10 Emerging Technologies.

With the advent of synthetic biology, especially in the past several years, a few cases involved in the production of pharmaceuticals and new biofuels[12] have been become milestones in this field. The first major practical achievement was the large-scale production of artemisinin by yeast at integrated renewable products company Amyris Inc.[13] Artemisinin, an efficient anti-malarial drug produced by the sweet wormwood plant *Artemisia annua*, has been used in China for more than 2000 years in the treatment of malaria patients.[14] However, the unstable source of affordable plant-derived artemisinin has resulted in price fluctuations and shortages.[15] As shown in Fig. 1A, Paddon et al. developed a process for the production of artemisinin by fermentation of simple inexpensive carbon

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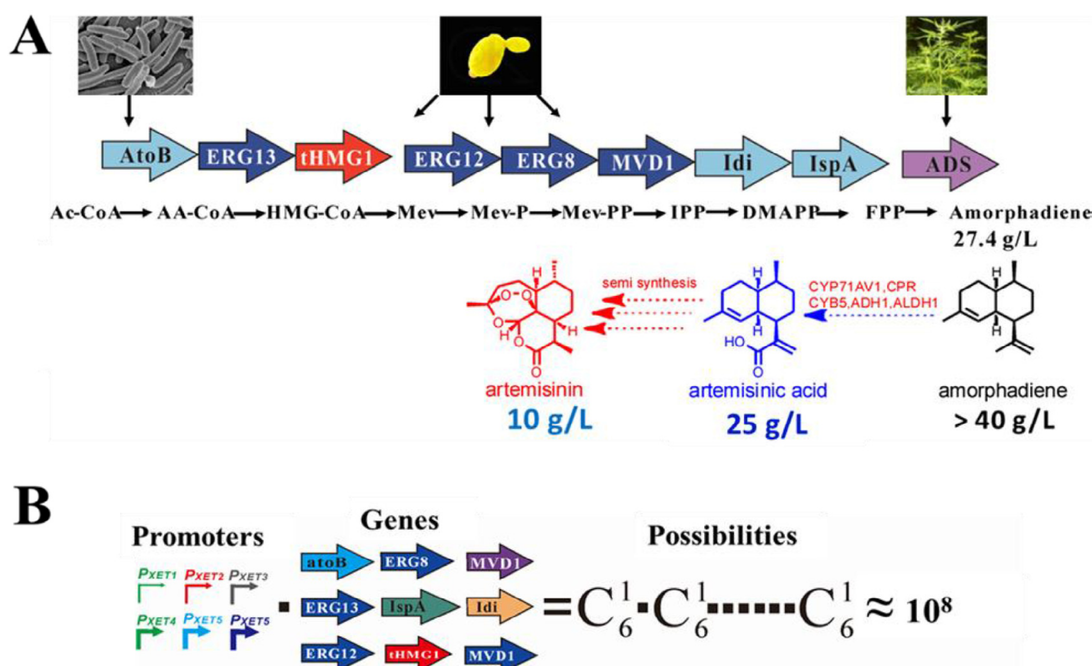


**Fig. 1.** Prospects and challenges of synthetic biology in the construction of high-efficiency microbial cell factories. (A) High-efficiency biosynthesis of the artemisinin precursor in yeast. The genes expressed encode the following enzymes: AtoB, acetoacetyl-CoA thiolase; ERG13, HMG-CoA synthase; tHMG1, truncated HMG-CoA reductase; ERG12, mevalonate kinase; ERG8, phosphomevalonate kinase; MVD1, mevalonate diphosphate decarboxylase; Idi, isopentenyl diphosphate (IPP) isomerase; IspA, farnesy diphosphate (FPP) synthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 enzyme that converts amorphadiene to artemisinic alcohol; CPR, cytochrome P450 reductase; CYB5, cytochrome b5; ADH1, artemisinic alcohol dehydrogenase; ALDH1, artemisinic aldehyde dehydrogenase. CYP71AVA1, CPR1, CYB5, ADH1 and ALDH1 derived from *A. annua* could oxidize amorphadiene to artemisinic acid. Genes colored light blue are derived from *E. coli*, dark blue genes are derived from *S. cerevisiae*, red genes is derived from *Staphylococcus aureus*, purple genes are derived from *A. annua*. (B) Challenges to the optimization of a biosynthetic module. The synthetic biology components, such as kinds of promoters and enzymes, could be constructed like building blocks, and the optimal pattern have to be selected from millions of combinations.

substrates using engineered *Saccharomyces cerevisiae* to produce artemisinic acid, followed by extraction and chemical conversation to artemisinin. The production of artemisinic acid was increased from 1.6 g $L^{-1}$ to 25 g $L^{-1}$.[13] Another landmark work was the total biosynthesis of opioids in yeast.[16,17] Opioids, derived from the opium poppy (*Papaver somniferum*), are the primary drugs used for pain management and palliative care. Recently, Smolke's group at Stanford University expressed more than 20 enzymes derived from rodents, plants and bacteria in a engineered host and, finally, realized the complete biosynthesis of opioids from glucose.[18] In these cases, synthetic biological approaches have been used to optimize both the host and pathways to maximize the production of targeted products. Although synthetic biology allows us to freely manipulate the components (e.g. promoters, enzymes, modules, etc.), just like building blocks, an optimal pattern has to be selected from millions of combinations (Fig. 1B). A common approach, for example, is to investigate as many mutants as possible; however, if a high-throughput method cannot be generated or a large mutant selection is too expensive, it would be difficult to obtain satisfactory results. It is worth noting that the formation of artemisinic acid requires enormous manpower and financial resources.[19,20]

Traditional metabolic engineering has made great advances in the optimization and innovation of industrial fermentation, including the biosynthesis of a taxol precursor in microbes,[21] conversion of lignocellulosic biomass to ethanol[22] and application of amino acid-producing bacteria.[23] The heterologous synthesis of a taxol precursor in *Escherichia coli* was one of the most famous works in the field of metabolic engineering. Taxol is a potent anticancer drug produced by the Pacific yew tree *Taxus brevifolia*.[24] Ajikumar et al. reported integration of a native upstream methylerythritol phosphate (MEP) pathway and a heterologous downstream terpenoid-forming pathway allowed taxadiene, the first committed taxol intermediate, to be obtained in large amounts from *E. coli* by fermentation.[21] In the bioenergy field, there are improved production rates of advanced biofuels, including butanol, hydrocarbons and terpene-based biofuels.[25–27] However, one important challenge for traditional metabolic engineering is the identification of gene targets of major importance for the improvement of specific cellular functions.[28] Additionally, owing to the lack of biochemical information and genetic background of the targeted metabolic pathways, many engineering works have not achieved the expected results.

A strategy called targeted engineering was proposed in an attempt to overcome these problems and challenges. For this strategy, the biosynthetic pathway is reconstituted *in vitro* and then the contributions of cofactors, substrates and enzymes are analyzed systematically. Subsequently, *in vivo* engineering could be guided by the information gained from *in vitro* assays. This approach might offer some opportunities to create cell factories based upon constructed biological components. Here, we present a review of targeted engineering and its application.

## 2. *In vitro* reconstitution guide for building a high-efficiency synthetic pathway

Over the past several decades, most of the multi-enzyme systems, for example bacterial fatty acid synthases, have been investigated extensively at the genetic and enzymatic level.[29–31] However, due to complex regulation of fatty acid synthesis, it is not easy to manipulate enhanced production of specific fatty acids.[30,32–35] In 2010, Liu et al. developed a cell-free system that could be used for direct quantitative investigation of fatty acid biosynthesis and regulation in *E. coli*.[36] The strong dependence of fatty acid synthesis on

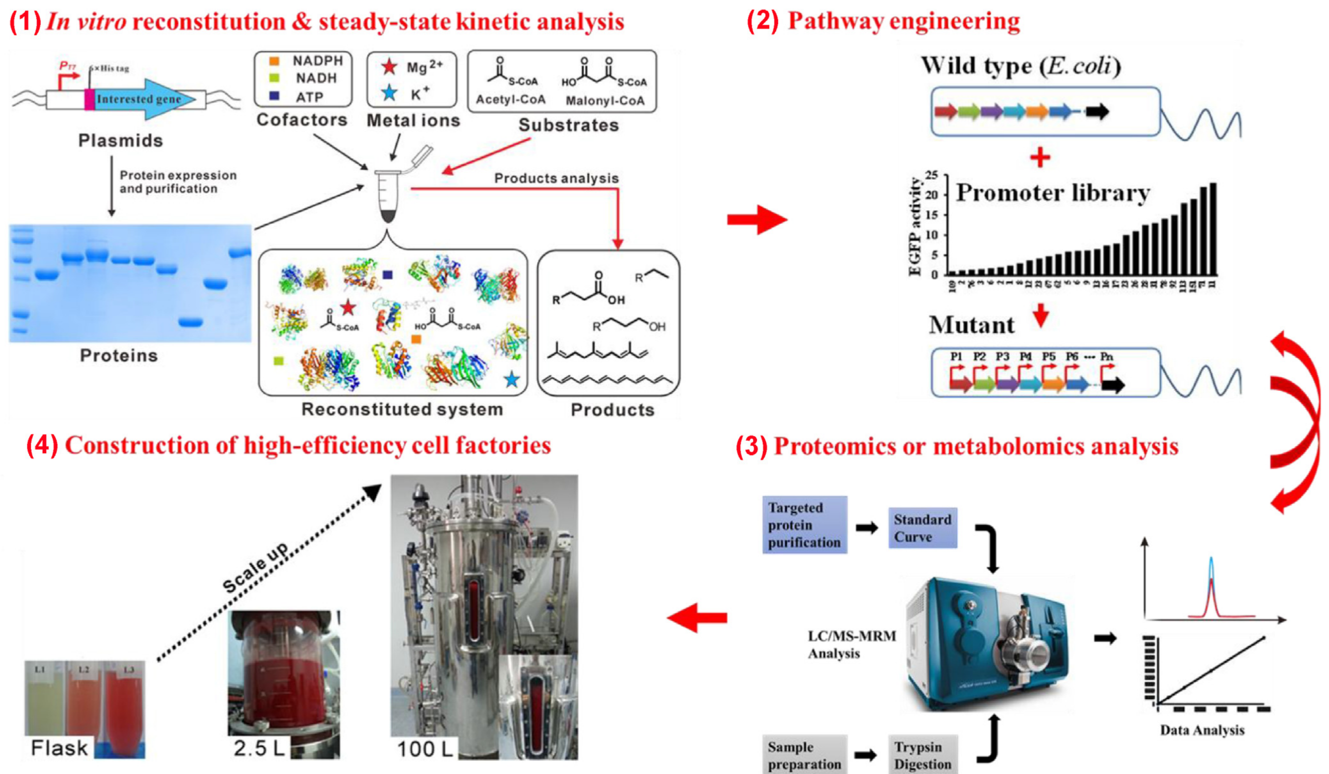


**Fig. 2.** *In vitro* reconstitution guide for building a high-efficiency synthetic pathway. The four parts of targeted engineering are (1) *in vitro* reconstitution of a biosynthetic pathway and steady-state kinetic analysis, (2) rational design, strict regulation and pathway engineering, (3) monitoring metabolic status and targeted proteomics analysis, and (4) construction of high-efficiency cell factories.

malonyl-CoA availability and several important phenomena in fatty acid synthesis were verified by the use of this system. That particular study introduced a new concept, that the *in vitro* quantitative analysis of a multi-enzyme system could guide subsequent engineering work.[8] In 2011, Yu et al. described the *in vitro* reconstitution of *E. coli* fatty acid synthases using eight purified protein components and reported detailed kinetic analysis of this reconstituted system.[37] This highlighted the utility of a cell-free system for investigating the properties of fatty acid synthases under steady-state conditions.

Inspired by the results of these earlier studies, we provide thorough instructions on how to build a high-efficiency synthetic pathway under the guidance of *in vitro* reconstitution; namely, targeted engineering. Unlike the traditional metabolic engineering procedure, targeted engineering does not construct a series of mutants, directly. First, the proteins involved in the pathway of interest are overexpressed and purified. The entire pathway is reconstituted in an Eppendorf tube without any background and then the effect of each component is analyzed systematically by *in vitro* reconstitution assays. In the second step, a few mutants are constructed with the guidance of information gained from the *in vitro* assays. In the third step, the metabolic status of each mutant is analyzed at both the protein and intermediate levels. It will be clear which step is inefficient based upon the accumulation of intermediates and the optimized system gained from *in vitro* assays. Deviation between the observed data and the optimum conditions provide targets for further engineering (Fig. 2). The procedures of targeted engineering are discussed in detail in four parts below: (1) *in vitro* reconstitution of a biosynthetic pathway and steady-state kinetic analysis; (2) rational design, strict regulation and pathway engineering; (3) monitoring metabolic status and targeted proteomics analysis; and (4) construction of high-efficiency cell factories.

### 2.1. In vitro *reconstitution of a biosynthetic pathway and steady-state kinetic analysis*

Biosystems can be classified as microbial or cell-free according to the biocatalysts used; further, cell-free systems can be based on cell extracts and purified enzymes.[5] Several decades ago, biochemists developed a cell-free system as a tool for investigation of bacterial fatty acid metabolism.[38–40] Compared to the complexity of living systems, *in vitro* cell-free systems could provide unprecedented freedom to modify and control biochemical systems for technological application and to understand the design principles of biological circuits.[41] To date, cell-free systems have been used as powerful tools for basic research and purified enzyme-based *in vitro* systems have contributed to biological technology research. For example, the best known *in vitro* system, which has been used widely in molecular biology, is the *in vitro* DNA amplification procedure; namely, the polymerase chain reaction (PCR).[42] In addition, the use of more complicated and elaborate cell-free systems, including *in vitro* transcription[43] and *in vitro* translation, have been reported.

An *in vitro* reconstituted system is based on the enzymes involved in the targeted biosynthetic pathway and the biochemical information for each component is a prerequisite for the *in vitro* reconstitution of the targeted biosynthetic pathway. In addition, all necessary enzymes must be over-expressed and purified with a high level of activity to mimic *in vivo* conditions. To estimate the initial relative protein contents of the targeted pathway in the native host, the mRNA level of each subunit and relative protein levels *in vivo* can be measured using quantitative PCR (qPCR) and western blot

analysis, respectively[37] and the concentration of each protein can be defined as a reference. Combined with cofactors, including ATP, $NADH/NAD^+$, $NADPH/NADP^+$ and metal ions, the *in vitro* system can be reconstituted in a reaction buffer.[44]

Using the *in vitro* reconstituted system, the contributions of each protein component, substrate and cofactor can be titrated by monitoring targeted products. In particular, the major factors for improvement of product formation can be determined easily. The steady-state kinetic, biochemical parameters and accumulation of intermediates can be detected, and on this basis, the relative optimized protein concentrations and the metabolic bottleneck(s) of the biosynthesis pathway can be revealed.[37] In addition, the potential of the targeted metabolic pathway can be estimated on the basis of *in vitro* data. Subsequently, *in vivo* engineering can be guided by the information gained from the *in vitro* assays.

### 2.2. Design, engineering or assembling of the targeted metabolic pathway

The targeted metabolic pathway can be designed and engineered using the well-established techniques of traditional metabolic engineering. However, it will be more straightforward when the manipulated targets are based on the information gained from the *in vitro* assays; for example, genetic manipulation of the major factors. Using gene manipulation, e.g. gene knockout or over-expression, the metabolic bottleneck could be bypassed, which should improve the biosynthetic activity of the targeted pathway. This approach could be an excellent supplement to the current randomized high-throughput methods for the generation of pathways and targeted screening by decreasing variables and providing guidelines for further engineering. Second, the optimal ratio of each protein in the pathway can be deduced and subsequent engineering requires fine-tuning of gene expression and coordination of each component within the pathway. Third, the expression levels of target genes can be controlled precisely. These important concepts are illustrated below by the presentation of a case report.

Promoter engineering can help to generate the dynamic range necessary to enable fine-tuned gene expression for metabolic application.[45] According to the data obtained from the *in vitro* system, the promoter library can be used to fine-tune gene expressions in the pathway. In addition to the traditional bacteriophage T7 promoter-based promoter library[46] and an *ermE* or *kasOp* promoter-based promoter library,[47–49] the novel sensor-regulator systems for dynamic regulation (or dynamic sensor-regulator system) have also been developed. These promoters could be used as a tool to balance metabolism and thereby increase the titers or yields of targeted products and stabilizing production hosts.

During the process of targeted pathway engineering or assembly, in addition to the traditional enzyme digestion and ligation methods, many more powerful and efficient approaches could be applied. You et al. developed a sequence-independent simple cloning method without the need for restriction or ligation enzymes.[50] This method can be used for subcloning up to three DNA fragments into any location on a plasmid. In addition, the Gibson method can be used for assembling multiple large DNA fragments.[51,52] The assembly of large, high (G + C) bacterial DNA fragments can be done in yeast.[53] Recently, with the emergence of the CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein) system as a new technique, many powerful CRISPR-based tools have been developed for gene-editing.[54–56]

### 2.3. Monitoring metabolic status and targeted proteomics analysis

As well as the engineering work described above, control can be achieved by precise measurement of the relative levels of proteins and intermediates in engineered mutant hosts using a modified targeted proteomics method and MS-based intermediate analysis.[57,58] Compared to traditional metabolic engineering, each stage in the evolution of the pathway can be controlled rationally and evaluated against objectively determined rather than empirically chosen milestones. By monitoring the key intermediates in the engineered mutants, flux through the upstream and downstream modules can be adjusted to avoid accumulation of toxic intermediate metabolites or diversion of feedstock to unproductive metabolism.[21] Additionally, MS-based intermediate analysis can help to elucidate the underlying metabolic mechanism and to identify any new metabolic junction in the engineered host. On the other hand, by using MS-based proteomics techniques, the expression level of each enzyme involved in targeted pathway could also be quantitatively analyzed.[59] Combined with metabolic status and proteomics analysis, these data could provide guidelines for further pathway engineering.

### 2.4. Construction of highly efficient cell factories

In the *in vitro* reconstitution system, we focused mainly on the contribution of cofactors, substrates and components involved in the targeted metabolic pathway. To construct highly efficient cell factories, however, it is necessary to integrate information from the *in vitro* and *in vivo* assays. The *in vivo* and *in vitro* data allow more engineering work to further promote the product formation using, as well as the modification mentioned above, precursor supply, redox balance, co-factor engineering, etc.

In addition, the selection or construction of appropriate host strains is very important in the synthesis of targeted products. Bacterial hosts, including *E. coli*, *Bacillus subtilis*, *Pseudomonas* sp., *Corynebacterium* sp. and *Streptomyces* sp., have either a long-standing or more recent application to the production of biodiesel, bulk chemicals and therapeutic natural compounds etc.[9,60] The host should be evaluated carefully according to biological properties that would either hinder or facilitate product biosynthesis.

## 3. Targeted engineering of fatty acid and its derivatives

Fatty acids are central hydrocarbon intermediates in the biosynthesis of biodiesel from renewable sources. Biosynthesis and regulation of fatty acids have been investigated extensively in *E. coli*. Here, we discuss several examples of how the concepts of targeted engineering can be used for guiding optimization of the biosynthesis of fatty acids and derivatives; for example, fatty alcohols and biodiesel (Fig. 3).

### 3.1. Fatty acids and new biodiesel

As mentioned above, much engineering guided by *in vitro* reconstitution assays has been used to enhance the biosynthetic efficiency of fatty acids in *E. coli*.[36,37] Subsequent engineering work could use this high-performance platform for downstream product innovation, including biosynthesis of high quality biodiesels.[61–63]

*S. cerevisiae* has been found more suitable than *E. coli* for bioengineering and has been engineered to produce biofuels.[27,64,65] *S. cerevisiae* is also an important industrial host for production of enzymes, pharmaceutical and nutraceutical ingredients and, recently, commodity chemicals.[9] Li et al. engineered *S. cerevisiae* for overproduction of fatty acids.[66] Acetyl-CoA carboxylase from yeast was titrated into the yeast cell-free extract with acetyl-CoA using an *in vitro* assay. The acetyl-CoA carboxylase proved to be a rate-limiting step of fatty acid synthesis and phosphorylation of acetyl-CoA carboxylase might influence its activity in *S. cerevisiae*. It is known that discovery of the limiting step is crucial for developing a “cell factory” for the overproduction of fatty acids using type I fatty

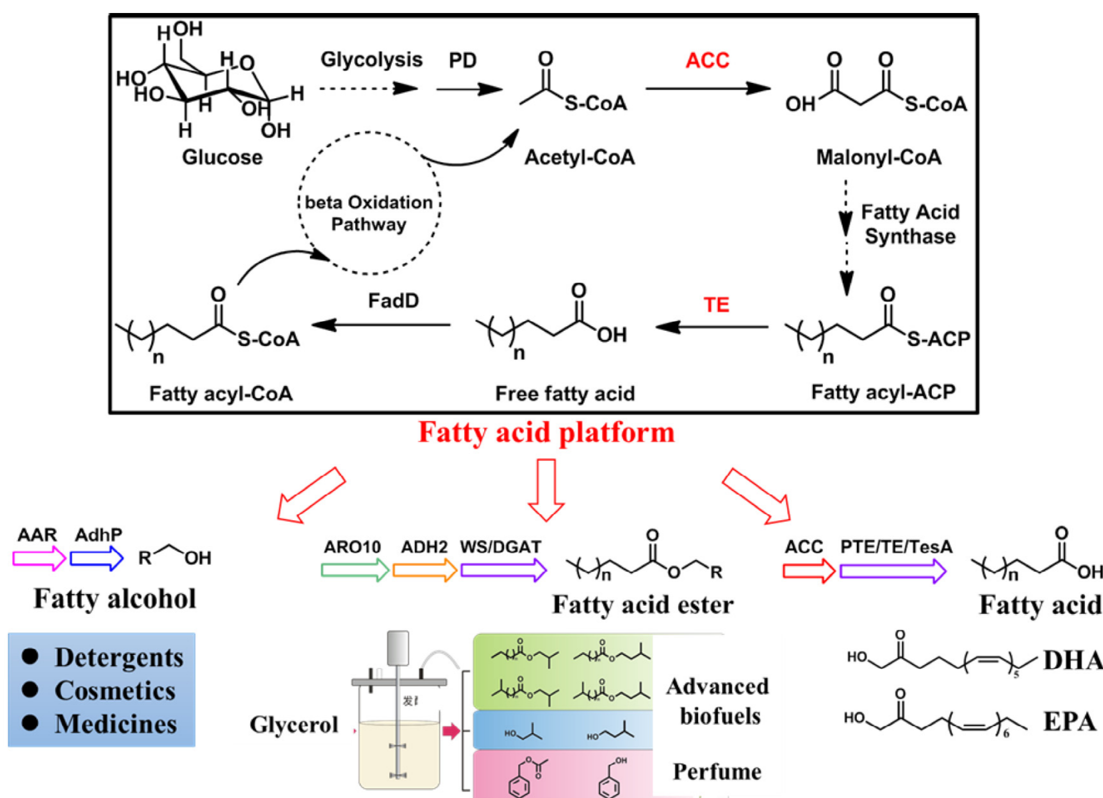


**Fig. 3.** High-performance fatty acid platform for downstream product innovation. PD: pyruvate dehydrogenase; ACC: acetyl-CoA carboxylase; FadD: fatty acyl-CoA synthetase; AAR: fatty acyl-ACP reductase; AdhP: ethanol dehydrogenase/alcohol dehydrogenase; ARO10: 2-keto acid decarboxylase; ADH2: alcohol dehydrogenase; WS/DGAT: diacylglycerolacyltransferase; ARO10: 2-keto acid decarboxylase; ADH2: alcohol dehydrogenase; PTE, TE, and TesA: thioesterases from *S. cerevisiae*, *Cinnamomum camphorum*, and *E. coli*.

acids synthase in yeast or other fungi. This result provided a rationale for future study of this crucial step.

### 3.2. Fatty alcohols

Fatty alcohols are important chemical raw materials and have been used in the manufacture of detergents, skin care products, cosmetics and medicines.[67,68] Traditionally, fatty alcohols are produced by direct extraction from plant material or chemical synthesis from fossil sources. The use of microbial fermentation to produce fatty alcohols from sustainable resources could reduce the dependence on fossil fuels. Low yield and productivity, however, are key problems hampering industrial application of fatty alcohol biosynthesis or microbial fermentation.[69]

Fatty alcohols are reduced from fatty acyl-acyl carrier proteins (ACPs), fatty acyl-CoAs or fatty acids by the enzymes fatty acyl-CoA/ACP reductase or carboxylic acid reductase. Fatty acyl-CoA/ACP reductase is a key enzyme found in many organisms.[70] The most economical strategy for ATP consumption would be for fatty acyl-CoA/ACP reductase to recognize and use fatty acyl-ACPs as a substrate. Thus, the *in vitro* system has been reconstituted to evaluate this hypothesis. First, on the basis of substrate preferences, *Simmondsia chinensis* (jojoba) FAR,[71] *Acinetobacter calcoaceticus* Acr1,[72] *Oryza sativa* DPW[26] and *Synechococcus elongatus* AAR,[73] four fatty acyl-CoA/ACPs reductase have been selected and purified. According to earlier work,[37] the individual proteins of fatty acid synthase, ACP and TesA, were overexpressed and purified for *in vitro* reconstitution. Using the *in vitro* system, the data indicated AAR showed great potential for fatty alcohol production. Both *in vitro* and *in vivo* results demonstrated that the activity and expression level of fatty acyl-CoA/ACP reductase is the rate-limiting step in the current protocol.[74] Guided by this information, the producer has been engineered efficiently and the production and productivity of fatty alcohols were 750 and 0.06 g $L^{-1} h^{-1}$, respectively. This case establishes a promising *in vitro* reconstitution-based synthetic pathway for industrial microbial production of fatty alcohols. The possibility and potential capacity for the targeted metabolic pathway could be easily evaluated. This strategy would definitely help to avoid the construction of numerous mutants to test our hypothesis, though it seems like a trial-and-error screening *in vitro*.

## 4. Targeted engineering of terpenoid overproduction

Terpenoids are one of the most diverse families of natural products and include more than 25,000 structures identified in microorganisms, plants and insects.[75] The mevalonate (MVA) pathway and methylerythritol-phosphate (MEP) pathway are responsible for the synthesis of the two isoprenoid building blocks[76,77] isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). To date, the production of terpenoid metabolites in bacteria has achieved great success in the production of drug precursors and small chemical molecules.[27,78] We have focused mainly on the important new jet fuel precursor farnesene and nutritional compounds. These compounds are synthesized from the building blocks DMAPP and IPP. *In vitro* reconstitution has proved an efficient tool for optimizing the IPP/DMAPP supply system, which could be used as a terpenoid overproduction platform (Fig. 4).

### 4.1. Farnesene

As shown in Fig. 4, terpenoid metabolites have many applications, including medicinal (taxol and artemisinin), nutraceutical

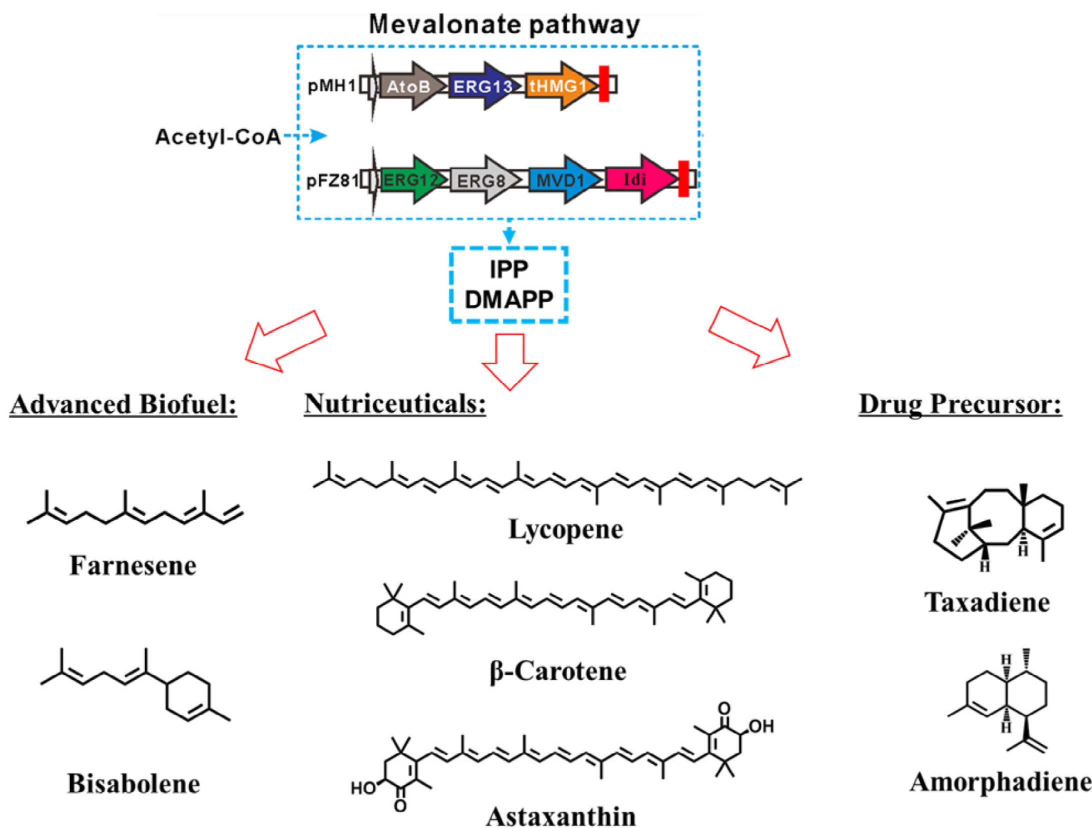


**Fig. 4.** Mevalonate pathway for downstream product biosynthesis. pMH1 contains the genes encoding the first three enzymes of the mevalonate pathway, AtoB, ERG13, and tHMG1; pFZ81 contains the other four genes in the mevalonate pathway, ERG12, ERG8, MVD1, and Idi. pMH1 and pFZ81 both use the single lac promoter in the pBBR1MCS backbone to control expression of the indicated three or four genes. The mevalonate pathway involves the conversion of three equivalents of acetyl-CoA into one equivalent of IPP or DMAPP (dimethylallyl pyrophosphate). Using the highly efficient IPP and DMAPP platform, many downstream products, such as advanced biofuel, nutriceuticals and drug precursor, could be readily derived.

(lycopene and carotenoids) and industrial (isoprene) products, and even as precursors of farnesene, the next generation jet fuel.[10,21,79] Farnesene is a model molecule within the metabolic network of terpenoids and many other products; for example limonene, carotenoids and lycopene, could be engineered by this strategy.

To reconstitute the farnesene biosynthetic pathway, nine genes have been cloned or synthesized based on the gene sequence from three different species.[80] Acetoacetyl-CoA thiolase (*atoB*), *idi* and farnesyl pyrophosphate synthase (*ispA*) genes were amplified from *E. coli* genomic DNA. 3-Hydroxy-3-methylglutaryl-CoA synthase (*erg13* or *hmgs*), a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase (*thmg1*),[81] mevalonate kinase (*erg12*), phosphomevalonate kinase (*erg8*), and mevalonate pyrophosphate decarboxylase (*mvd1*, also known as *erg19*) genes were amplified from *S. cerevisiae*. An α-farnesene synthase gene has been derived from *Malus × domestica*.[82] These nine genes have been overexpressed and purified using the pET28a plasmid. For steady-state analysis, the *in vitro* system has been constituted; the *in vitro* assay indicated Idi and ERG13 have important roles in terpenoid overproduction and show us the optimized ratio of each protein for farnesene biosynthesis. The information from this *in vitro* reconstituted system guided us to optimize farnesene production in *E. coli* by quantitatively overexpressing each component. Through targeted engineering, farnesene has been produced at 1.1 g $L^{-1}$ in shake flask fermentation.[80] Additionally, the mass spectrometry (MS)-based intermediate analysis showed us that, in the engineered high-producing strain, the substrate (acetyl-CoA), energy and cofactor limited the production of farnesene. Based on the analysis of these data, the next round of metabolic engineering work could be used for construction of a highly efficient cell factory for production of farnesene.

### 4.2. Lycopene

The strategy for biosynthesis and overproduction of farnesene inspired development of biosynthesis of the nutritional product lycopene in *E. coli*.[44,83] Lycopene is one of the major precursors of downstream carotenoids and is produced by many plants and microorganisms. It is of special interest due to its antioxidative,[84] anticancer[85,86] and anti-inflammatory activities.[87] Zhu et al. used the MVA pathway developed for the biosynthesis of farnesene system[80] and combined it with the lycopene biosynthesis pathway. An extra copy of the isopentenyl diphosphate isomerase (Idi) gene was used and the production of lycopene was 1.23 g $L^{-1}$ in a 150 L bioreactor.[44] The success of this work indicated *in vitro* information is universal and can be used as guidance for similar metabolic pathway engineering.

### 4.3. Astaxanthin

In addition to lycopene, products such as astaxanthin could be produced efficiently on the terpenoid overproduction platform. Astaxanthin is a highly valued keto-carotenoid, used widely in aquaculture, cosmetic, and functional foods.[88,89] In order to improve the efficiency of astaxanthin biosynthesis, Ma et al. constructed a highly efficient targeted engineering carotenoid synthesis platform in

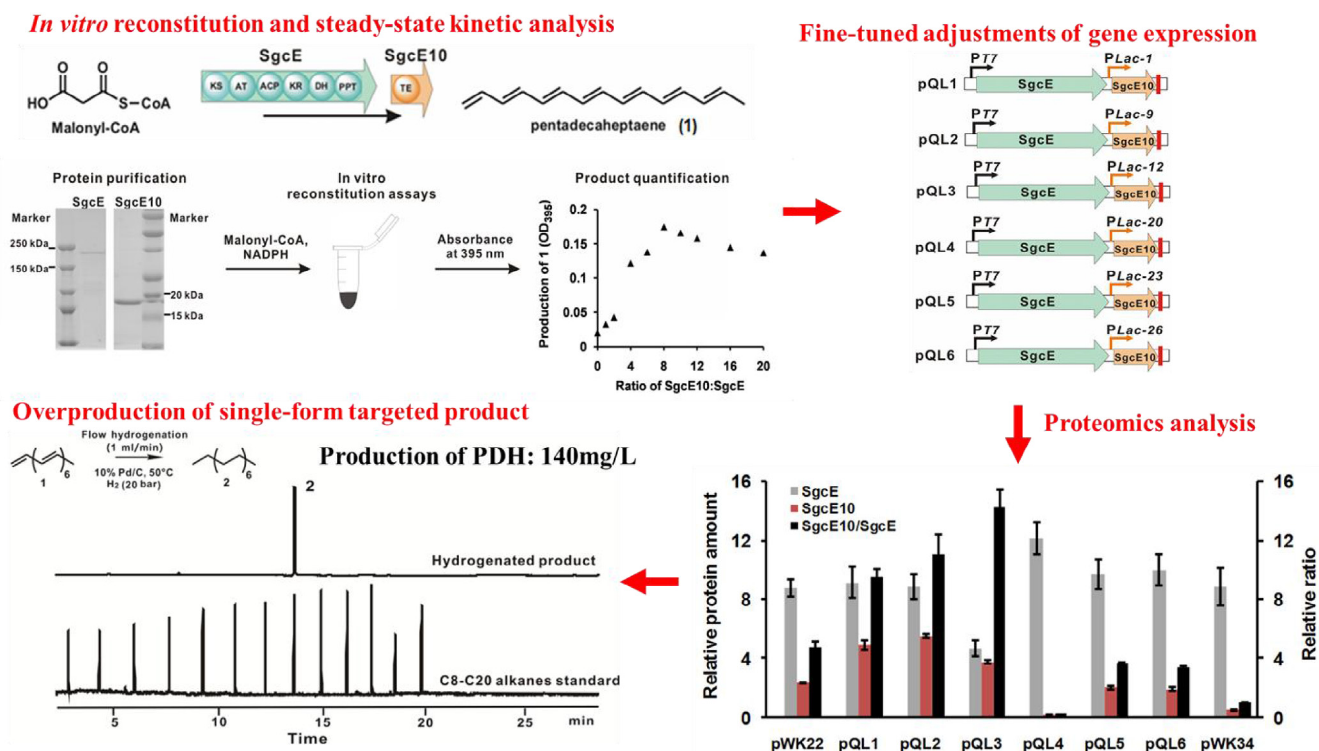


**Fig. 5.** Reconstitution *in vitro* assays: use of an iterative polyketide pathway for valued chemicals in single form. 1) *In vitro* reconstitution of the pathway for pentadecaheptaene (PDH) production. PDH can be biosynthesized from malonyl-CoA via the iterative polyketide pathway. Purified SgcE (203 kDa) and SgcE10 (18.5 kDa) proteins can be used for *in vitro* reconstitution. *In vitro* reconstitution assays processed with malonyl-CoA and NADPH, and titration products PDH quantified by measuring absorbance at 395 nm. 2) Fine-tuned adjustments of targeted gene expression. Based on the results of the titration studies, several *in vivo* constructs engineered for fine-tuned SgcE10 expression:SgcE is under the T7 promoter and SgcE10 is under selected promoters of various strength. 3) Targeted proteomics analysis. Modified MRM-MS proteomics analysis of SgcE and SgcE10 expression in engineered strains. Relative amounts of SgcE and SgcE10 were determined by summing peptide peak areas and normalizing with BSA (bovine serum albumin). 4) Overproduction of single-form targeted product. Analysis of hydrogenated product compared with the C8–C20 alkane standard with GC–MS. Automated flow chemical hydrogenation was performed on the H-Cube. The engineered strain BL21(DE3)/pQL1 showed the best PDH production (140 mg $L^{-1}$).

*E. coli*, and the production of astaxanthin in a heterologous host reached 8.64 mg $g^{-1}$ dry cell weight.[83]

## 5. Re-edit the microbial metabolic pathway: utilize the polyketide pathway for valued chemicals

As reported, polyketide syntheses (PKS) and fatty acid syntheses are remarkably similar with regard to their underlying mechanisms.[90,91] However, PKS can produce many different structures for several reasons. First, various CoA-units can be selected and elongated in the PKS assembly line by basic β-keto-synthase (KS), acyltransferase (AT), and ACP domains. Second, keto-reductase (KR), dehydratase (DH) and enoylreductase (ER) have various reducing roles at the β-keto position organized from all to none of their oxidative activities, thereby producing carbon chains with diverse levels of oxidation.[92] Third, the various types of thioesterase involved in PKS release have a role in directing final product structure. Our goal is to edit and design the PKS genes and thereby use the PKS pathway for production of valuable chemicals, including long and short alkane, dicarboxylic acid etc.

Alkanes and alkenes are important primary components for the formation of biodiesel. There are several pathways for engineering alkane and alkene production derived from the fatty acid system as reported earlier.[73,93–97] However, because of the nature of the fatty acid biosynthesis mechanism, all products reported in these studies are obtained in mixtures. As shown in Fig. 5, Liu et al. attempted to engineer the alkane biosynthesis pathway using iterative type I PKS SgcE and the cognate TE SgcE10 in *E. coli*, with the goal of overproducing single form pentadecaheptaene (PDH) followed by its hydrogenation to pentadecane (PD).[46] Using the *in vitro* reconstitution assays, we found the production of PDH was strongly dependent on the SgcE10:SgcE ratio and a ratio of 8 afforded maximum PDH production. The level of expression of SgcE10 was subsequently fine-tuned using a T7 promoter-based synthetic promoter library. In addition, the cellular concentration of SgcE10 and SgcE was monitored using an MS-based targeted proteomic approach.[57,80] Finally, the single form of C15 alkane was achieved and the highest titer was reached at 140 mg $L^{-1}$, and the best SgcE10:SgcE ratio calculated *in vivo* was closer to that obtained from the *in vitro* assay. In this study, the *in vivo* findings supported the physiological relevance of the *in vitro* observations, suggesting the utility of developing *in vitro* reconstitution systems for *in vivo* engineering.

## 6. Conclusion and perspectives

In these cases, target engineering has shown its potential for metabolic engineering. It is expected to be widely used as there are several advantages compared to traditional metabolic engineering. Firstly, it is much more efficient compared to direct *in vivo* engineering because we can adjust each component freely and precisely. Instead of constructing hundreds of derivate strains to test their contributions by using genetic methods, e.g. overexpression, deactivation and down-regulation, a series of *in vitro* assays can be set up easily by adding an exact amount of each component as designed into the system. Secondly, the clear background of the *in vitro* system makes it useful to explore the maximum potential of the pathway of interest. Each component can be added as designed,

which can be very close to the ideal condition to make the pathway work as fast as possible and as smoothly as it can, which is not possible for *in vivo* engineering. These results help to evaluate the industrial practical possibility of the pathway of interest. Thirdly, the quantified data of each step make it clear for further engineering. The modified targeted proteomics and analysis of intermediates has enriched the targeted engineering because each step can be performed upon the basis of the quantified data. Combinations of these pieces of information will identify the next target.

The complexity of a naturally occurring *in vivo* system often is a major problem for engineering; these systems are products of evolutionary forces and have redundant and often overlapping regulatory elements. During the targeted engineering of a specific pathway, the *in vitro* system cannot always mimic the *in vivo* conditions precisely. Interferences occurring *in vivo*, e.g. the existence of competition or a branched pathway, phosphorylation or acetylation modification of targeted proteins[66] etc., are barriers to pathway engineering. Therefore, an essential interpretation of the targeted pathway is a prerequisite for successful *in vitro* reconstitution. It should be noted this approach is not suitable for a pathway containing proteins with certain attributes, e.g. poorly soluble, difficult to purify, susceptible to loss of activity *in vitro*. In addition, the effect of accumulated intermediates on the whole cell system cannot be addressed in the clear background of the *in vitro* system; the ratio of cofactors of different forms titrated by i*n vitro* reconstitution is sometimes not consistent as it is *in vivo*. To construct highly efficient cell factories, both the pathway of interest and the whole cell system should be well balanced.[98] Consequently, by monitoring metabolic status (or metabolomics analysis) and proteomics analysis, this approach could help provide more guidance on metabolic engineering of the whole cell system, such as redox, energy and cofactor metabolism.

It is a challenge to express proteins precisely as expected *in vivo*, which means it is difficult to achieve the exact optimized pathway *in vivo*. The targeted engineering is still valuable for the *in vivo* engineering as it can provide quantified data for key factors of the pathway of interest. Targeted engineering will be more useful after a more accurate expression technique is available. Meanwhile, with the widespread application of "-omics" techniques and computational biology techniques, many genome-scale metabolic models have been constructed as tools for various applications, including metabolic engineering, pathway rerouting and systems biology. Combined with targeted engineering, the steady-state kinetic data and the overall flux of a targeted pathway obtained from *in vitro* reconstitution could facilitate the construction of high-quality metabolic models. The use of a synthetic biology approach in the post-genomic era could artificially design many new pathways for the synthesis of targeted products. The biosynthesis procedures or route of targeted product often can be divided into several synthetic modules upon the basis of their catalyst function. Each synthetic module would be easy to optimize by reconstitution. As a result, modular synthesis of pharmaceutical compounds will likely become the focus of interest, and targeted engineering could be of great benefit to highly efficient modular design and optimization.

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