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Current Medicinal Chemistry, **2020,** *27,* **1-9**

REVIEW ARTICLE

Strategies for the Biosynthesis of Pharmaceuticals and Nutraceuticals in Microbes from Renewable Feedstock

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Abstract: ***Backgrounds*:** Abundant and renewable biomaterials serve as ideal substrates for the sustainable production of various chemicals, including natural products (*e.g.*, pharmaceuticals and nutraceuticals). For decades, researchers have been focusing on how to engineer microorganisms and developing effective fermentation processes to overproduce these molecules from biomaterials. Despite many laboratory achievements, it remains a challenge to transform some of these into successful industrial applications.

***Results*:** Here, we review recent progress in strategies and applications in metabolic engineering for the production of natural products. Modular engineering methods, such as a multidimensional heuristic process markedly improve efficiencies in the optimization of long and complex biosynthetic pathways. Dynamic pathway regulation realizes autonomous adjustment and can redirect metabolic carbon fluxes to avoid the accumulation of toxic intermediate metabolites. Microbial co-cultivation bolsters the identification and overproduction of natural products by introducing competition or cooperation of different species. Efflux engineering is applied to reduce product toxicity or to overcome storage limitation and thus improves product titers and productivities.

***Conclusion*:** Without dispute, many of the innovative methods and strategies developed are gradually catalyzing this transformation from the laboratory into the industry in the biosynthesis of natural products. Sometimes, it is necessary to combine two or more strategies to acquire additive or synergistic benefits. As such, we foresee a bright future of the biosynthesis of pharmaceuticals and nutraceuticals in microbes from renewable biomaterials.

ARTICLE HISTORY

Received: July 16, 2018
Revised: September 06, 2018
Accepted: October 18, 2018

DOI:
10.2174/0929867327666200212121047

Keywords: Natural products, secondary metabolites, metabolic engineering, synthetic biology, modular engineering, dynamic regulation, microbial co-cultivation, efflux engineering.

1. INTRODUCTION

The tremendous diversity of natural products is unmatched by those produced by chemical synthesis. Of 1562 new chemical entities approved by FDA between 1981 and 2014, 60% were natural products or their analogs [1]. As medicines and nutraceuticals, these natural products have high economic values and command large markets. Currently, fermentation processes, chemical synthesis or the combination of the two approaches (or semi-synthesis) supply the bulk of the natural products. For example, antibiotics are predominantly produced by fermentation using bacteria (*e.g.*, *Streptomyces*) and fungi (*e.g.*, *Penicillium*) [2, 3]. Compared to total chemical synthesis, microbial fermentation has several benefits: efficient production of enantiopure compounds, critical for pharmaceutical applications, and the use of sustainable biomaterials that are environmentally friendly, avoiding the use of toxic solvents and catalysts [4].

However, microbial fermentation has limitations and the major one is the relatively low titers and yields in production. Currently, biological conversion rates are usually lower than those of chemical conversion and bio-products are often more diluted [5]. The challenges of biomanufacturing of natural products lie partially in the complexity of biological systems, such as intricate cellular metabolism and multilayer regulation networks at the transcriptional, translational and post-

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0929-8673/20 $65.00+.00

translational levels [6, 7]. To overcome these challenges, metabolic engineers and synthetic biologists have developed innovative methods and toolboxes. These methods either bypass the biological complexity or elegantly confine it to more controllable subsystems. Here, we review the recent advances in metabolic engineering strategies and their applications, focusing on the production of pharmaceuticals and nutraceuticals.

2. STRATEGIES IN METABOLIC ENGINEERING

In the 90s, there was limited information about genes, genomes and metabolism of many organisms. This severely limited the development of sophisticated tools and methods to engineer cells. It is arguable that two recent and unprecedented technological advancements have shaped the landscape of biotechnology. One of these advancements is the development of novel DNA sequencing technologies, which has enabled the determination of genome sequences of numerous organisms [8]. Since the first genome was sequenced by the classical Sanger method [9], the next-generation sequencing (NGS) technologies have provided remarkable means to determine sequences at breathtaking speed and at rapidly declining cost. The current third-generation nanopore technology is providing even longer read lengths and at even lower cost [10].

The second advancement is DNA synthesis technologies that enabled the generation of large pieces of DNA and the assembly into genomes at an affordable cost [11]. The cost of synthesis per base pair was as high as $10 in 1998 as compared to the current cost of less than $0.02.

These unprecedented technological advancements have affected the rate of discoveries in fundamental sciences and knowledge gained to direct the biological engineering of cells and organisms. Specifically, we have now deciphered numerous biosynthetic pathways of desired natural products in more detail, that allow us to employ targeted and rational engineering approaches to improve, rewire and even redesign microbes more effectively and efficiently.

Here we will discuss four specific strategies for the metabolic engineering of microbes to produce natural products from a variety of biomaterials. The first three (modular engineering methods, dynamic control of pathways and microbial co-cultivation) are directly related to metabolic pathway design and optimization. The fourth one is related to detoxification or desaturation of end products by using efflux transporters.

2.1. Modular Engineering Methods of Metabolic Pathways

Metabolic pathway design/optimization is arguably the most direct and effective way to overproduce different chemicals. Previously, a relatively small number of genes are manipulated as there are limited tools available for genetic engineering. With the advancements in DNA sequencing, synthesis and genome editing technologies, it is nowadays relatively easy to construct a large number of genes to synthesize complex natural products, such as antitumor taxol precursors [12, 13], analgesic opioids (*e.g*., morphine and hydrocodone) [14-16], antioxidant astaxanthin [17] and lycopene [18, 19], antimalarial artemisinin [20] and its precursor amorphadiene [21-24]. Yet, it is not trivial to control the expression of multiple genes in order to redirect and maximize the carbon fluxes towards final products. This challenge to regulate gene expression is mainly because in cells, different genes can have different expression levels and the enzymes have different kinetic parameters (*e.g*., hundreds even thousands fold difference in turnover numbers k_{cat}/K_m) and half-lives. In view of the complexity of biological regulations, it becomes routine to design and use synthetic promoters and 5' untranslated regions (5'UTRs) including ribosome binding sites (RBSs), thus minimizing endogenous cellular regulations. Such libraries of regulatory elements can be easily characterized using fluorescence proteins [25]. In addition, it is challenging to measure *in vivo* enzymatic kinetics activities to enable the construction of predictable mathematical models [26]. Thus, combinatorial approaches using a diverse library of different regulatory elements that control pathway genes are more pragmatic than purely model-guided pathway designs.

Generally, there are two common ways to produce such a combinatorial expression library. The first is to construct a library where each gene has the full combination of different regulatory elements (promoters, 5'UTRs, *etc*.). With this design, Smanski *et al*. built genetic permutations of the nitrogen fixation gene cluster (16 genes) in *Escherichia coli* that recovered 57% of wild-type activity of *Klebsiella oxytoca* [27]. This approach is comprehensive but labor- and cost-intensive and requires a high throughput system for both construction and screening of libraries. Besides, the library size increases exponentially with the number of genes in the pathway. The second way is to categorize the multiple genes into distinct modules and to control these modules independently instead of individual genes. One typical example is the multivariate

modular metabolic engineering (MMME) method that divides the whole pathway into the upstream methylerythritol-phosphate pathway module and downstream terpenoid module. By controlling the modules independently, the production of taxadiene (a precursor to Taxol) was improved by 15,000-fold [12]. Such modular approaches can be optimized using statistical experimental design to further decrease the experimental workload. Zhang *et al*. successfully used an experimental design and regression models to optimize a four-module system for amorphadiene production [22].

These modular methods significantly reduce the size of combinatorial libraries to be explored. However, it has a limitation that the expression of intra-modular genes may not be controlled. To resolve this, Zhang *et al*. developed a superior modular approach, a multidimensional heuristic process (MHP, Fig. **1A**), to learn and identify optimal control sub-systems. Similar to other modular methods, MHP first assembles combinatorial libraries using predefined regulatory elements. In the MHP, modules are controlled separately at different dimensions. First, two dimensions are gene copy number and transcriptional controls that cover all the genes along biosynthetic pathways. The third dimension is tuning the translation efficiency of a single gene in a specific module by distinct 5'UTRs. Lastly, enzyme variants are integrated into the system to enhance performance. Consequently, MHP not only reduces the combinatorial library size but maintains the flexibility of controlling gene expression individually. MHP expands the dimensions in control and solution space and simultaneously improves resolution. As a proof-of-concept, MHP was successfully applied to improve the production of astaxanthin, linalool and nerolidol to high titers and yields [17].

2.2. Dynamic Control of Metabolic Pathways

In addition to modular engineering approaches, another effective strategy is the use of sensor-response promoters to achieve dynamic control of metabolic pathways. Dynamic pathway regulation is built on a biosensor capable of identifying signal molecules (such as intermediate metabolites) and a responding regulator that controls enzyme expressions accordingly (Fig. **1B**) [21]. To our knowledge, the first example was reported in 2000 by Liao's group. They developed a dynamic control system adapted from Ntr regulon. The system controls the expression of metabolic pathway enzymes to produce lycopene (a nutraceutical) according to acetyl phosphate concentration [28]. A second example is the development of stress-responsive promoters to toxic intermediate metabolites. As the accumulation of farnesyl pyrophosphate (FPP) is known to be toxic to *E. coli* [29], the idea then is to down-regulate FPP synthetic pathway when this metabolite accumulates and to up-regulate the FPP consumption pathway to produce terpenes. Based on the genome-wide transcriptional analysis, Dahl *et al*. identified FPP stress-responsive promoters and applied them to improve amorphadiene production by dynamically regulating the FPP synthetic and consumption pathways [21]. In addition, Xu *et al*. developed a dynamic control system for the production of fatty acids using malonyl-CoA-responsible sensors [30] and the example has been briefly summarized previously [31]. Gupta *et al*. devised a quorum-sensing system that could dynamically control the expression of target genes according to cell density [32]. With this system, they identified the optimal point to redirect glycolysis flux into the production of myo-inositol or glucaric acid which improved the titers and yields greatly. For more examples of the dynamic control strategy, a recently-published review article can be reviewed [33].

Despite the methodological difference, modular pathway engineering and dynamic pathway regulation share a common goal – reducing the accumulation of toxic intermediates and maximizing the carbon flux to products. The former has advantages such as simplicity of design and ease of analyses with experimental design and system modelling [22, 34], but lacks in the dynamic response to biomass or intermediate metabolites. The latter has advantages of the accumulation of toxic intermediate metabolites dynamically, thereby decreasing metabolic burden in the cells. However, it can be a challenge to design suitable dynamic controls for complex biosynthetic pathways, which require the coordination of multi-sensors to attain a finely tuned system.

2.3. Microbial Co-cultivation

In microbial ecosystems, different species communicate and interact in complex manners. Combinations for two successful interacting partners can be classified into any of these non-exclusive patterns: win-win relationship (mutualism), loss-win interactions (predator-prey and host-parasite relationship), commensalistic relationship (one partner benefits but not affecting the other), amensalism (one partner is harmed without any advantage to the other) and loss-loss relationship [35]. Co-cultivation of microorganisms in a fermenter mimics some form of the natural microbial community but

usually more simplified with commonly two species, due to the difficulty in maintaining a stable ratio of the species over time. In this situation, many silent gene clusters of secondary metabolites are activated, and thus producing significantly more natural products than isolated cultivation [36]. As a result, microbial co-cultivation has been widely used for the identification of novel natural products where cryptic biosynthetic pathways may be activated to produce antitumor compounds and antibiotics [37-39].

In addition, co-cultivation has been applied to overproduce secondary metabolites (Fig. **1C**). Zhou *et al*. designed a mutualistic system between *E. coli* and *Saccharomyces cerevisiae* to produce oxygenated taxanes precursors to anti-cancer Taxol [13]. The mutualism was established with *E. coli* producing acetate from xylose to feed yeast directly. As acetate accumulation inhibits *E. coli* growth, the consumption of acetate by yeast benefits both *E. coli* and yeast. Broadly, the microbial consortium could be applied to produce other valuable metabolites using sugar mixtures [40] or even complex cellulosic biomass [41]. As it segments the long biosynthetic pathway into the two species, the main advantage of the co-culture system is that each pathway module can be constructed and optimized in parallel and thus significantly reducing workload. In addition, it can take advantage of the unique properties of different microbes [13] and the co-utilization of sugar mixtures [40].

It is, however, not trivial to maintain the stability of the mix population in microbial consortia, especially with polycultures (>2 strains) [42] and at the industrial fermentation scale. In addition, effective efflux of the intermediate metabolite from one host and its effective uptake by another host are critical prerequisites for the success of co-culture systems. These features will constrain the application of co-culture approaches.

2.4. Efflux Transporter Engineering

The accumulation of toxic intermediate metabolites poses a challenge to metabolic engineering. It restricts the production capacity per cell due to limited intracellular space and also reduces biomass in bioreactors. A promising solution to this challenge is to export toxic intracellular products from microbial cells. Natural evolution has endowed microbes the capability to secrete toxic xenobiotics using efflux transporters. This can be similarly exploited to export some of the toxic metabolic intermediates. Interestingly, a large number of proteins in the cellular proteomes are membrane transporters involved in the influx and efflux of chemical compounds of diverse structures. Among the 5690 proteins in the proteome of *S. cerevisiae*, about 300 (~5%) code for known or predicted membrane transporters [43]. In *E. coli*, about 33% and 7% of inner membrane proteins are influx and efflux transporters, respectively [44].

Efflux transporters are classified into five different families, the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family [45]. The energy of ABC transporters comes from ATP hydrolysis, while the others are driven by proton motive forces or Na^+, K^+ gradients. Different families of efflux transporters commonly have different substrates, and some (*e.g*., ABC and RND families) can have very diverse substrates. In *E. coli*, AcrAB-TolC (RND families) is well studied and is capable of secreting a large number of substrates, including many antibiotics (chloramphenicol, lipophilic β-lactam antibiotics, tetracycline, nalidixic acid, novobiocin, and rifampin, *etc*.), acriflavine, ethidium bromide, bile salts and short-chain fatty acids. Hence, the AcrAB-TolC system or the engineered forms of the complexes have been used to improve the efflux of terpenes and biofuels [23, 46-48].

The functional relevance of a certain efflux transporter for the desired product is commonly identified by two approaches (Fig. **1D**). The first is a competitive growth method where a pool of cells expressing different efflux transporters, natives or mutants, is exposed to toxic substances. Those cells expressing the appropriate efflux transporters will then outgrow the others and will gradually dominate the population. The cells are then isolated and sequenced to identify the transporters. With this approach, Dunlop *et al*. identified the pumps that could improve the tolerance of *E. coli* to geranyl acetate, geraniol, α-pinene, limonene or farnesyl hexanoate [46]. In addition, the overexpression of a pump from *Alcanivorax borkumensis* increased the limonene production. Similarly, Foo and Leong identified several ArcB variants from a single-site mutant library that has superior secretion capability of octane and α-pinene [47].

The second strategy is the genome-wide scanning of native efflux transporters by single-gene knockout/knockdown or transcriptomic analysis. By disrupting a single gene (*e.g*., by a transposon, CRISPR-Cas9 or other homology recombination methods), the effect on metabolite tolerance or the production of intracellular and extracellular metabolites is compared between

wildtype and mutant strains. Thus, this allows the identification of endogenous efflux transporters that are responsible for the secretion of metabolites. Further validation can be carried out by the overexpression of these transporters to increase the production and productivities of metabolites. With this strategy of single-gene knockout, Zhang *et al*. identified that tolC is responsible for the secretion of amorphadiene in *E. coli*. The overexpression of tolC and its related multidrug-resistant transporters improved the production of amorphadiene [23]. Transcriptomic studies have also identified transporters capable of secreting isopentenol in *E. coli* [49], *p*-hydroxybenzoate in *Pseudomonas* [50] and alkanes in *S. cerevisiae* [51].

Efflux transporter engineering is now a useful practice to reduce the toxicity of some metabolites and has been used to enhance carotenoid production in *E. coli* [17, 52]. However, to identify suitable efflux pumps for a particular toxic product can be challenging as we have yet to structurally predict and match the products with particular pumps. In addition, membrane transporters are known to be difficult to engineer as the overexpressed mutants are often insoluble and tend to aggregate intracellularly. Thus, fine-tuning of the expression of efflux transporters is critical to maximizing product secretion but yet to avoid toxicity when overexpressed.

3. SYNERGISTIC APPLICATIONS OF DIFFERENT STRATEGIES

Every method has its advantages and limitations. It is sometimes necessary to combine two or more orthogonal strategies to acquire additive or synergistic benefits. There are many successful examples; two particular strategies are highlighted here: (1) combining enzyme engineering and metabolic pathway optimization and (2) integrating genetic engineering and abiotic optimization.

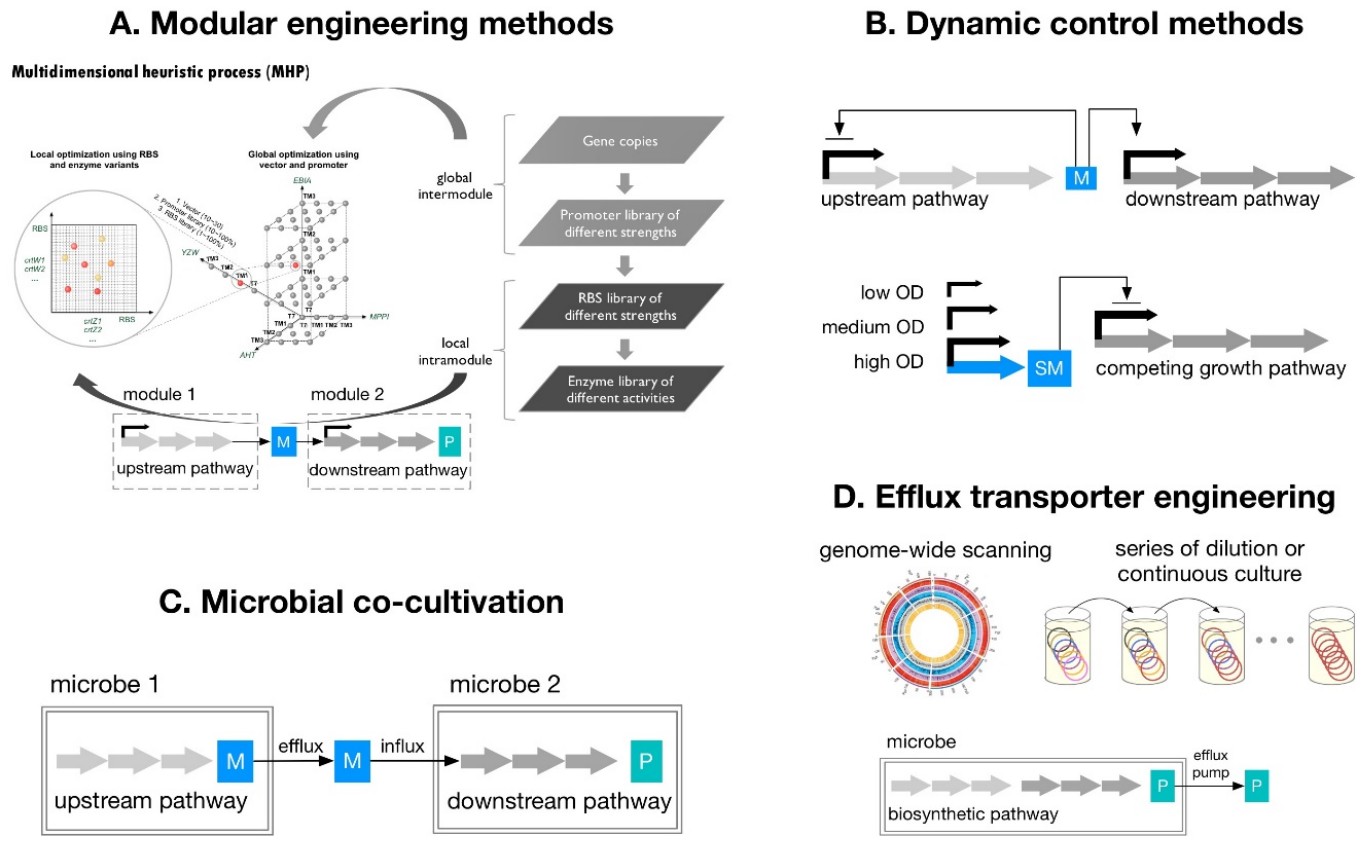

Fig. (1). Different metabolic engineering strategies. (**A**) Modular engineering methods, adapted from the multidimensional heuristic process [17], where the biosynthetic pathway genes are segmented into different modules. (**B**) Dynamic pathway control methods using stress-responsive promoters or quorum-sensing circuits [21, 32] to realize autonomous adjustment. (**C**) Microbial co-cultivation, where biosynthetic pathways are divided and expressed in two or more co-cultivated microbes. (**D**) Efflux transporter engineering, where products are secreted into media aided by appropriate efflux pumps. Abbreviation: M- metabolite, SM – signal molecule, P- product. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

3.1. Integrated Enzyme Engineering and Metabolic Pathway Optimization

Enzyme engineering, such as directed evolution strategy, has been widely used to improve enzyme activity, stability, or to change selectivity. Such a method is very powerful and can improve side activity or selectivity of enzymes with non-natural substrates. Oscar *et al*. adopted directed evolution to improve the thermostability and alkali tolerance by $>10^6$-fold of carbonic anhydrase [53]. A mammalian paraoxonase 1 was evolved to acquire a 10^5-fold increase in catalytic activity on cyclosarin [54]. The metabolic engineering toolbox often is limited by the availability of efficient natural enzymes. For cases where the key enzyme has very low intrinsic activity, improving the specific enzymatic activity by protein engineering is superior to merely overexpress the native enzyme. The strategy of combining enzyme engineering and metabolic pathway engineering has been applied in the production of many natural products. For example, such a strategy was used to improve the production of levopimaradiene, a precursor to pharmaceutically important ginkgolides, where a 2600-fold increase in titer was achieved [55]. Zhang *et al*. also adopted a similar approach to improve the production of apocarotenoids with a 1000-fold and 3700-fold increase in yields of α- and β-ionone, respectively [19].

In addition, enzyme engineering can also be integrated into metabolic engineering for designing synthetic pathways with desired features (such as novel applications, higher theoretical yields or using more abundant cofactors). By introducing three point mutations, a methylsuccinyl-CoA dehydrogenase that originally uses flavoproteins and ubiquinone for electron transfer was mutated into a methylsuccinyl-CoA oxidase that uses oxygen as electron acceptors directly. This novel methylsuccinyl-CoA oxidase was successfully used in a synthetic pathway for carbon dioxide fixation [56]. Similarly, a pyruvate dehydrogenase-bypass pathway was constructed in *S. cerevisiae* that enabled a higher yield and reduced oxygen requirement of farnesene production [57]. These are excellent examples to demonstrate the power of the strategy of combining enzyme engineering and metabolic engineering.

3.2. Integrating Bioprocess Development with Strain Engineering

Many great achievements in laboratories fail when scaling up into industrial processes. One of the main reasons is the lack of low-cost, robust and compatible bioprocesses for the strains. Thus, it is critical to develop a robust and compatible bioprocess for the engineered strains at an early stage. An effective strategy is engineering microbial strains to acquire competitive advantages for the use of xenobiotic substrates (*e.g*., carbon sources or nitrogen sources), thus eliminating contamination from other microbes and assuring robustness in bioprocesses. Shaw *et al*. developed the strategy "robust operation by utilization of substrate technology" (ROBUST) [58]; with ROBUST, they engineered an *Escherichia coli* strain able to assimilate melamine and the yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* able to assimilate nitrogen from cyanamide and phosphorus from potassium phosphite. These strains, when grown in these special media, outperformed contaminating strains in bioreactors. Similarly, Zhang *et al*. developed a chemically defined medium for a phosphotransferase system deficient *Escherichia coli*, which had a superior performance for the production of isoprenoids [18]. The success of these examples evidenced the importance of the strategy integrating bioprocess development with strain engineering in translating into successful industrial production.

CONCLUSION

Global trends towards green and sustainable developments are driving the innovation in biotechnology, especially metabolic engineering and synthetic biology. We foresee even more petrochemical processes that will gradually be replaced by bioprocesses (fermentation and enzymatic reactions) that are sustainable, green, of high product purity and low wastes and also require lower capital investments [5]. Here, a couple of recent metabolic engineering strategies are discussed, with special emphasis on their applications in the production of pharmaceuticals and nutraceuticals. Particularly, we underscored the importance of targeted metabolic pathway design and optimization. Modular engineering approaches, dynamic control and microbial co-cultivation are different strategies to address the unmet needs for the production of valuable substances from biomaterials. In addition, the efflux transporter engineering method is also highlighted that can alleviate the intracellular toxicity of some accumulated products. We further highlighted the effectiveness of integrating different strategies, such as combining enzyme engineering and metabolic engineering and integrating bioprocess development (abiotic conditions) with strain engineering. In addition, many other combinations can be conceived to produce synergistic benefits, such as integrating modular engineering approaches and efflux

transporter engineering. As such, we expect a bright future for the biosynthesis of natural products using microbial cells.

LIST OF ABBREVIATIONS

NGS	=	Next generation sequencing
5' UTR	=	5' Untranslated regions
RBS	=	Ribosomal binding site
MMME	=	Multivariate modular metabolic engineering
MHP	=	Multidimensional heuristic process
FPP	=	Farnesyl pyrophosphate
ABC family	=	ATP-binding cassette family
MFS family	=	The multidrug and toxic-compound extrusion family
SMR family	=	Small multidrug resistance family
RND family	=	The resistance nodulation division family
ROBUST strategy	=	Robust operation by utilization of substrate technology

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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