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

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ABSTRACT

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 biomaterials9 and drug precursors10,11 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 biofuels12 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
substrates using engineered *Saccharomyces cerevisiae* to produce artemisinic acid, followed by extraction and chemical conversion to artemisinin. The production of artemisinic acid was increased from 1.6 g L^{-1} to 25 g L^{-1}. Another landmark work was the total conversion of artemisinic acid to artemisinin. The production of artemisinic acid was increased by the Pacific yew tree *Taxus brevifolia*. However, due to complex regulation of fatty acid synthesis, it is not easy to manipulate enhanced production of specific fatty acids. In the bioenergy field, there are improved producing bacteria. Traditional metabolic engineering has made great advances in the optimization and innovation of industrial fermentation, including the biosynthesis of a taxol precursor in microbes, conversion of lignocellulosic biomass to ethanol and application of amino acid-producing bacteria. 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*. 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. In the bioenergy field, there are improved production rates of advanced biofuels, including butanol, hydrocarbons and terpene-based biofuels. However, one important challenge for traditional metabolic engineering is the identification of gene targets of major importance for the improvement of specific cellular functions. 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. However, due to complex regulation of fatty acid synthesis, it is not easy to manipulate enhanced production of specific fatty acids. 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*. The strong dependence of fatty acid synthesis on
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 in vitro quantitative analysis of a multi-enzyme system could guide subsequent engineering work. 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. 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. Several decades ago, biochemists developed a cell-free system as a tool for investigation of bacterial fatty acid metabolism. 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. 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). In addition, the use of more complicated and elaborate cell-free systems, including in vitro transcription 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\textsuperscript{27} and the concentration of each protein can be defined as a reference. Combined with cofactors, including ATP, NADH/NAD\textsuperscript{+}, NADPH/NADP\textsuperscript{+} and metal ions, the in vitro system can be reconstituted in a reaction buffer.\textsuperscript{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.\textsuperscript{17} 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.\textsuperscript{36} 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\textsuperscript{46} and an ermE or kasOp promoter-based promoter library,\textsuperscript{37–40} 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.\textsuperscript{48} 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.\textsuperscript{32,49} The assembly of large, high (G + C) bacterial DNA fragments can be done in yeast.\textsuperscript{50} 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.\textsuperscript{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.\textsuperscript{37,58} 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.\textsuperscript{51} 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{61,62} Subsequent engineering work could use this high-performance platform for downstream product innovation, including biosynthesis of high quality biodiesels.\textsuperscript{61–63} S. cerevisiae has been found more suitable than E. coli for bioengineering and has been engineered to produce biofuels.\textsuperscript{27,64–65} S. cerevisiae is also an important industrial host for production of enzymes, pharmaceutical and nutraceutical ingredients and, recently, commodity chemicals.\textsuperscript{6} Li et al. engineered S. cerevisiae for overproduction of fatty acids.\textsuperscript{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
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.\(^6^7,^6^8\) 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.\(^6^9\)

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.\(^7^0\) 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,\(^7^1\) *Acinetobacter calcoaceticus* Acr1,\(^7^2\) *Oryza sativa* DPW,\(^7^3\) and *Synechococcus elongatus* AAR,\(^7^4\) four fatty acyl-CoA/ACP reductase have been selected and purified. According to earlier work,\(^3^7\) 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.\(^7^4\) 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.\(^7^5\) The mevalonate (MVA) pathway and methylerythritol-phosphate (MEP) pathway are responsible for the synthesis of the two isoprenoid building blocks, 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.\(^2^7,^7^8\) 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...
(lycopene and carotenoids) and industrial (isoprene) products, and even as precursors of farnesene, the next generation jet fuel. 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. Acetoacetyl-CoA thiolase (atoB), idi and farnesylpyrophosphatesynthase (ispA) genes were amplified from E. coli genomic DNA. 3-Hydroxy-3-methylglutaryl-CoA synthase (erg13 or hmg), a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase (thmg1), 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. 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.

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. 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, anticancer and anti-inflammatory activities. Zhu et al. used the MVA pathway developed for the biosynthesis of farnesene system 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. 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. In order to improve the efficiency of astaxanthin biosynthesis, Ma et al. constructed a highly efficient targeted engineering carotenoid synthesis platform in
E. coli, and the production of astaxanthin in a heterologous host reached 8.64 mg g\(^{-1}\) dry cell weight.

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

As reported, polyketide synthases (PKS) and fatty acid synthases are remarkably similar with regard to their underlying mechanisms. 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. 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. 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 over-producing single form pentadecaheptaene (PDH) followed by its hydrogenation to pentadecane (PD). 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. Finally, the single form of C15 alkane was achieved and the highest titer was reached at 140 mg L\(^{-1}\), and the best SgcE10:SgcEratio 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 derivative 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 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, 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 in 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. 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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