Trends in Biotechnology

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Focus on Microbial Consortia

Review

Modular Metabolic Engineering for Biobased Chemical Production

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Microorganisms can manufacture a wide range of biobased chemicals that are useful for diverse industrial applications. However, the overexpression of heterologous enzymes in recombinant strains often leads to metabolic imbalance, resulting in growth retardation and suboptimal production of the target compounds. Here we discuss the recent development of modular metabolic engineering approaches that enable the global fine-tuning of engineered pathways by modularizing the synthetic pathway in single or multiple hosts. In particular, we highlight applications with microbial consortia. To build a vibrant biobased economy, multivariate modular metabolic engineering (MMME), modular coculture engineering (MCE), and spatiotemporal and integrative genome-scale metabolic modeling can be exploited to expedite strain optimization and improve the production of a broad variety of high-value biobased chemicals.

Building a Biobased Economy with Modular Engineering

The production of bulk and fine chemicals biologically from renewable resources has proved to be an attractive route to replace nonsustainable petrochemical-based chemical production [1]. It is estimated that the production of biobased chemicals can yield an annual revenue of US $10–15 billion in the global chemical industry by 2020 [2]. In particular, specialty chemicals (e.g., natural products) that are difficult or impossible to produce by traditional chemical methods represent a promising starting point to establish a biobased economy.

The development of recombinant DNA technology along with the use of **model organisms** (see Glossary) has substantially improved our ability to program the microbial cell factories with the desired phenotypes. However, the engineering of endogenous genes and insertion of heterologous pathways into recombinant strains often result in metabolic imbalance, leading to growth retardation and suboptimal production of the target compounds [3]. This drawback is especially evident with compounds that require long and complex synthesis pathways (e.g., natural products such as isoprenoids and flavonoids), owing to overwhelming metabolic stresses from overexpressing a large set of pathway genes [4].

Fortunately, natural systems have provided metabolic engineers with clues to overcome this obstacle. To balance metabolic fluxes in nature, higher organisms partition metabolic pathways into various modules via organelle compartmentalization, while microscopic organisms achieve the same objective via microbial consortia [5]. This concept of modularizing synthetic pathways has provided new strategies for the systematic optimization of engineered strains. In modular metabolic engineering, enzymes in pathways are grouped into a series of interacting modules to constrain genetic design and reconstitute metabolic balance. Distinct from the traditional metabolic engineering approaches that separately assess parts of the synthetic pathway sequentially, modular metabolic engineering holistically examines the entire synthetic pathway and cooperatively modulates the expression levels of each pathway module to enable global

Highlights

Microbial cell factories for the production of biobased chemicals are a promising route to achieve a sustainable future. However, it is essential to address metabolic imbalances caused by engineered pathways in microbial hosts.

Multivariate modular metabolic engineering (MMME) and modular coculture engineering (MCE) can reconstitute the metabolic balance by modularizing the synthetic pathways and globally fine-tuning the expression levels of pathway modules in single or multiple hosts.

The cross-cultural modular design of MCE significantly reduces the time and difficulty of reconstituting long synthetic pathways in a single host and polycultures enable the synthesis of complex compounds.

Computer-aided genome minimization coupled with designing orthogonal metabolic pathways (independent from biomass synthesis) can open new paths to optimize microbial strains in a modular synthetic community.

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fine-tuning of the metabolic network [4]. For example, enzymes with comparable catalytic turnover rates are often assembled into an operon. Subsequently, the expression levels of enzymes in a module are tuned simultaneously to equalize the turnovers of different modules, directing the metabolic flux towards the production of the target chemical. In addition, pathway modules that have been constructed and optimized can be further rewired in a plug-and-play fashion to produce diverse compounds, significantly reducing the time and resource investment in metabolic engineering [6,7]. In parallel, as modules and microbial cocultures become more elaborate, new computational tools are required to process the increasing scales of data and solve the mathematical formulations used to model the biology of multiple metabolic modules.

In the past few years, significant progress has been made in applying modular engineering strategies to address metabolic imbalances in microbial hosts to promote overall cell fitness and product yield. Initially, through modularizing the synthetic pathways in a single host, **MMME** has emerged as an effective strategy to reconstitute metabolic balance and improve metabolite production [4]. The modular pathway design not only expedites strain optimization but also facilitates pathway refactoring to improve the production of a wide range of biobased chemicals. More recently, the modularity concept has been further extended to segregate the synthetic pathways into two or more hosts by **MCE** [7]. The synergetic effects of multiple constituent strains offer advantages over a single strain, thereby further elevating the productivity of biobased chemicals. In this review we focus on the concept of modularity and present the recent development of modular metabolic engineering for biobased chemical production, placing particular emphasis on MCE. Furthermore, we discuss how the recent development of computational and analytical tools for modular pathway analysis, design, and optimization are systematizing the fields of MMME and MCE and expediting the development of modular strain and coculture engineering for efficient biobased chemical production (Figure 1, Key Figure).

MMME: A Focused Combinatorial Engineering Approach

Throughout evolution, microorganisms have developed a tightly regulated and balanced metabolism [8]. However, such a balanced metabolism is often missing in engineered strains since their native pathways have been reconfigured to overproduce value-added metabolites. As a consequence, metabolic fluxes within the biochemical network of engineered hosts are often imbalanced, resulting in **pathway bottlenecks** that may penalize cell fitness and pathway productivity [9,10]. Therefore, fine-tuning of the engineered pathways is essential to unlock the full potential of cell factories in biobased chemical production.

Early metabolic engineering efforts typically relied on rational engineering approaches (Box 1). Although moderate strain improvements were achieved, these approaches generally lacked a holistic consideration of the interconnectedness of cellular metabolism and focused on only examining individual parts of a pathway, neglecting the fact that interactions within a pathway are mostly complex and nonlinear [11]. Consequently, improvements were often limited to the local yield maxima of a pathway. To achieve the global optimal yield, combinatorial engineering (Box 1) can be implemented to generate a large phenotype space by randomly and simultaneously optimizing multiple enzymes within a pathway, enabling global fine-tuning of pathways [12]. However, evaluating the performance of such a large metabolic space requires a sensitive high-throughput screening assay, which is often lacking for many desired molecules [13].

To circumvent the challenges associated with combinatorial engineering, MMME has emerged as a focused, combinatorial engineering approach to generate a rationale-based but relatively

Glossary

Cellular resources: energy molecules [e.g., NAD(P)H, ATP] and various pathway-dependent building blocks (e.g., nucleotides, lipids, amino acids).
CRISPR–Cas9: a genome-editing technique that can effectively alter the genome of both model and nonmodel organisms. A typical CRISPR–Cas9 system comprises a Cas9 nuclease that cleaves double-stranded DNA, guide RNA that provides targeting specificity for the Cas9 nuclease, and a repair module that stitches the double-stranded break.
CRISPR interference (CRISPRi): a genetic perturbation technique derived from the CRISPR–Cas9 system. It utilizes a deactivated Cas9 nuclease and a customizable single guide RNA to silence target genes at the transcriptional level.
Dynamic flux balance analysis (DFBA): a variant of FBA that accounts for the accumulation of biomass and metabolites in the environment. It then computes the corresponding redistribution of metabolic fluxes over time. DFBA can also model diverse fluctuations in the environment (e.g., pH dynamics, fed-batch systems). DFBA is useful for modeling and predicting metabolic behavior over changing concentrations of the feedstocks and products as well as at different stages of population density.
Flux balance analysis (FBA): a technique that computes the optimal distribution of metabolic fluxes in a given metabolic network as provided by its GSM. Constraints are applied to optimize a predefined reaction in the GSM. The distribution of fluxes and prediction of growth rates can be analyzed when optimizing the reaction that synthesizes biomass. FBA can be applied to many research questions in biotechnology. For example, the optimization objective can be a reaction of interest that would increase the production rate of biobased chemicals. The impact on the flux distribution of metabolites by single or multiple gene knockouts and other metabolic engineering strategies can also be investigated through FBA.
Genome-scale metabolic model (GSM): a mathematical

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Key Figure

Modular Engineering Approaches for Biobased Chemical Production.

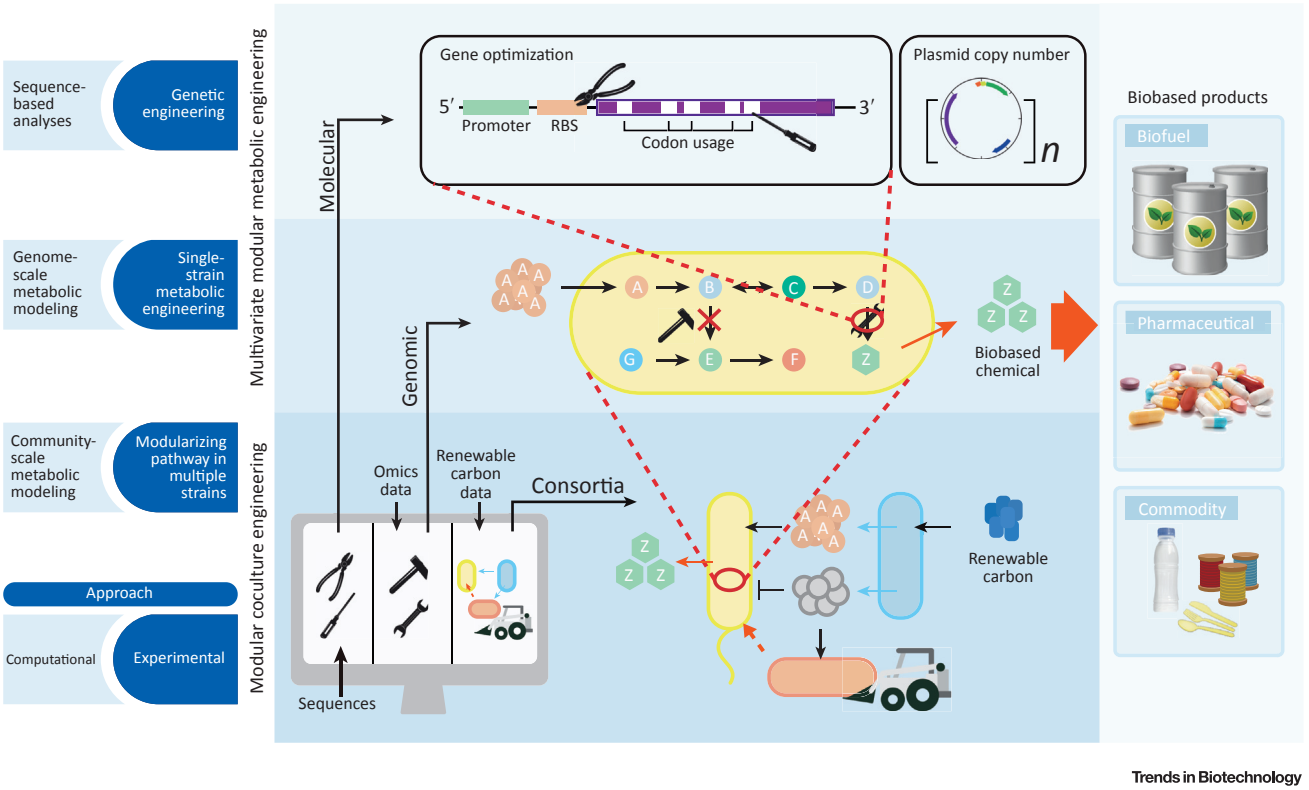


Figure 1. Experimental and computational approaches are deployed in concert in multivariate modular metabolic engineering (MMME) and modular coculture engineering (MCE) for effective biobased chemical production.

Box 1. Rational Engineering and Combinatorial Engineering

The rational engineering strategy requires detailed knowledge of the metabolic pathways so that metabolic flux is correctly directed towards pathways of interest by overexpression of the rate-limiting enzymes, gene deregulation, and/or knocking out competing pathways. However, one inherent limitation of this strategy is that after one rate-limiting bottleneck in a pathway is eliminated, new and unexpected constraints are often simultaneously generated somewhere else in the same or a different pathway due to the interdependency within and between pathways [10]. Although multiple rounds of strain construction, selection, and optimization could improve strain performances, such an iterative design–build–test–learn cycle is often labor intensive, time consuming, and expensive.

By contrast, unlike the knowledge-driven approaches, the combinatorial engineering strategy requires minimal knowledge about the metabolic pathway of interest. This strategy typically relies on random mutagenesis to enable sampling of a large phenotype space for the identification of strains that exhibit the desirable phenotype. A high-throughput assay is required to screen the large phenotype space to identify the superior strain in the mutant library.

representation of a metabolic network. The model comprises a matrix of stoichiometric coefficients corresponding to the biochemical reactions and compounds constituting the metabolism of an organism. Reactions in the model are primarily derived from and explicitly associated with genes in the genome of an organism. Subsequent manual refinement, imposition of constraints based on physiological experiments, and integration of omics data can improve the prediction power of GSMs.

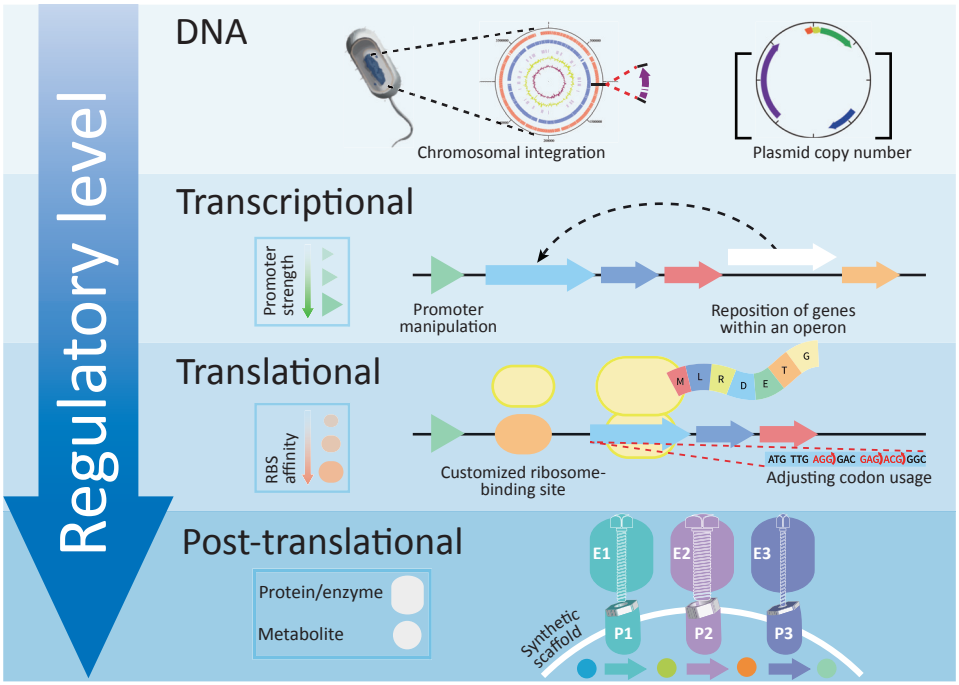


Figure 2. Commonly Used Tools for Modulating the Relative Expression of Pathway Modules. To minimize intrinsic constraints (e.g., plasmid-associated metabolic burden, allele segregation, instability) associated with a single regulatory tool and to enable tighter modular expression in multivariate modular metabolic engineering (MMME), the above regulatory tools are often employed in concert (e.g., altering promoter strength and plasmid copy number simultaneously is most widely implemented in MMME studies).

narrowed combinatorial space [4]. This approach partitions a complex pathway into simpler distinct modules that allow parallel optimization based on moderate *a priori* knowledge about the synthetic pathway, such as metabolite biochemistry (e.g., toxicity) [14], pathway branching [15], and catalytic turnover [16]. Subsequently, the expression levels of each module are cooperatively modulated towards an optimally balanced pathway where the input and output of the connected modules are synchronized to sustain sufficient enzymatic activity and minimize metabolic load on excessive protein synthesis. The relative expression levels of the pathway modules can be finely tuned by various regulatory tools (Figure 2). The principles related to MMME have recently been reviewed in the literature [12,13] and are not emphasized here.

MCE: A Spatial Pathway Modularization Approach

Despite the progress in engineering single strains, the construction and optimization of biosynthetic pathways in a single host are still hampered by several intrinsic limitations. First, the difficulty of gene cloning in a single host increases with the number of exogenous genes that have to be introduced, mainly due to the challenges of expressing multiple cassettes or plasmid construction for various genes [4]. Second, even if the desired genes can be constructed in a single host, constitutive expression of a large number of heterologous enzymes can easily overexploit **cellular resources** [3], thereby overloading the host with an excessive metabolic burden that impairs cell viability and product synthesis. Third, a single host is restricted to a monocellular environment, which often fails to meet all of the special conditions required by the enzymes involved [17]. Fourth, undesirable interference can occur among reactions in the

Model organisms: typically include organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* that have been extensively studied. They are ideal for research or industrial applications due to their intrinsic characteristics and ease of experimental manipulation.

Modular coculture engineering (MCE): an approach that segregates a complex pathway into a series of modules and isolates the individual pathway modules into different strains to improve the desired biosynthesis performance. As a result of the synergetic effects of multiple constituent strains, MCE provides a viable and attractive option for microbial consortium engineering of synthetic pathways.

Multivariate modular metabolic engineering (MMME): an approach that partitions a complex pathway into simpler distinct modules to enable parallel optimization, simultaneous variation of the expression of each module, and the assembly of various modules to generate an engineered strain library. Finally, by using a multivariate statistical analysis, an optimal strain with a balanced metabolic flux can be efficiently determined.

Pathway bottlenecks: pathway constraints such as redox imbalance from unmatched precursor or cofactor specificity, feedback inhibition due to accumulation of toxic or unstable intermediates, and diversion of resources to competing pathways.

sRNA devices: synthetic small regulatory RNA tools that can manipulate post-transcriptional regulation by interacting with the 5′ untranslated region of the mRNA.

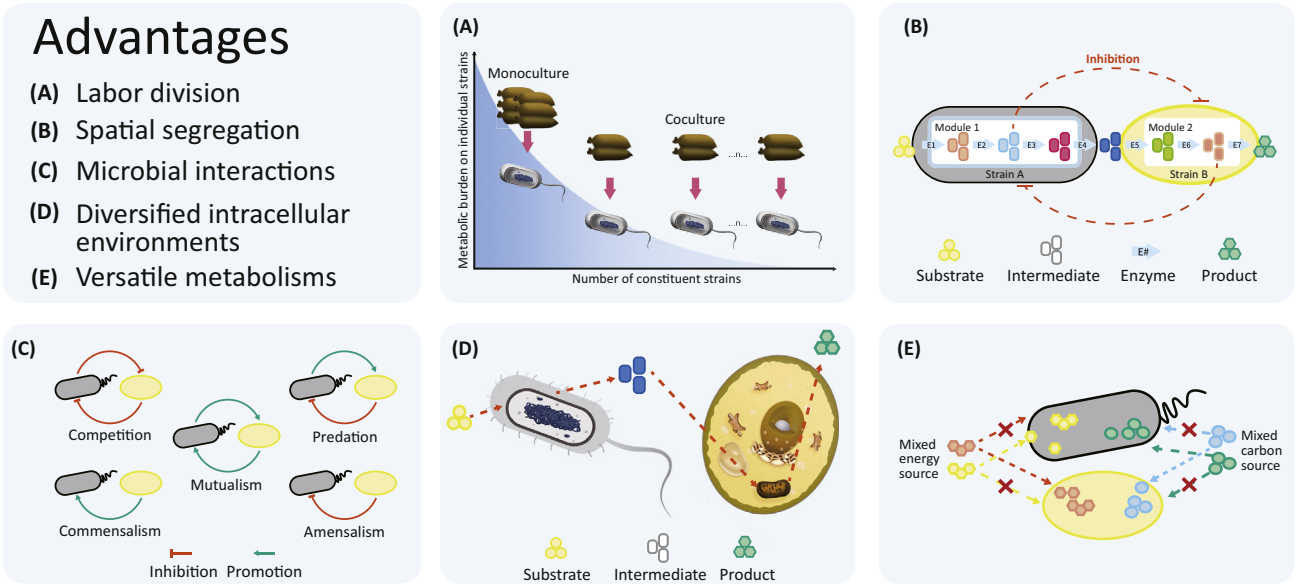


Figure 3. Advantages of a Multihost System over a Single-Host System.

synthetic pathway when an intermediate of one reaction negatively influences another metabolite [18].

As a spatial pathway modularization approach, MCE can potentially overcome all of these barriers. The principle of coculture-based strategy is to modularize heterologous metabolic pathways and assign pathway modules to different hosts in a system for the optimal functioning of the complete pathway [7]. Different from MMME, the individual pathway modules are physically arranged into separate hosts; therefore, the metabolic reactions are isolated from one another in different cells. Compared with the single-host approach, such a multistrain system significantly reduces the time and difficulty of reconstituting long synthetic pathways, as the cross-cultural modular design allows parallel construction of the separate hosts carrying the partial pathways and reduces the number of genetic modifications in each host. The employment of multiple strains with unique functions and characteristics offers distinct advantages over a single-host system, including labor division that substantially lightens the metabolic burden on constituent individuals [19], spatial segregation that prevents negative cross-influences among pathway modules [18], beneficial microbial interactions that promote cell fitness and productivity [7], diversified intracellular environments that accommodate the functional expressions of a larger variety of heterologous enzymes [7], and versatile metabolisms that allow greater functional flexibility [20] when different species are incorporated as workhorses (Figure 3).

Host Selection and Pathway Division

One dilemma in MCE is whether to employ strains derived from the same or different species, as either choice has its own merits and defects. Ideally, a multispecies system can exploit the strengths of each species, such as their unique physiochemical properties and biosynthesis capabilities. For instance, while bacteria (e.g., *Escherichia coli*) can rapidly produce proteins, yeast (e.g., *Saccharomyces cerevisiae*) excel at manufacturing sophisticated eukaryotic

proteins that require advanced protein-folding machineries [21]. In a study by Zhou and colleagues, these advantages were combined by coculturing *E. coli* and *S. cerevisiae* together for oxygenated taxane production, resulting in a yield (33 mg/l) that was significantly higher than the monospecies *E. coli–E. coli* counterpart (0.8 mg/l) [7]. However, the drawback of a multispecies system is that the consortium composition can be unstable as one species can easily outgrow another due to different cell growth rates [17]. Although adjusting the growth conditions (e.g., pH, temperature, oxygen level) or inoculum ratio can partially mitigate the problem of culture instability [22], more complex approaches are needed to maintain growth compatibility, such as introducing interspecies metabolic interactions via cross-feeding [19].

In terms of microbial chassis, current MCE studies have mainly deployed traditional model organisms to take advantages of their aerobic and fast-growing characteristics in addition to well-developed genetic tools [8]. However, with the development of more sophisticated genome editing tools [e.g., **sRNA devices**, clustered regularly interspaced short palindromic repeats and the associated protein Cas9 (**CRISPR–Cas9**), **CRISPR interference (CRISPRi)**], the host candidates have now been expanded to nonmodel organisms that were traditionally difficult to manipulate but have a unique proficiency or cellular environment for specific enzymatic functions. One good example is that the *Clostridium* species-mediated acetone–butanol–ethanol (ABE) fermentation pathway was previously engineered into an *E. coli–E. coli* coculture system for *n*-butanol production, achieving the highest titer of 5.5 g/l from glucose [23]. Recently, however, using CRISPRi and an optimized electroporation technique, the ABE fermentation pathway was successfully engineered in a twin-clostridial consortium, increasing the *n*-butanol production to 11.5 g/l [24].

One critical consideration of the MCE design is that the conjunctive intermediates connecting different pathway modules are required to travel efficiently between cells so that the partial pathways in separate cells can be reconnected as one complete pathway [25]. However, some intermediates (e.g., coenzyme A, phosphorylated compounds) have no or limited ability to cross cell membranes, disqualifying them as suitable conjunctive molecules. In this situation, transporter engineering offers a chance to enhance the transportability of the target intermediates [26]. However, when transporter engineering of the target intermediates is challenging or infeasible, an easier solution is to rationally divide the pathway so that other transportable molecules act as the conjunctive intermediates.

Microbial Interactions as Strategies to Improve Production

Microbial interactions are ubiquitous in natural consortia and are crucial in determining the functionality, stability, and dynamics of communities [27]. In MCE, microbial interactions can be exploited to improve the viability and productivity of cells. In general, mutualistic interactions are built between the employed strains as such a relationship can benefit the interacting strains, thus promoting the overall desired performance [19]. To establish a mutualistic coculture, the interacting partners can be designed to lean on each other for the exchange of essential nutrients or detoxification of inhibitory substances. For instance, although oxygenated taxanes could be produced from an *E. coli–S. cerevisiae* coculture when grown on glucose, the cell growth and taxane titers of *E. coli* were largely inhibited by the accumulated ethanol produced by *S. cerevisiae*. To tackle this problem, the sole carbon source was switched to xylose, which can be utilized only by *E. coli* and not by *S. cerevisiae*. Thus, *S. cerevisiae* obligately relied on the acetate produced by *E. coli* as the sole carbon source for growth without producing the toxic ethanol. Meanwhile, acetate consumption by *S. cerevisiae* also mitigated acid inhibition in the coculture. As a result, this mutualistic coculture increased oxygenated taxane production by

15.5 times over that of a control without a mutualistic design [7]. In addition to mutualism, exploration of other modes of interaction (e.g., competition, predation, amensalism, commensalism) for the design and optimization of modular coculture is also possible when special traits or controllable mechanisms are required to improve target compound production [27].

MMME and MCE Expedite Strain Optimization and Facilitate Pathway Refactoring

In recent years, MMME and MCE have been successfully applied to enable global fine-tuning of cellular reactions to substantially enhance the production of a wide range of value-added chemicals including alcohols (e.g., *n*-butanol, monolignol) [18,23,24,28], acids (e.g., *cis*,*cis*-muconic acid, 4-hydroxybenzoic acid, 2-keto-L-gulonic acid, 3-amino benzoic acid) [14,20,29–31], and natural products (e.g., isoprenoids, flavonoids) [4,6,7,16,22,32–39], which are important in the biofuel, chemical and pharmaceutical industries (Table 1 and 2). By taking advantage of the modularity of microbial partnerships, the overall titer, yield, and productivity of the target products in the cocultures have been significantly improved compared with their monoculture counterparts.

Table 1. Selected MMME Studies for Biobased Chemical Production

Product	Carbon source	Organism	Number of modules	Number of engineered strain library	Engineering approach to modulate the expression of pathway modules	Titer (mg/l)	Refs
N-Acetylglucosamine	Glucose	*Bacillus subtilis*	3	7	Expressed various combinations of synthetic small regulatory RNAs and Hfq protein; promoter manipulation	8300	[15]
Fatty acids	Glucose	*Escherichia coli*	3	17	Plasmid copy number variation; promoter manipulation; varying antibiotic resistance marker; altering ribosome-binding sites	8100	[14]
Miltiradiene	Glucose	*Saccharomyces cerevisiae*	2	9	Plasmid copy number variation	61.8	[35]
Taxadiene	Glucose	*E. coli*	2	32	Plasmid copy number variation; promoter manipulation; chromosomal integration	1000	[4]
Isoprene	Arabinose	*E. coli*	2	84	Promoter manipulation	17.5	[36]
Medium-chain fatty acids	Glucose	*E. coli*	2	24	Plasmid copy number variation; promoter manipulation	3800	[31]
(2*S*)-Pinocembrin	Glucose	*E. coli*	4	4	Plasmid copy number variation; promoter manipulation; gene codon usage	40	[37]
(2*S*)-Pinocembrin	Glucose	*E. coli*	4	17	Plasmid copy number variation; promoter manipulation	432.4	[38]
Resveratrol	L-Tyrosine	*E. coli*	3	18	Plasmid copy number variation; promoter manipulation	35	[16]
(2*S*)-Naringenin	Glucose	*E. coli*	3	13	Plasmid copy number variation; promoter manipulation	100.6	[6]
Pinosylvin	Glucose	*E. coli*	2	20	Plasmid copy number variation; promoter manipulation	281	[39]
β-Carotene	Glucose	*E. coli*	5	12	Interchanging promoters and mRNA-stabilizing regions	2100	[69]

Table 2. Selected MCE Studies for Biobased Chemical Production

Product	Carbon source	Organism	Titer (mg/l)	Remark	Refs
Oxygenated taxanes	Xylose	*Escherichia coli–Saccharomyces cerevisiae*	33		[7]
Monoacetylated dioxygenated taxane			1.1	First report of microbial production of a monoacetylated, dioxygenated taxane from xylose	
Ferruginol			18	Exceeded the highest titer reported, of 10 mg/l by *S. cerevisiae*	
Nootkatone			4		
Nootkatol			30		
cis,*cis*-Muconic acid	Glucose/xylose mixture	*E. coli–E. coli*	4700[a]	Achieved 19-fold titer increase over monoculture	[20]
4-Hydroxybenzoic acid			2300[a]	Equaled the highest titer reported previously	
Thebaine	Glycerol	Four *E. coli*	NR	Achieved yield of 2.1 mg/l, which is 300-fold higher than the previously reported yeast system [70]	[34]
Hydrocodone			NR	Achieved yield of 0.4 mg/l	
Afzelechin	Glucose	Three *E. coli*	26.1	*De novo* production of flavan-3-ol and anthocyanidin-3-*O*-glucoside for the first time in a microbial host	[25]
Callistephin		Four *E. coli*	9.5		
Flavan-3-ol	*p*-Coumaric acid	*E. coli–E. coli*	40.7	Achieved 970-fold titer increase over monoculture; first application of empirical modeling techniques to enhance production titer of a coculture system	[22]
Caffeyl alcohol	*p*-Coumaryl alcohol	*E. coli–E. coli*	401 854.1[a]	Achieved 12-fold titer increase over monoculture	[18]
Coniferyl alcohol			124.9	First report of microbial production of caffeyl alcohol and coniferyl alcohol	
n-Butanol	Glucose	*E. coli–E. coli*	5500	Achieved twofold titer increase over monoculture and 69% of the theoretical yield	[23]
Acetone	Alkali-extracted, deshelled corncobs	*Clostridium cellulovorans* DSM 743B–*Clostridium beijerinckii* NCIMB 8052	4250	Achieved 87.2% total solvent titer increase over the author's previously established twin-clostridial consortium without genetic manipulation of *C. cellulovorans* DSM 743B	[24]
n-Butanol			11 500		
Ethanol			6370		
Styrene	Glucose	*Streptomyces lividans–Streptomyces lividans*	29		[33]
	Cellobiose		16		
	Xylo-oligosaccharide		5.5		
cis,*cis*-Muconic acid	Glycerol	*E. coli–E. coli*	1016 2000[a]	Achieved 17-fold titer increase over monoculture	[41]
Resveratrol	Glycerol	*E. coli–E. coli*	22.6	First MCE report for the biosynthesis of resveratrol from glycerol	[32]
Perillyl acetate	Glucose	*E. coli–E. coli*	21.7	Achieved 12-fold titer increase over monoculture	[71]
2-Keto-L-gulonic acid	D-Sorbitol	*Gluconobacter oxydans–Ketogulonicigenium vulgare*	76 600	Achieved 89.7% of the theoretical yield	[30]
3-Amino benzoic acid	Glucose	*E. coli–E. coli*	48		[29]
Succinate	Glucose/xylose mixture	*E. coli–E. coli*	40 000		[40]

NR, not reported.
[a]Scale-up production in a bioreactor.

Furthermore, the modules already optimized in MMME can be efficiently combined with new modules to reprogram the biosynthesis pathway through part swapping and combinatorial optimization to accelerate the production of new compounds. For example, the malonate assimilation and coumaroyl-coenzyme A production modules employed in the resveratrol synthetic pathway were reorganized with a new pathway module comprising chalcone isomerase and chalcone synthase to enable the *de novo* synthesis of (2*S*)-naringenin in *E. coli*. [6]. Similarly, because of the modular nature of the coculture design in MCE, the established pathway modules in a host can also be easily modified or reassembled into a different synthetic pathway to produce other valued-added products. For example, replacing a few heterologous genes of the oxygenated taxane-producing pathway modules enabled the production of sesquiterpene and other diterpenes in the same coculture, significantly expediting the pathway refactoring processes for these value-added products [7]. In another example, by merely swapping the downstream module in the *cis*,*cis*-muconic acid-producing coculture, 4-hydroxybenzoic acid can be produced via the same upstream module contained in cells synthesizing 3-dehydroshikimic acid as a precursor [20].

In theory, employing more constituent members could enable the modular coculture system to generate more structurally complex compounds and perform more complex functions because the system versatility can be expanded and the pathway-associated burdens can be shared by more dedicated members. However, most recent MCE studies [7,18,20,22–24,28–30,32,33,40–42] have been limited to a system with only two strains, which is simpler to design, construct, and control than a system with more than two hosts. The deployment of modular polycultures remains in the early stage, but three recent studies have clearly demonstrated the feasibility of modularizing synthetic pathways in multiple hosts. Nakagawa and colleagues developed a sequential polyculture comprising four *E. coli* strains for opiate biosynthesis, achieving a yield of 2.1 mg/l, which is 300 times higher than a yeast monoculture [34]. Although the stepwise segregated fermentation strategy utilized could enable greater control over the process, it comes at a price of losing beneficial microbial interactions and creating several scale-up concerns such as increased capital costs for multiple reactors and higher process operation and maintenance costs. In another example, Jones and colleagues constructed a *de novo* anthocyanin-producing pathway by distributing 15 heterologous genes across four *E. coli* strains [25], which were cultured together as a consortium in a consolidated process. In addition, although the final product was not a chemical compound, Liu and colleagues established a three-species (*E. coli*, *Bacillus subtilis*, and *Shewanella oneidensis*) microbial consortium to convert glucose to electricity [43], illustrating the promise of polycultures with multiple different species for biobased chemical production.

Challenges of Implementing MMME and MCE

Although MMME offers a viable option to reconstitute metabolic balance, the key experimental challenge of implementing this approach is that the construction of multiple modules in a single host needs to be carefully designed to avoid overexploitation of cellular resources (e.g., the energy metabolism for ATP synthesis in a host is limited). However, MMME typically relies on a plasmid-based approach to construct multiple pathway modules. The maintenance of high-copy-number plasmids and multiple pathway modules can significantly increase ATP expenditure, resulting in deleterious effects on cell fitness and product yield. Therefore, MMME is applicable only when the energy metabolism of the host is sufficient to supply the energy expenditure of each pathway module [9]. To this end, metabolic engineers should consider exploiting native pathways as partial synthetic pathways and refraining from constructing pathway modules that require excessive energy expenditure [44]. In addition, the application of chromosomal integration approaches (e.g., chemically inducible chromosomal evolution)

[45] for modular pathway construction can potentially reduce the energy burden due to plasmid maintenance.

Although MCE distributes the metabolic burden between multiple strains and exploits the cellular resources of more than a single strain, a stable and reliable artificial microbial consortium is more difficult to design and maintain than a single-host system. The coexistence and phenotypic stabilization of the constituent strains are the main challenges. To overcome them, systematic analysis and computational modeling can be applied to assess and predict the compatibility of the constituent members and the module output of a coculture system.

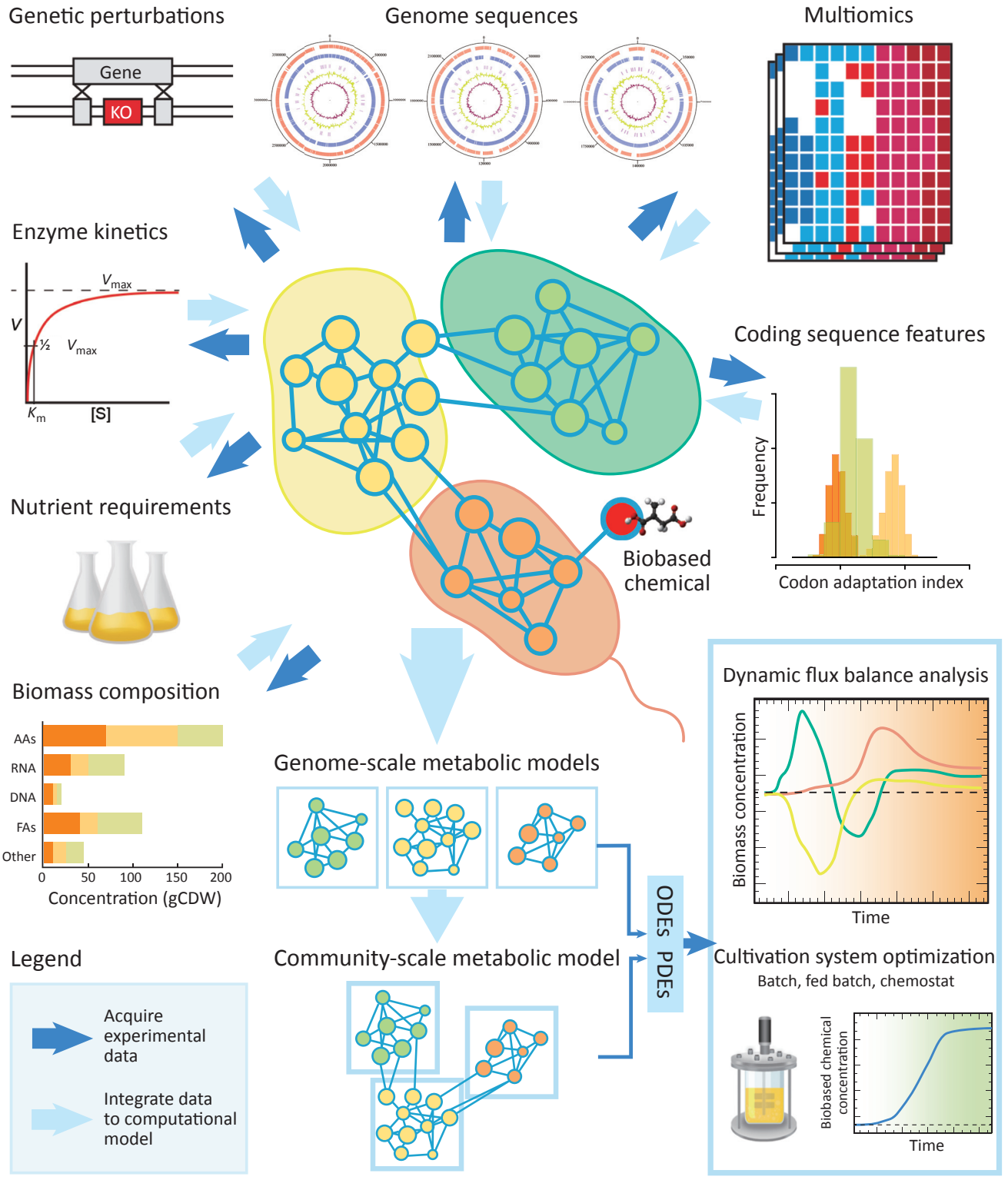
Computational Approaches for Modular Strain and Coculture Engineering

In addition to the recent progress in experimental approaches, major advances in metabolic engineering have been made possible by the rapid development of **genome-scale metabolic models (GSMs)**. Recent innovations in the construction and optimization of GSMs have enhanced our ability to simulate phenotypes based on genomic information [46]. Furthermore, the scaffolded nature of GSMs (Figure 4) has inspired the development of new methods for the integration of transcriptomic or proteomic [47], codon usage [48], and kinetic [49,50] data to improve the quantitative prediction of flux distribution. Because of the advances in the predictive power of GSMs, implementing GSMs to analyze complex bioreactor production systems is now possible (Box 2). Metabolic models have also provided a stable foundation to build novel engineering strategies in modular cell design [51]. Here we review the computational advances that are crucial for the implementation of innovative strategies for biobased chemical production with an emphasis on the *in silico* design of modular strains and modular microbial communities.

Augmenting GSMs to Identify Unexplored Engineering Strategies

In recent years, GSMs have been expanded to account for cellular molecular processes, such as basal protein production and the macromolecular expression cost associated with peptide biosynthesis [52]. These macromolecular expression models predict phenotypes by incorporating mathematical descriptions of transcription, tRNA charging, and translation. Likewise, other modulators of metabolism, such as enzyme abundance and protein turnover, can also be included as an additional layer of constraint. For example, the construction of an enzyme-constrained model of *S. cerevisiae* allowed the identification of unintuitive engineering strategies for biobased chemical production [49]. According to the model, while succinate production could be increased through gene knockouts and biomass coupling in *S. cerevisiae*, enhanced farnesene production can be achieved only by boosting enzyme activity and abundance [49].

GSMs can also be harnessed to identify optimal targets for modular metabolic engineering. UP Finder is a computational package that makes use of the genetic information in GSMs to find gene expression patterns that are associated with higher production of the desired metabolic compounds [53]. As an application of this method, GSMs of *E. coli* and *Synechocystis* sp. PCC 6803 were used to predict gene targets that could be overexpressed for increased production of farnesyl pyrophosphate and fatty acyl-ACP, respectively [53]. In another recently developed approach, prediction of transcriptional repressors was combined with GSMs and strain-optimization algorithms to enable the adjustment of metabolic modules that control the production of the desired biobased chemicals (e.g., shikimic acid, muconic acid) [54].



(See figure legend on the bottom of the next page.)

Box 2. GSMs and Biobased Chemical Production in Bioreactors

Outstanding applications of GSMs in biobased chemical production beyond the bench scale are provided by the development of models that account for the dynamics and complexity of the production systems. By integrating a GSM and spatiotemporal modeling of a complex bioreactor, Chen and colleagues were able to model the dynamics of the ethanol-producing strain *Clostridium ljungdahlii* in a bubble-column bioreactor and to optimize the theoretical biofuel production of the microorganism by identifying initial feedstock conditions that could increase the ethanol titer [65]. In a second work, a GSM and dynamic reactor modeling were applied to optimize production in photobioreactors [66]. In this case, the computational model was capable of predicting the intracellular accumulation of metabolites and identifying fed-batch cultivation strategies that optimized β-carotene production from varying feeding conditions of light and nitrogen [66]. Future applications of GSMs for biobased chemical production are expected to be driven by the improved accuracy provided by the genome-scale kinetic metabolic models. The integration of kinetic parameters, such as the enzymatic efficiency for catalysis of product formation [protein turnover number (k_{cat})], as another layer of constraints in computational models of metabolism can refine the accuracy of GSMs to predict metabolism dynamics derived from genetic alterations [50,67]. However, the development of genome-scale kinetic metabolic models carries an implicit and still unattended demand for large-scale and high-throughput screening techniques to obtain kinetic parameters experimentally [68]. With continuous improvements in modeling, collaborations between computational and experimental researchers are expected to increase in the biotechnology industry for scaling up and augmenting the prospect of biobased chemical production.

In silico Design of Enhanced Modular Cell Systems

The construction of more comprehensive GSMs has opened new possibilities towards the design of cellular systems with higher modularity. Novel *in silico* platforms for the design of modular systems have adopted the metabolic engineering strategy in which biobased chemical production can be either dependent [51] or independent [55] of cellular growth.

For *E. coli*, growth-dependent modular cells can be designed through two recently developed approaches: MODCELL [51] and MinGenome [56]. MODCELL aims to identify *in silico* auxotrophic cells that can grow only when complementary metabolic modules (designated as production modules) are provided to the cells [51]. The production modules can harbor the metabolic pathways involved in biomass synthesis as well as those required to produce a predefined biobased chemical. MODCELL has been applied computationally in the production of short-chain alcohols and esters [51] and experimentally in ethanol production [57]. By contrast, MinGenome incorporates the concept of genome minimization [56]. In this approach, the modular cells of *E. coli* are designed by coupling its metabolic model to data concerning transposons, operons, promoters, and transcription factors. MinGenome has successfully predicted the gene deletions required to produce a reduced (minimal) genome suitable for biobased chemical production [56]. For *S. cerevisiae*, modular cells have been designed in two steps. First, exogenous metabolic pathways are appended to a GSM; second, **flux balance analysis (FBA)** and strain-optimization algorithms are applied to predict combinations of gene deletions that yield enhanced modular strains for biobased chemicals [58].

In contrast to the growth-dependent strategy, modular systems can also be designed by incorporating orthogonal pathways in the metabolic network. The orthogonality concept comprises modification of the global network structure to reduce the metabolic association between the synthesis of the biomass and the target metabolite [55]; thus, biobased chemical production is decoupled from the metabolism required to sustain growth. Orthogonal pathway

Figure 4. Metabolic Models for Biobased Chemical Production. A genome-scale metabolic model (GSM) is an open-ended platform to represent the metabolism of an organism based on its enzymatic potential. Improvements in the prediction power of these models can be achieved through the integration of data derived from diverse experimental approaches such as genetic perturbations (e.g., gene knockouts, auxotrophy), multiomics experiments (e.g., transcriptomics, proteomics, metabolomics, fluxomics), molecular analyses (e.g., codon usage bias, translation efficiency), medium composition adjustment, and enzyme kinetics. Spatial and temporal environmental fluctuations can be formulated using ordinary differential equations (ODEs) and partial differential equation (PDEs). Coculture analysis, modeling, design, and optimization can be achieved by incorporating multiple GSMs. Hence, GSMs are multiscale platforms, enriched by systems and synthetic biology, for investigating and optimizing biobased chemical production.

design has been applied to *E. coli* to demonstrate its advantages over the growth-dependent strategy for the production of succinic acid, isobutanol, adipic acid, ethanol, 1,4-butanediol, and 2,3-butanediol [55].

Analyzing Synthetic Microbial Communities

In addition to the vast potential of GSMs for single organisms, computational studies of microbial consortia are also possible by coupling multiple GSMs to produce community-scale metabolic models (CSMs) (Figure 4). CSMs have been successfully applied to analyze synthetic microbial consortia. For instance, the community composition that maximizes the yield and production rate of methane was assessed through FBA of metabolic models that incorporate single, two, and three species [59]. However, FBA is unable to simulate the complex dynamics of microbial consortia, and strategies to engineer microbial communities can be overlooked when the analyses are limited to FBA. Overcoming the limitations of FBA, the dynamics in time and space of microbial ecosystems can be modeled by implementing **dynamic FBA**. Such a spatiotemporal modeling platform can be exemplified by COMETS [60] and BacArena [61]. Based on DFBA, COMETS has accurately predicted the unexpected metabolic interplay in two- and three-strain synthetic communities of *E. coli*, *Salmonella enterica*, and *Methylobacterium extorquens*. By contrast, based on individual-based modeling and FBA, BacArena has been applied to unveil heterogeneous phenotypes in a two-strain community, single-species biofilm, and multispecies human gut community.

Designing Synthetic Microbial Communities

In silico methods can also be employed to uncover engineering strategies to design microbial consortia with the desired outcomes. For example, DFBA has revealed a synthetic coculture design (yeast and microalgae) that has promising cost reduction for the industrial production of biodiesels [62]. However, *a priori* knowledge about the uptake kinetic parameters of the two organisms was required to find the appropriate engineering strategy, which is not ideal for finding unexplored designs of synthetic microbial consortia.

Conversely, novel computational approaches can assume no previous knowledge about the metabolism of individual strains composing the community. Instead, they harness genomic information to find partners with the suitable metabolic potentials and produce designs of synthetic microbial communities with predefined functions. This capability can be found in CoMiDA [63] and MultiPus [64]. CoMiDA can identify strains required to construct a synthetic consortium that produces a desired biobased chemical based on the genomes of the species and nutrients available. MultiPus, by contrast, takes advantage of the topological information on metabolism found in GSMs to identify consortia capable of producing metabolites of interest. MultiPus has been used to study the theoretical production of penicillin, cephalosporin C, and 1,3-propanediol by various modular synthetic microbial communities [64].

Concluding Remarks and Future Perspectives

Biobased chemical production via modular metabolic engineering holds promise to revolutionize the chemical industry. MMME and MCE are effective modularization strategies to reconstitute metabolic balance in single or multiple hosts. The modular design of these approaches not only significantly expedites strain optimization for higher product yield and titer but also facilitates pathway refactoring for the production of a wide range of biobased chemicals in a plug-and-play fashion. Both MMME and MCE have benefited from insights generated by *in silico* approaches for the construction of more efficient and versatile industrial microbial strains. However, to industrialize biobased products on a large scale, both the economic and the

Outstanding Questions

What types of biobased chemicals have the greatest potential to replace their counterparts currently produced through the chemical route? Can we accurately predict their chance of market success based on applications, production costs, and environmental impacts?

What are the most effective computational approaches that can complement experimental modular metabolic engineering to facilitate the development of novel cell factories to produce valuable compounds that are difficult or infeasible via chemical methods?

With the technical advances in genome editing and metabolic engineering, will the engineering of nonmodel organisms open new opportunities to further enhance the titer, yield, productivity, and range of biobased chemicals?

Can we improve the predictability of the experimental outcome of modular systems by linking genome minimization to orthogonal pathway design?

Can we improve control over modular synthetic communities by coupling mathematical formulations of dynamic controls (e.g., biosensors, quorum sensing, synthetic transcription factors) to metabolic models of consortia?

technical aspects of pathway design, reconstruction, and optimization need to be carefully considered (see Outstanding Questions).

Recognizing the synergic effects of microbial consortia, metabolic engineers are now striving to further explore the deployment of multiple constituent strains to produce more structurally complex compounds. Meanwhile, the advance of synthetic and genetic engineering tools will expand the number of workhorse chassis to include more specialized strains with unique proficiency. The increased number of constituent strains and possible host candidates along with the possible pathway constructs will inevitably lead to more sophisticated coculture systems that require extensive *in silico* analysis for rational design, construction, and optimization. To this end, the increasing comprehensiveness and predictive power of computational tools derived from advances in systems and synthetic biology will provide metabolic engineers with insights to explore new and more complex techniques for creating robust modular cocultures to produce high-value compounds in a biobased economy.

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