Toward fine-tuned metabolic networks in industrial microorganisms

Ning Li a,b,c, Weizhu Zeng a,b,c, Sha Xu a,b,c, Jingwen Zhou a,b,c,*

a Key Laboratory of Industrial Biotechnology, Ministry of Education and School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China
b National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China
c Jiangsu Provisional Research Center for Bioactive Product Processing Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu, 214122, China

ARTICLE INFO

Keywords: Fine-tuned regulation Protein engineering Upregulation Downregulation Dynamic regulation

ABSTRACT

There are numerous microorganisms in nature capable of synthesizing diverse useful compounds; however, these natural microorganisms are generally inefficient in the production of target products on an industrial scale, relative to either chemical synthesis or extraction methods. To achieve industrial production of useful compounds, these natural microorganisms must undergo a certain degree of mutation or effective fine-tuning strategies. This review describes how to achieve an ideal metabolic fine-tuned process, including static control strategies and dynamic control strategies. The static control strategies mainly focus on various metabolic engineering strategies, including protein engineering, upregulation/downregulation, and combinatorial control of these metabolic engineering strategies, to enhance the flexibility of their application in fine-tuned metabolic networks. Then, we focus on the dynamic control strategies for fine-tuned metabolic networks. The design principles derived would guide us to construct microbial cell factories for various useful compounds.

1. Introduction

Microorganisms are widely used for the industrial production of useful chemicals. In the early stage of industrial biotechnology, high-producing strains are screened from the natural environment; however, natural microorganisms usually produce low titers and yields of target products. Additionally, accumulation of byproducts not only decreases the yield, but also makes the downstream process difficult. Mutagenesis and efficient screening techniques can significantly enhance microorganism performance [1]; however, the types of the products capable of being produced remain limited. To address these issues, engineering the microorganisms to achieve efficient production of target compounds has progressed, and the advent of genetic engineering has greatly promoted the development of the fermentation industry. Because microbial fermentation involves the actions of multiple enzymes in the microbial metabolic network, Stephanopoulos [2] and Bailey [3] proposed the concept of metabolic engineering. A major challenge in this field is the construction of appropriate metabolic pathways and regulation of the expression levels of specific genes in these pathways.

In earlier studies, directing metabolic flux to target products involved the use of simple metabolic engineering methods, including knockout and overexpression [4], to modify specific pathways. These methods achieved effective accumulation of target products, while reducing byproduct biosynthesis, resulting in high-performance strains. With continued metabolic modification of strains, researchers found that additional modifications could not further improve target-product accumulation, and that such modifications could lead to serious inhibition of cell growth, thereby adversely affecting target-product biosynthesis. Therefore, the balance of cofactors and energy became a topic of interest [5].

To achieve fine-tuned gene regulation in the metabolic network, various fine-tuned methods have become research hotspots in metabolic engineering and synthetic biology. However, fine-tuned metabolic networks rely on powerful and effective methods and has become a significant challenge. The rapid development of metabolic engineering and synthetic biology allowed the emergence of a variety of gene-regulation methods, some of which are used for gene fine-tuning (e.g., RNAi [6], multivariate modular engineering [7], and CRISPRi [8]). In this review, we first describe what is an ideal fine-tuned metabolic process and the current challenges to building the fine-tuning of metabolic networks, then summarized the relevant static strategies, including protein engineering strategies, upregulation/downregulation strategies and combinatorial control of these metabolic engineering strategies. Finally, we focus on the dynamic regulation of these strategies to realize the construction of a fine-tuned metabolic networks. The design principles derived would guide us to construct microbial cell factories for various useful compounds.
factories for various useful compounds.

2. Current challenges to the fine-tuning of synthetic metabolic networks

An ideal metabolic fine-tuned process needs the cooperation of many elements, including sensor, transducer, actuator, resulting in a sensor-transducer-actuator system [9]. The sensor can sense environmental change, then the transducer convert signal to the actuator, the actuator actuat transcriptional events to compensate for metabolic activity. Many of these synthetic systems make up an ideal fine-tuning synthetic metabolic network (Fig. 1). The rapid development of metabolic engineering and synthetic biology has allowed the biosynthesis of multiple compounds; however, challenges still remain. The biosynthetic pathways of some compounds remain unknown or are unattainable in some microorganisms. Therefore, an important task involves constructing biosynthetic pathways. Additionally, rate-limiting steps and regulatory factors need to be discovered in order to allow fine-tuning of metabolic pathways (Fig. 2).

2.1. Metabolic pathway construction

Natural existed microorganisms can biosynthesize a wide variety of compounds. However, many of them can only produce little desired compounds and be hard to metabolic engineering, even there are many compounds that fail to find the appropriate biosynthetic pathways in microorganisms. It is premature to discuss how to fine-tune the metabolic network of these compounds which fail to find the appropriate biosynthetic pathways. Therefore, to obtain strains capable of high yields of target compounds, construction of biosynthesis pathways is the first step. Currently, enzymes involved in biosynthesis of target compounds are mostly screened from different sources [10]. The heterologous expression of enzymes may result in products suffering from improper folding, lack of activity, and poor specificity [11], all of which remain challenges to metabolic pathway construction. In addition to enzyme screening and selection, protein engineering provides avenues to address these challenges [12].

**Fig. 1.** Ideal fine-tuning metabolic engineering processing. A complete metabolic network contains multiple elements, including the production of target-product, enzyme, cofactor, and an energy source. The concentration of these elements affects the expression level of related genes or the activity of related enzymes. Fine-tuning of which plays an important role in improving target-product accumulation and reducing the byproduct synthesis.
Fig. 2. Current challenges for the fine-tuning of metabolic networks. (A) Construction of metabolic pathway, includes screening and identification of enzymes involved in the pathway. (B) Identifying rate-limiting steps. (C) Construction of dynamic regulation system, includes response to the changes of target products and by-products, then dynamically up-regulate or down-regulate the related gene expression.
2.2. Identifying rate-limiting steps

Target-compound yield is dependent on enzyme-specific rate-limiting steps. To fine-tune metabolic networks, it is necessary to identify these steps and engineer alternative pathways to address their shortcomings in order to maximize target-product yield. Using CRISPR technology, it is possible to identify whether specific genes play an important role [13], and determination of intermediate metabolites also represents an intuitive method of identifying rate-limiting steps.

The development of -omics techniques enables detection and analysis of various factors and cellular metabolic components. Such methods include transcriptome-based identification of rate-limiting steps for feedback inhibition of yeast in order to increase lipid production [14], proteomics-based identification of two rate-limiting activities associated with inefficient isopenotenol production [15], and comparative metabolomics to identify key metabolites involved in FK506 production [16]. Monitoring metabolic dynamics is an important step in understanding how cells respond to environmental changes on a real-time scale, which was accomplished by combining high-resolution mass spectrometry to identify evidence of switch-like feedback inhibition in amino acid biosynthesis [17]. Static and dynamic -omics methods can diagnose rate-limiting steps but can only analyze specific metabolic pathways. Complex cellular environments limit their ability; therefore, development of more powerful tools is necessary.

2.3. Dynamic regulation system construction

Currently, many regulatory strategies for fine-tuning metabolic networks focus on the static level, which leads to imbalanced metabolism under various environmental conditions. Dynamic regulation can maintain balanced metabolic flux. QS is a commonly used dynamic regulatory technique [18], but it might not be sufficient for the diversity of metabolic networks. Besides, other methods such as riboswitches and biosensors also are used to construct the dynamic regulation system [19,20]. Development of methods for dynamic regulation is a slow process, and understanding native dynamic regulation networks in microorganisms is the basis for this development and very complicated. We believe that development of a dynamic metabolic regulatory system will maximize the synthesis of target products in microorganisms.

3. Static control strategies

3.1. Protein-engineering strategies

Target products can be synthesized efficiently in microorganisms according to specific metabolic pathways; however, some hosts lack enzymes related to target-product biosynthesis or harbor enzymes exhibiting low activity and high degrees of promiscuity. Here, we discuss acquisition of optimal enzymes using protein-engineering methods, including protein bioprospecting and directed evolution and rational engineering of proteins (Fig. 3). These protein-engineering strategies will help researchers to construct a series of biosynthetic pathways of compounds and even improve their biosynthesis efficiency [21].

3.1.1. Screening of specific proteins from the natural environment

Many useful compounds exist only in higher-level organisms, and wide availability of such compounds is limited by currently inefficient and high-cost extraction methods. Therefore, to produce these products efficiently by microorganisms, de novo designing optimal pathways for the products by screening of specific proteins from natural environment are essential [22]. However, the enzymes from different sources have different properties in the host; screening of the sources is also essential. To develop a pinoresinol bioconversion cascade in Escherichia coli, suitable enzymes needed to be identified for scavenging H₂O₂. Lv et al. [23] evaluated all available E. coli peroxidases from PeroxiBase and used these as queries against the E. coli BL21(DE3) genome sequence through BLAST, ultimately identified two peroxidases displaying efficient activity from eight that were screened from 20 homologous sequences. After genome sequencing, alignment, verification, and annotation of the Ketogulonicigenium vulgare WSH-001 genome, different combinations of five L-sorbosone dehydrogenases (SDHs) and two L-sorbosone dehydrogenases (SNDHs) from K. vulgare WSH-001 were introduced into Gluconobacter oxydans WSH-003 for conversion of D-sorbitol to 2-keto-L-gulonic acid to replace the conventional two-step fermentation process with a one-step process, resulting in a good combination of SDH and SNDH [24]. The cited study successfully constructed the metabolic pathway for target products by using currently existing enzymes. However, these successful examples were limited by the library of natural enzymes. Furthermore, insolubility of heterologous proteins can be a major obstacle to efficient biosynthesis of target products. For these limitations, additional efficient enzyme-engineering strategies need to be developed.

3.1.2. Directed evolution of proteins

Introducing exogenous biosynthetic pathways into microorganisms can enable target-product production, such as the cases of caffeyl alcohol [25] and dihydrochalcones [26]. However, it is possible that some enzymes are incapable of such synthesis due to low substrate-binding capacity, temperature sensitivity, low catalytic efficiency, and promiscuity, resulting in low or no production of target compounds. Protein engineering describes the process of altering the structure of an existing protein to improve its properties [27], and includes three steps: selection of mutation sites, application of mutation methods, and evaluation of modification results. In cases of minimal knowledge regarding the protein structure, there will be a lack of a theoretical basis for the selection of mutation sites; therefore, directed evolution represents a suitable strategy.

Directed evolution [28] is a method that involves simulating and accelerating the natural evolution of enzymes in the laboratory. First, a mutation library of protein-coding genes is constructed using random-mutation methods, such as DNA shuffling [29], a staggered extension process [30], random-priming in vitro recombination [31], error-prone polymerase chain reaction (epPCR) [32], and rapidly efficient combinatorial oligonucleotides for directed evolution [33]. An efficient screening method was established for screening dominant mutants from a mutant library according to the intended experimental purposes. The tyrosine phenol lyase (TPL) can be used in the biocatalytic synthesis of L-DOPA, and TLP with high catalytic activity have been reported from several microbes, but substrates and product strongly inhibited TPL activity, resulting in the production of L-DOPA lower than expected. Using TPL derived from Erwinia herbicola, Zeng et al. [34] constructed a mutant library by epPCR. Then a high throughput screening method was established to screen high activity TPL, resulting in a L-DOPA high-producing strain. Additionally, to rapidly screen target mutants, a suitable reporter strain was established to perform screening in combination with fluorescence-activated cell sorting [35]. Directed evolution strategy can obtain enzymes with better performance, but it is also time-consuming and laborious. To reduce the workload, the most important strategy is either constructing an efficient screening method or reducing the size of the library.

3.1.3. Rational engineering of proteins

To obtain a protein that meets specific requirements, screening and directed evolution of existing proteins are common methods. Moreover, directed evolution does not require a thorough understanding of the structure and catalytic mechanism of the target enzymes, making it useful for engineering of many proteins. After a blind random mutation, a large library is constructed, followed by screening, with the screening workload determined the efficiency of the method. Therefore, a rational method that can reduce the size of the library needs to be established in order to spend the least amount of time achieving the desired goal. For
this process, a thorough knowledge of the target protein is necessary for selection of important regions for engineering. Given the increased availability of protein structures, rational design and rational engineering methods have been widely applied to augment de novo protein design [36–38]. One-carbon assimilation pathway is a very important part of ecosystem, but it is difficult to introduce heterologous one-carbon assimilation pathway into the organisms that lack it, so that one carbon compound is the sole carbon source. Siegel et al. [39] created a new enzyme named formolase by de novo computational design which can catalyzes the carboligation of three formaldehyde into the central metabolite dihydroxyacetone phosphate (DHAP), resulting in a new pathway. Although protein engineering can change enzyme performance, the degree of the change is sometimes limited, suggesting that further improvements to these methods are required.

3.2. Upregulation strategies

To improve the metabolic flux of specific pathways in
microorganisms, the most common method used involves enhanced expression of the specific genes involved in this pathway. Fig. 4 describes upregulation strategies, including vector-based, promoter-based, ribosome-binding site (RBS)-based, and codon-based strategies, that are involved in altering gene copy number, transcription initiation, translation initiation, and translation extension, respectively.

3.2.1. Vector-based strategies

Genes encode proteins following transcription to mRNA. Some organisms harbor multiple gene copies, which can facilitate high levels of expression under specific conditions. Therefore, increasing gene-copy number in the genome might represent a feasible strategy for upregulating expression levels. However, genome-specific increases in copy number are inefficient. Plasmids are a key aspect of genetic engineering, using a series of plasmids harboring different copy numbers to increase the gene-copy number [40], modular optimization strategies have improved the titer of some compounds, such as fatty acids [41] and terpenes [42]. To optimize (2S)-pinocembrin production from d-glucose, Wu et al. [43] used three modules in four vectors (pCDFDuet-1, pETDuet-1, pACYCDuet-1, and pRSFDuet-1) harboring different copy numbers. Guo et al. [44] designed a metabolic strategy using plasmids for the overexpression of two subunits of pyruvate dehydrogenase components in order to reduce the accumulation of pyruvate. Moreover, to increase the supply of 5-aminolevulinate, a precursor for haem synthesis, Zhao et al. [45] increased the expression of synthetic genes using high-copy plasmids to achieve higher accumulation of haem. Although multi-plasmid strategy can regulate gene-expression levels by increasing gene-copy number, this can also put a burden on strains.

3.2.2. Promoter strategies

Regulation of gene expression can be fine-tuned at various levels, including at the transcription and translation levels [46]. Transcriptional regulation alters metabolic flux by controlling the transcript abundance of key genes at their promoter regions [47]. Recently, increasing efforts have focused on promoter alteration, which attempts to modulate transcriptional capacity through mutation or direct insertion of a different promoter [48]. Different promoters affect transcription differently, making their selection a critical component of metabolic engineering design [49]. Previous studies screened promoters displaying different strength, followed by selection according to the requirements of gene expression in metabolic pathways [50]. Strong promoters can overexpress specific genes. The T7 promoter [51] is a high-strength phage-derived promoters often used for overexpression of heterologous proteins in prokaryotes, whereas the strong constitutive promoters TEF [52], GPD [53], and HXT7 [54] are typically used for metabolic engineering purposes in Saccharomyces cerevisiae. Hu et al. [55] screened promoters by proteomics analysis, resulting in overexpression of D-sorbitol dehydrogenase in Gluconobacter oxydans following incorporation of the new promoter and resulting in increased titers and productivity of l-sorbose synthesis from D-sorbitol. Upregulation of gene-expression levels can be achieved using specific promoters, with different strengths of the promoters capable of regulating expression levels. However, different promoters have different properties in different strains, making their use frequently strain-specific. Additionally, promoter screening is time-consuming, thereby making its practice a limiting factor in engineering processes.

3.2.3. RBS-based strategies

RNA translation involves four steps: initiation, elongation, termination, and ribosome turnover. Translation initiation is the rate-limiting step and determined by multiple interactions, including hybridization of 16S ribosomal RNA to the ribosome binding site (RBS) and binding of translation RNA (tRNA) to the start codon [56]. Efficient translation initiation is primarily determined by the RBS, which comprises the sequence upstream of AUG/GUG in an mRNA transcript and that can be accurately recognized by the ribosome [57]. Therefore, gene upregulation can be achieved by RBSs exhibiting high ribosomal-binding capacity. To upregulate gene expression via 104 promoters with varying strengths, Zhou et al. [58] integrated strong transcriptional promoters harboring strong RBSs. To improve recET expression in
Corynebacterium glutamicum, Wang et al. [59] incorporated RBSs with different strength and ultimately achieved heterologous expression of recET at an appropriate level. By incorporating high-strength RBSs, specific gene expression can be upregulated to fine-tune the regulation of metabolic flux; however, results can differ between strains like promoter. Therefore, the universality of this strategy requires further improvement.

3.2.4. Codon-based strategies

Protein translation occurs following generation of gene transcripts. Promoter strength and mRNA stability determine the levels of intracellular mRNA, which affect translation efficiency [60]. Synonymous codon usage in protein-coding sequences are not random, and codon-usage bias is a distinct feature of the genome of each organism [61]. Recent studies report codon usage as a factor involved in mRNA stability [62]. Besides, each codon is matched to a specific tRNA, and limited concentrations of tRNAs targeting rare codons in mRNAs will reduce the translation rate. Therefore, codon distribution in mRNA influences protein translation and ultimately determines the level of gene expression [63]. Codon optimization can increase protein expression, suggesting that codon usage plays an important role in gene expression [64]. In addition to the influence on gene expression, non-optimal codon usage can toxic to strains. Mittal et al. [65] found many synonymous variants that are toxic to E. coli. Unlike previous studies of the effect of synonymous mutations, the effect is independent of translation, but it depends on the production of toxic mRNA molecules. Finally, the toxicity was prevented by reducing mRNA level. These phenomena showed that codon optimization can improve gene expression, but sometimes it can also cause the production of toxic mRNA, which is not conducive to gene expression.

3.3. Downregulation strategies

During target-product synthesis, some metabolic pathways compete with others for metabolic flux, making it necessary to limit the metabolic flux of competing pathways. Downregulation genes is a conventional method used to accomplish this. In addition to some strategies such as promoter strategies and codon-based strategies similar to up-regulation strategies, there are sRNA-based strategies and CRISPR-based strategies, with Fig. 5 showing strategies used for this activity.

3.3.1. sRNA-based strategies

Regulatory RNAs are key modulators of gene expression through their interactions with DNA, RNA, proteins, and metabolites at every step of gene expression. These regulatory RNAs include synthetic sRNA, RNA interference (RNAi), and antisense RNA (asRNA). sRNA includes a MicC scaffold and a target-specific sequence. The MicC scaffold recruits the Hfq protein, which binds the scaffold to impede translation of the target mRNA bound to sRNA via Watson–Crick base-pairing [66]. RNAi involves activity by the RNA-silencing complex (RISC) to silence translation of specific mRNAs and can be used to fine-tune the regulation of metabolic flux following binding of small-interfering RNA to mRNA targets, thereby restricting RISC-mediated translation. Moreover, asRNA binding of mRNA targets impedes ribosome binding to the mRNA, thereby blocking translation [67].

The 6S RNA in E. coli represents an sRNA that interacts directly with α70-RNA polymerase and is involved in downregulating the expression of many genes [68]. In E. coli, > 80 sRNAs have been identified [69,70], resulting in construction of a database and investigation into their use as regulatory mechanisms in synthetic biology. Clostridium is a promising microbial host for the production of valuable industrial chemicals; however, genetic manipulation of Clostridium is very difficult. Cho et al. [71] developed an sRNA-based system to fine-tune gene expression in Clostridium acetobutylicum, where knockdown of the adhE1 and pta genes decreased acetic acid production and enhanced butanol production. For prokaryotes, such as E. coli, use of sRNA allows rapid downregulation of gene expression [72]; however, in higher-order eukaryotes, RNAi is used to fine-tune gene expression by reducing

Fig. 5. Strategies for downregulating genes in industrial microorganisms. (A) For prokaryotes, gene downregulation can be achieved using CRISPR, CRISPRi, weak-promoter replacement, weak-RBS replacement, rare-code substitution, sRNA, and antisense RNA at the genome, transcription, and translation levels. (B) For eukaryotes, gene downregulation can be achieved using CRISPR, CRISPRi, regulatory sequence modification, weak-promoter replacement, rare-code substitution, and RNA interference at the genome, transcription, and translation levels.
mRNA levels. Crook et al. [73] optimized a yeast RNAi system to construct a series of hairpin RNA-expression cassettes and then used RNAi to quickly identify metabolic pathways associated with itaconic acid production, which also reduced the cost of the design-build-evaluate cycle in yeast. Wu et al. [74] established an efficient asRNA system that balanced the need for malonyl-CoA between cell growth and target-product biosynthesis. Despite its effectiveness, sRNA specificity remains a limitation; therefore, further development of RNA-based strategies is necessary.

3.3.2. CRISPR-based strategies

CRISPR is used as a defensive system for prokaryotes, including bacteria and archaea [75]. In 2013, Cong et al. [76] engineered two different type II CRISPR systems and created CRISPR/Cas9 for use in humans and mice. Mali et al. [77] engineered the type II bacterial CRISPR system in human cells, whereas Hwang et al. [78] reported the ability of the CRISPR-Cas9 system to function in vivo to induce targeted genetic modifications in zebrafish. This method remains a precise technique for genome editing in various biological systems, including humans [79], mice [80], yeast [81], Bacillus subtilis [82], E. coli [83], and Caenorhabditis elegans [84]. CRISPR enables rapid genome editing in order to obtain knockout strains, thereby enabling its use to fine-tune gene expression. In the CRISPR/Cas9 system, Cas9 is a nuclease capable of cleavage of target DNA. The CRISPRi system includes a catalytically inactive Cas9 protein (dCas9), with the dCas9–single-guide RNA capable of binding target DNA but be not able to cut it, resulting in hindered RNA polymerase activity [85]. Sander et al. [86] used CRISPRi to silence candidate genes and increase arginine production.

CRISPR has been a great benefit to synthetic biology and other fields; however, there remain issues concerning off-target effects. Recent versions of the Cas9 protein have been designed, including Sniper-Cas9 [87], eSpCas9(9.1) [88], and SpCas9-HF1 [89], that reduce the off-target efficiency of the CRISPR system. To enhance CRISPR-Cas9 accuracy, Chen et al. [90] designed a new hyper-accurate Cas9 variant (HyCas9) to investigate the mechanism of target discrimination, finding that minor changes in the REC3 domain of Cas9 affected differences in on- and off-target editing. It is expected that continued optimization of the CRISPR system will enhance its role in synthetic biology and metabolic engineering.

3.4. Combinatorial control strategies

For multi-gene pathways, it is very difficult to balance metabolic flux. Flexible use of the above up/down regulation strategies would be an important and innovative method. To increase fatty acid production in E. coli, Xu et al. blocked the fatty acid degradation pathway and relieved the allosteric inhibition of acetyl-CoA carboxylase, but only a natorial control.

ditional RBS strength. These results indicated that the balance of the metabolic pathways is not controlled by a single factor, but are often regulated coordinately by several factors. Therefore, the combination of several different factors capable of regulating a system will constitute an adequate construction of a dynamic control system.

4. Dynamic control strategies

Metabolic engineering and synthetic biology endow many microbes with the ability to produce a wide variety of useful compounds. To reduce metabolic flow to non-target products, previous studies focused on direct overexpression and knockdown/knockout of target genes [93]. However, some genes are essential for cell growth, and their knockdown/knockout adversely affects cell growth/survival [94]. However, excess overexpression of a gene can also burden microorganisms [95]. Therefore, it is important to use appropriate metabolic regulation methods to regulate genes at the right time in order to avoid unintended consequences.

4.1. Identifying naturally existing dynamic regulatory networks

Microorganisms have evolved exquisitely regulated networks and exhibit a variety of metabolic activation, repression, and regulation activities induced by the surrounding environment [96]. Quorum sensing (QS) is a dynamic regulatory system in bacteria used to coordinate complex population-wide phenotypes through space and time [97]. QS constitutes cell-cell communication achieved through a group of signaling molecules called autoinducers, such as 3-oxohexanoylhomoserine lactone ( AHL) synthesized by LasI [98], and that allows microorganisms to sense their population and actuate gene transcription. Additionally, microbial communities can react to changes in metabolite/substrate levels in a process called substrate-feedback regulation. Cellular malonyl-CoA levels represent a metabolic signal that regulates the expression of genes involved in mediating fatty acid synthesis, such as fabA and fabB in E. coli [99] and fabA in Bacillus subtilis [100]. Furthermore, microorganisms harbor a variety of other regulatory systems that could potentially be utilized in engineering applications.

4.2. Reconstruction of dynamic regulation networks

There are many native biological systems in microorganisms, we can mimic those and use a dynamic regulatory network to eventually construct a microbial cell factory. However, the native biological systems control the metabolic networks of the original strains, and are difficult to fine-tune regulate the new metabolic network directly [101]. Therefore, it is necessary to modify such systems in a way suitable for other strains. Gupta et al. [102] integrated parts of the QS system from Pantoea stewartii into the E. coli genome, resulting in dynamic control of endogenous essential genes involved in glycolysis and aromatic amino acid biosynthesis. Victor et al. [103] reengineered the native QS regulon to guide high-level production of rhamnolipid biosurfactant in Burkholderia thailandensis E264. To address the severe metabolic burden on host cells after genetic modification, QS can be linked with an RNAi module to enable target-gene silencing after reaching high cell-population density [104]. These examples show the applications of QS to enable dynamic regulation of target genes following introduction of an exogenous QS system.

In addition to QS, substrate feedback plays an important role in regulating metabolic networks. Recently, De et al. [105] created chimeric biosensors for the desired luteolin-specific response by introducing five compatible vectors, with varying promoter strength, plasmid copy number, antibiotic resistance marker, and additional RBS strength. These results indicate that balance of the multiple gene pathways metabolism can be achieved through combinatorial control.

Regulation of gene expression can be described as a form of logical gate. Genetic logic gates enable several input signals to be integrated [106] and can integrate multiple input signals, followed by their integration and translation into a specific activity involving one or more
genes. This could be used to determine levels of dissolved oxygen and substrates in order to determine gene-expression profiles. Wang et al. [107] constructed a novel real-time control system for gene regulation in E. coli that included an enhancement element (duplex DNA aptamers and an upstream promoter) and a repression element (RNA aptamers and an upstream RBS). Ligation (thrombin) binding to the duplex DNA aptamers initiated target-gene expression, whereas ligand (vascular endothelial growth factor) binding to the RNA aptamer repressed mRNA translation. Combining the two approaches in one gene regulatory system allowed control based on different concentrations of multiple ligands. Logic gate regulation can integrate signals and respond to changes in environmental conditions; however, the discovery and modification of regulatory factors and related trans-acting elements restricts its development.

One central goal of synthetic biology is to integrate multiple gene circuits and achieve dynamic control in living biological systems. As a carbon donor, many value-added compounds have been synthesized by regulating the metabolism of malonyl-CoA. Malonyl-CoA is a precursor of fatty acid biosynthesis, the regulation of the malonyl-CoA source pathway and sink pathway is very necessary for fatty acid production. Xu et al. designed a T7-based malonyl-CoA that can sense malonyl-CoA based on the trans-regulatory protein FapR and the cis-regulatory element fapO [108]. Next another malonyl-CoA sensor based on pGAP also was engineered based on FapR and fapO [101]. The pGAP-based malonyl-CoA sensor was markedly different from the T7-based malonyl-CoA. When the level of malonyl-CoA was low, FapR activated the transcriptional activity of the pGAP-based promoter and repressed the transcriptional activity of the T7-based promoter, then the transcription levels were opposite when the malonyl level was high. By intergating the malonyl-CoA-regulated T7 and pGAP promoter, a logic gate regulation of fatty acid production was constructed in E. coli, resulting in an oscillatory malonyl-CoA pattern and a balance between cell growth and fatty acid production.

5. Future perspective

Building a variety of useful compound-producing strains to replace chemical synthesis is a goal of metabolic engineers and synthetic biologists. However, the main problems they are facing are the construction of target product biosynthesis pathway and the improvement of biosynthesis efficiency. For the construction of biosynthesis pathway, the selection of enzymes that can catalyze specific reactions is the basis. In addition, the specificity and catalytic activity of the enzymes have an important influence on the biosynthesis efficiency. In order to improve the biosynthesis, metabolic engineers and synthetic biologists are also trying to replace those complex and inefficient biosynthesis pathways with simpler and more efficient ones, all of which would be achieved through the above mentioned protein-engineering strategies [109].

Through protein engineering, we can construct the target biosynthesis pathways and slightly improve the biosynthesis efficiency. In addition to the performance of enzymes, the imbalance of metabolism also affects the biosynthesis efficiency. Therefore, the biosynthesis efficiency problem still needs the assistance and cooperation of fine-tuned strategies to get a more perfect solution. Previous studies have shown that static control strategies can solve the problem of metabolic imbalance, while dynamic control strategies can solve it better [110]. Many systems are based on synthetic promoters and inducible transcription factors, which are limited by the number of metabolite-responsive transcriptional factors. No biological process has the opportunity to equip such systems. Although similar dynamic control systems can be used in different strains, specific methods should be constantly constructed and improved. The metabolite-responsive transcriptional factors identified by traditional sequencing, comparison and analysis can not meet the needs of the rapid development of synthetic biology. With the maturity of next-generation sequencing technology, the improvement of high-throughput screening technology and the extensive application of artificial intelligence, it will be faster and more efficient to screen and identify highly specific and efficient enzymes, construct and optimize the dynamic regulatory system if we can reasonably use these technologies [111].

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2017YFC1600403), the National Science Fund for Excellent Young Scholars (21822806), the National Natural Science Foundation of China (31670095, 31770097), the Fundamental Research Funds for the Central Universities (JUSRP1701A), the National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-08).
Glucobacter oxydans WSIH-003 for the direct production of 2-keto-L-gulonic acid from D-sorbitol. Metab Eng 2014;24:30–7.

[25] Chen ZY, Sun XX, Li Y, Yan YJ, Yuan QP. Metabolic engineering of Escherichia coli for microbial production of monoglucitol. Metab Eng 2017;39:102–9.

[26] Eichenberger M, Leikin BJ, Folly C, Fischer DI, et al. Metabolic engineering of Saccharomyces cerevisiae for de novo production of dihydroxycarbonates with known antioxidant, antiadipic, and sweet tasting properties. Metab Eng 2017;39:80–9.

[27] Kudnalska RJ, Bomscheuer UT. Finding better protein engineering strategies. Nat Chem Biol 2009;5:603.

[28] Bassalo MC, Liu RM, Gilt RJ. Directed evolution and synthetic biology applications to microbial systems. Curr Opin Biotechnol 2016;39:126–33.

[29] Stemmer WP. Rapid evolution of a protein in vitro by DNA shuffling. Nature 1994;370:75.

[30] Zhou SS, Ding RP, Chen J, Du GC, et al. Obtaining a panel of cascade promoter-5'–UTR complexes in Escherichia coli. ACS Synth Biol 2017;6:1065–75.

[31] Wang B, Hu Q, Zhang Y, Shi R, et al. A RecET-assisted CRISPR-Cas9 genome editing in Corynebacterium glutamicum. Microbiol Cell Factories 2018;17:63.

[32] Gogolub H, Pipel P. Determinants of translation efficiency and accuracy. Mol Syst Biol 2011;7:481.

[33] Isemura T. Codon usage and tRNA content in unicellular and multicellular organisms. Mol Biol Evol 2011;28:123–34.

[34] Rojano Nismura AM, Haning K, Janovsky J, Vasquez KA, et al. Codon selection affects recruitment of ribosome-associating factors during translation. ACS Synth Biol 2019;8:329–42.

[35] Yu CH, Dang Y, Zhou Z, Wu C, et al. Codon usage influences the local rate of translation elongation to co-translational protein folding. Mol Cell 2015;59:774.

[36] Bornslev S, Selmi T, Fland S, Dietmann S, Fyre M. Codon usage optimization in pluripotent embryonic stem cells. Genome Biol 2019;20:10.

[37] Mittal P, Brindle J, Stephen J, Piotkin JB, Kudina G. Codon usage influences fitness through RNA toxicity. Proc. Natl. Acad. Sci. U.S.A. 2018;115:8369–44.

[38] Eriksen M, Sneppen K, Pedersen S, Mitarai N. Occlusion of the ribosome binding site connects the translational initiation frequency, mRNAs stability and premature transcription termination. Front Microbiol 2017;8.

[39] Zhou SH, Ding RP, Chen J, Du GC, et al. Obtaining a panel of cascade promoter-5'-UTR complexes in Escherichia coli. ACS Synth Biol 2017;6:1065–75.

[40] Wintrode PL, Miyazaki K, Arnold FH. Cold adaptation of a mesophilic subtilisin-Asp by directed evolution. Proteins 2005;60:132–41.

[41] Stemmer WP. Rapid evolution of a protein in vitro by DNA shuffling. Nature 1994;370:75.

[42] Tang YL, Ren WK, Li FN, Liu G, et al. Methionine de carbolizing enzymes from poultry. J Ind Microbiol Biotechnol 2013;40:1423.

[43] Zhao XR, Choi KR, Lee SY. Metabolic engineering of Saccharomyces cerevisiae. Angew Chem, Int Ed Engl 2017;56:6396.

[44] Wu JJ, Du GC, Zhou JW, Chen J. Metabolic engineering of Escherichia coli for high-level production of (2S)-naringenin from L-tyrosine in Escherichia coli. Appl Environ Microbiol 2014;80:7283–92.

[45] Eichenberger M, Lehka BJ, Folly C, Fischer D, et al. Metabolic engineering of Escherichia coli for high-level production of (2S)-naringenin from L-tyrosine in Escherichia coli. Appl Environ Microbiol 2014;80:7283–92.

[46] Tang YL, Ren WK, Li FN, Liu G, et al. Methionine de carbolizing enzymes from poultry. J Ind Microbiol Biotechnol 2013;40:1423.

[47] Zhao XR, Choi KR, Lee SY. Metabolic engineering of Saccharomyces cerevisiae. Angew Chem, Int Ed Engl 2017;56:6396.

[48] Stemmer WP. Rapid evolution of a protein in vitro by DNA shuffling. Nature 1994;370:75.

[49] Tang YL, Ren WK, Li FN, Liu G, et al. Methionine de carbolizing enzymes from poultry. J Ind Microbiol Biotechnol 2013;40:1423.

[50] Eriksen M, Sneppen K, Pedersen S, Mitarai N. Occlusion of the ribosome binding site connects the translational initiation frequency, mRNAs stability and premature transcription termination. Front Microbiol 2017;8.

[51] Zhou SH, Ding RP, Chen J, Du GC, et al. Obtaining a panel of cascade promoter-5'-UTR complexes in Escherichia coli. ACS Synth Biol 2017;6:1065–75.

[52] Wang B, Hu Q, Zhang Y, Shi R, et al. A RecET-assisted CRISPR-Cas9 genome editing in Corynebacterium glutamicum. Microbiol Cell Factories 2018;17:63.
[95] Takeno S, Hori K, Ohtani S, Mimura A, et al. L-Lysine production independent of the oxidative pentose phosphate pathway by Corynebacterium glutamicum with the Streptococcus mutans gapN gene. Metab Eng 2016;37:1–10.

[96] Xu P. Production of chemicals using dynamic control of metabolic fluxes. Curr Opin Biotechnol 2018;53:12–9.

[97] Torres-Cerna CE, Morales JA, Hernandez-Vargas EA. Modeling quorum sensing dynamics and interference on Escherichia coli. Front Microbiol 2019;10.

[98] Chuang SK, Vila GD, Priblich KS, Gitis Z. Surface association sensitizes Pseudomonas aeruginosa to quorum sensing. Nat Commun 2019;10.

[99] Fujita Y, Matsuoka H, Hirooka K. Regulation of fatty acid metabolism in bacteria. Mol Microbiol 2007;66:829–39.

[100] Schujman GE, Padoletti L, Grossman AD, de Mendoza D. FapR, a bacterial transcription factor involved in global regulation of membrane lipid biosynthesis. Dev Cell 2003;4:663–72.

[101] Xu P, Li LY, Zhang FM, Stephanopoulos G, Koffas M. Improving fatty acid production by engineering dynamic pathway regulation and metabolic control. Proc. Natl. Acad. Sci. U.S.A 2014;111:11299–304.

[102] Gupta A, Reisman IMB, Reisch CR, Prather KLJ. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat Biotechnol 2017;35:273.

[103] Victor IU, Kwicieniec M, Tripathi L, Cobice D, et al. Quorum sensing as a potential target for increased production of rhamnolipid biosurfactant in Burkholderia thailandensis E264. Appl Microbiol Biotechnol 2019;103:6505–17.

[104] Williams TC, Averesch N, Winter G, Duda MR, et al. Quorum-sensing linked RNA interference for dynamic metabolic pathway control in Saccharomyces cerevisiae. Metab Eng 2015;29:124–34.

[105] De Paepe B, Maertens J, Vanholme B, De Mey M. Chimeric LysR-Type transcriptional biosensors for customizing ligand specificity profiles toward flavonoids. ACS Synth Biol 2019;8:318–31.

[106] Smanski MJ, Zhou H, Claesen J, Shen B, et al. Synthetic biology to access and expand nature’s chemical diversity. Nat Rev Microbiol 2016;14:135–49.

[107] Wang J, Cui X, Yang L, Zhang Z, et al. A real-time control system of gene expression using ligand-bound nucleic acid aptamer for metabolic engineering. Metab Eng 2017;42:85–97.

[108] Xu P, Wang W, Li L, Bhan N, et al. Design and kinetic analysis of a hybrid promoter-regulator system for malonyl-CoA sensing in Escherichia coli. ACS Chem Biol 2014;9:451–8.

[109] Li C, Zhang R, Wang J, Wilson LM, Yan Y. Protein engineering for improving and diversifying natural product biosynthesis. Trends Biotechnol 2020. https://doi.org/10.1016/j.tibtech.2020.01.001.

[110] Miano A, Liao M, Hasty J. Inducible cell-to-cell signaling for tunable dynamics in microbial communities. Nat Commun 2020;11:1193.

[111] Zeng W, Guo L, Xu S, Chen J, Zhou J. High-throughput screening technology in industrial biotechnology. Trends Biotechnol 2020. https://doi.org/10.1016/j.tibtech.2020.01.001.