Metabolic Flux Phenotyping of Secondary Metabolism in Cyanobacteria

Piyoosh K Babele[1*], Amit Srivastava[2], and Jamey D Young[3, 4*]

[1]College of Agriculture, Rani Lakshmi Bai Central Agricultural University Jhansi, 284003, Uttar Pradesh, India.

[2]University of Jyväskylä, Nanoscience Centre, Department of Biological and Environmental Science, 40014 Jyväskylä, Finland

[3]Department of Chemical and Biomolecular Engineering, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

[4]Department of Molecular Physiology and Biophysics, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

*****Correspondence*:*

piyoosh.bhu06@gmail.com (Piyoosh K. Babele), j.d.young@vanderbilt.edu (Jamey D. Young)

Abstract

Cyanobacteria generate energy from photosynthesis and produce various secondary metabolites with diverse commercial and pharmaceutical applications. Unique metabolic and regulatory pathways in cyanobacteria present new challenges for researchers to enhance their product yields, titers, and rates. Therefore, further advancements are critically needed to establish cyanobacteria as a preferred bioproduction platform. Metabolic flux analysis (MFA) quantitatively determines the intracellular flows of carbon within complex biochemical networks, which elucidate the control of metabolic pathways by transcriptional, translational, and allosteric regulatory mechanisms. The emerging field of systems metabolic engineering (SME) involves the use of MFA and other omics technologies to guide the rational development of microbial production strains. This review highlights the potential of MFA and SME to optimize the production of cyanobacterial secondary metabolites and discusses the technical challenges that lie ahead.

Keywords: Secondary metabolism; secondary metabolites; systems metabolic engineering; metabolic flux analysis; genome-scale metabolic models

Introduction

Among contemporary global issues, environmental protection is of primary importance for the sustained prosperity and progress of mankind. Excessive exploitation of natural resources and overdependence on fossil fuels have resulted in growing concerns over global warming, food insecurity, disease outbreaks, the energy crisis, and many other environmental issues. Therefore, alternative manufacturing approaches that are economically viable, carbon neutral, and technologically feasible are urgently required to meet sustainability goals [1,2]. The potential of microbial bioproduction for long-term economic expansion has been realized in the past few decades [3,4]. In particular, photosynthetic microbes such as cyanobacteria have shown tremendous potential to produce biofuel, bioplastic, and other industrial products [5,6]. Cyanobacteria require fewer resources for growth (e.g., atmospheric carbon dioxide, water, sunlight, and minimal nutrients), which provides economic and sustainability advantages over heterotrophic microbes [6]. The recent discovery of fast-growing cyanobacteria makes them comparable to heterotrophic bacteria in terms of doubling time and concomitant production. Unlike higher plants, they do not require arable land, and owing to their prokaryotic genome, they are more amenable to engineering than eukaryotic algae [7].

Cyanobacterial metabolism has evolved to support diverse and complex functions and can be divided into primary metabolism and secondary metabolism (Figure 1). Primary metabolism involves essential biochemical pathways that are required for the survival and growth of the organism. In cyanobacteria, primary metabolism involves the conversion of inorganic carbon dioxide and water into organic compounds (e.g., carbohydrates, proteins, lipids, and nucleic acids) using the process of photosynthesis to produce energy (ATP) and reducing power (NADPH) [8]. On the other hand, secondary metabolism refers to the production of specialized metabolites that are not essential for the basic functions of the cell but provide the organism with selective advantages in terms of survival, growth, and defense [9,10]. Cyanobacteria produce a variety of secondary metabolites that include terpenoids, flavonoids, polyketides, scytonemins, mycosporines, etc. In particular, many bloom-forming cyanobacteria produce a large variety of cyanotoxins in response to the combined pressures of climate change driven abiotic and biotic stresses with high ecological and human health risks [11,12]. The structural information and bioactivities of many such compounds can be accessed from CyanoMetDB, an open-access database dedicated to cyanobacterial secondary metabolites [13].

.

Metabolic flux analysis (MFA) enables the quantification of metabolic fluxes (see Glossary) within biochemical pathways, allowing the assessment of production and consumption rates of metabolites within an organism. MFA is one of the core metabolic engineering tools that has been continuously developed and widely applied to rigorously investigate cell metabolism and quantify the carbon flux distribution in central metabolic pathways [14]. MFA produces detailed flux maps that represent an integrated readout of the cellular phenotype resulting from transcriptional, translational, and allosteric regulatory mechanisms. However, the metabolic study of cyanobacteria is complicated by several factors. For example, light harvesting, carbon fixation, and other metabolic functions are spatially distributed within the cell. Moreover, dynamic regulatory mechanisms that respond to light availability and naturally oscillate with the diurnal cycle further complicate the study of metabolic flux. In addition, flux analysis under photoautotrophic conditions typically requires dynamic $^{13}CO_2$ labeling experiments and **isotopically nonstationary MFA (INST-MFA)**, which are more experimentally and computationally demanding than the steady-state MFA experiments used to characterize heterotrophic metabolism [5,10].

MFA belongs to a broader discipline called **systems metabolic engineering (SME)**, which integrates systems biology and synthetic biology with traditional metabolic engineering [3][4]. SME has been increasingly applied to identify gene targets for engineering the primary metabolism of cyanobacterial hosts for the production of various chemicals serving as potential biofuels and bioplastics [14,15]. So far, however, MFA and other SME approaches have not been widely used for investigating secondary metabolism.

Metabolic engineering of secondary metabolism in cyanobacteria

Cyanobacteria have shown great promise as biocatalysts for the direct conversion of CO_2 into commercial chemicals such as biofuels and bioactive compounds [4]. In order to create cyanobacterial strains with the ability to convert specific central metabolites into desired end products, several primary metabolic pathways have been engineered by either de-regulating genes or introducing heterologous genes [5]. Metabolic engineering has also been used in model species such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002 to increase the production of secondary metabolites (see Box 1) [16]. A number of studies have engineered cyanobacteria for production of terpene products, including isoprene [17, 18], limonene [19], farnesene [20], squalene [21], and astaxanthin [22], which have various applications as cosmetics, pharmaceuticals, and potential petrochemical replacements. These efforts typically

involve overexpressing enzymes in the native methylerythritol phosphate (MEP) pathway and/or knocking down enzymes in competing pathways. Other studies have focused on enhancing production of secondary products that serve as photoprotectants (e.g., mycosporine-like amino acids (MAAs) or storage polymers (e.g., polyhydroxyalkanoates (PHAs)). A study in *Synechocystis* sp. PCC 6803, found that production of shinorine-an MAA that protects against the harmful effects of UV radiation—can be increased up to 10 times (2.37 ± 0.21 mg/g dry biomass weight) by introducing the shinorine gene cluster from the filamentous cyanobacterium *Fischerella* sp. PCC 9339. Integrated transcriptional and metabolic profiling helped to identify the rate-limiting steps in the heterologous production pathway [23]. Koch et al. constructed a PHA-overproducing strain by deleting a regulatory protein (PirC/Sll0944) in *Synechocystis* sp. PCC 6803 that conferred a higher activity of phosphoglycerate mutase and resulted in increased poly-hydroxybutyrate (a promising bioplastic) accumulation under nitrogen and phosphorus depleted conditions [24,25]. However, the final product titers of secondary metabolites achieved in cyanobacterial cultures are typically in the mg/L range, which is too low for scalable commercial production. Therefore, there is a critical need to apply MFA (^{13}C-MFA and INST-MFA) and other systems biology strategies to improve production rates and final titers of these secondary metabolites by identifying bottleneck reactions that limit pathway flux.

Why metabolic flux analysis of cyanobacterial secondary metabolism is important?

MFA is an effective method for determining metabolic fluxes *in vivo* by applying a stoichiometric model of the biochemical reaction network to derive a comprehensive set of metabolite mass balances that can be used to calculate a unique flux solution from a limited set of measurements. Flux information obtained from MFA has been effectively applied to (i) characterize new host organisms, (ii) identify wasteful pathways that limit product yield, and (iii) identify metabolic bottlenecks that restrict production rate [26]. By quantifying fluxes at each major node of the metabolic network and determining how these fluxes become re-routed in response to targeted genetic or environmental perturbations, fundamental insights about network regulation can be obtained to guide further rounds of metabolic engineering.

Additionally, within secondary metabolism, it is crucial to identify and quantify the main competitive branch points that lead flux away from the secondary metabolites of interest. In this manner, the 'wasteful fluxes' leading to undesired side products can be pinpointed and subjected to metabolic engineering to improve product yield [34]. When applied in this way, MFA provides a platform for systematically identifying and removing metabolic bottlenecks, wasteful pathways, and

futile cycles that restrict the production of desired secondary metabolites. Furthermore, MFA provides fundamental insights into how metabolic flux is natively regulated in cyanobacteria, and how the existing control circuitry can be co-opted or disrupted in order to divert flux into non-native sink pathways. Finally, metabolic engineering of secondary metabolism can also improve the function or fitness of the host organism under industrially relevant cultivation conditions. A major reason for performing MFA in cyanobacteria is to quantify fluxes that supply the key intermediary precursors of secondary metabolites. For example, flux estimation at the phosphoenolpyruvate (PEP) and pyruvate nodes is critical for determining the amount of fixed carbon directed towards shikimate (the precursor for aromatic amino acids, alkaloids and MAAs) versus the TCA cycle. Similarly quantifying the flux around the acetyl-CoA node is important for determining the carbon directed towards fatty acids versus TCA cycle. Hasunuma et. al., (2019) applied ^{13}C labeling based metabolomics to understand the flux distribution in an astaxanthin producing recombinant strain of *Synechococcus* sp. PCC 7002. ^{13}C labeling of metabolites indicate higher flux distribution in the Calvin cycle and glycolysis due to overexpression of astaxanthin biosynthetic genes, and suggesting the role of central metabolism and MEP pathway to enhance the astaxanthin biosynthesis [27]. Based on dynamic ^{13}C labeling experiments and metabolite profiling, the MEP pathway in *Synechococcus elongatus* PCC 7942 was engineered (by overexpression of isopentenyl pyrophosphate isomerase), which resulted in the direct production of 1.26 g L^{-1} of isoprene from CO_2 [28]. Another study presented a feasible strategy to engineer *Synechocystis* sp. PCC 6803 for photosynthetic production of the isoprenoid limonene. Based on metabolic engineering strategies (genome-scale modeling and ^{13}C MFA) the pentose phosphate pathway (PPP) genes ribose-5-phosphate isomerase and ribulose-5-phosphate 3-epimerase were overexpressed, and a geranyl diphosphate synthase from *Abies grandis* (a conifer plant) was expressed to generate a limonene overproducing strain that accumulated a final titer of 6.7 mg L^{-1} [29]. Nirati et al. (2022) applied steady state ^{13}C-MFA to compare the carbon flux distribution between glucose-tolerant wild-type vs isoprene-producing recombinant *Synechocystis* sp. PCC 6803. Study pointed out striking difference in the Calvin cycle, glycogen metabolism (high in wild type) and anaplerotic pathway activity (high in recombinant strain) through phosphoenolpyruvate carboxylase and malic enzyme and thus suggested a possible role of ATP and NADPH in regulating the flux distribution in recombinant strain [30].Another example is the use of MFA to calculate fluxes around the 2-oxoglutarate (2OG) node, an important branch point for both carbon (C) and nitrogen (N) metabolism [31]. This C/N balance is key to cyanobacterial bloom formation and cyanotoxin production, and an imbalance in C/N metabolism alters the tradeoff

between primary and secondary metabolism resulting in cyanotoxin production [32,33]. MFA provides a quantitative depiction of overlapping C/N metabolic networks, which can link genome profiling to phenome analysis and reveal the pathways associated with bloom formation.

Genome-scale metabolic modeling and flux balance analysis

Genome-scale metabolic models (GSMs) predicting global metabolic flux distributions under given genetic and environmental conditions are important systems biology tools for metabolic engineering and strain development [35]. A GSM is a large-scale stoichiometric model that describes all the metabolic pathways using gene-protein-reaction associations experimentally and/or theoretically characterized through stoichiometric coefficients and mass balances of participating metabolites, simulated using mathematical optimization [36,37].

GSMs are based on stoichiometric relationships between biochemical reactions in a metabolic network and use linear programming to predict the metabolic fluxes in the network under different conditions [38]. GSMs are typically constructed using genome annotations and metabolic pathway databases and can include thousands of reactions and metabolites. GSMs provide a comprehensive view of the metabolic network and can be used to predict the behavior of the network under different environmental conditions. However, GSMs do not account for the kinetics of individual reactions and do not capture the dynamics of metabolic pathways [38]. This can limit their accuracy in predicting the behavior of cells under non-steady-state conditions or in response to external perturbations. In contrast, kinetic models are based on the detailed kinetic equations that govern individual biochemical reactions in a metabolic network and can account for the dynamics of metabolic pathways and the kinetics of individual reactions [39]. Kinetic models typically involve a large number of parameters, which can be difficult to measure experimentally. Kinetic models provide a more detailed and accurate description of the metabolic network and can be used to predict the behavior of cells under non-steady-state conditions or in response to external perturbations. However, the computational cost of simulating kinetic models can be high, and the models can be difficult to validate due to the large number of parameters involved [39]. Broddrick et al. (2016) manually curated and experimentally validated a GSM of *Synechococcus elongatus* PCC 7942 and discovered unique metabolic characteristics, such as the importance of a truncated, linear TCA pathway. They also highlighted poorly understood areas of metabolism as exemplified by knowledge gaps in nucleotide salvage [35]. Janasch et al. (2019) created a kinetic model of the CBB cycle of *Synechocystis* sp. PCC 6803 to investigate its stability and underlying control mechanisms [40].

These modeling approach relies on the assumption of a pseudo-steady state, which is best applied to simulating primary metabolism during exponential growth phase. Importantly, the GSM enables the prediction of metabolic flux values for the entire network of metabolic reactions using optimization techniques such as **flux balance analysis (FBA)** based on linear programming [41].

FBA is a widely used constraint-based approach for studying biochemical networks, in particular genome-scale reconstructions, or gap filling of possibly missing reactions in existing GSMs. FBA calculates the flow of metabolites through the metabolic network, thereby making it possible to predict the growth rate of an organism or the rate of production of a biotechnologically important metabolite based on a limited number of empirically derived constraints (Figure 2). FBA calculations and simulations use **constraint-based reconstruction and analysis (COBRA)** methods which can be performed using many available tools [42,43]. Metabolic questions that can be best addressed with GSM and FBA include, but are not limited to (i) prediction of the most efficient pathway that leads to the maximal product yield of a target compound and (ii) optimization of precursor supply and intracellular redox balances, typically through prediction of the effects of gene knockouts and over-expressions [44]. GSMs also serve as a platform for the integration and analysis of meta-omics and maintenance energy (ATP) turnover data [45]. As next generation sequencing tools and relevant meta-omics analyses continue to evolve, the quality and application scope of GSMs have also expanded accordingly, and together they have contributed to a better understanding of metabolism in innumerable organisms.

Due to its inability to predict changes in metabolite concentrations and its lack of kinetic parameters, FBA has certain limitations. Furthermore, it often suffers from incomplete annotation of the proteins in a genome. Several reactions may be predicted to have zero fluxes from FBA due to the inadequate nature of annotation since the reactions involving metabolites downstream or upstream of these reactions may not have been identified, thus leaving metabolic gaps. Except in some modified forms, FBA does not account for regulatory effects such as activation of enzymes by protein kinases or regulation of gene expression, so predictions by FBA may not always be accurate. Manual reconstruction of GSMs is a time-consuming procedure, in which a large number of gene-protein-reaction associations and many other sources of data and information must be considered. Significant progress has been made to automate the GSM reconstruction procedure including, but not limited to, better annotation of genome sequences, standardization and cross-referencing of different metabolic databases, and improved algorithms for detecting and filling metabolic gaps. Several software

programs for automatic GSM reconstruction have been developed and were described previously [36].

A number of GSMs of cyanobacteria have been presented in the last decade (reviewed in [16][44]), but they are still underrepresented in comparison to heterotrophic microorganisms. Also, >300 cyanobacterial genomes have been sequenced [46]; however, the construction of GSMs for non-model species is limited, and reconstructions and refinements of GSMs have only been performed for model species. Accurate modeling of cyanobacterial metabolism requires a new level of information, including modeling the processes of light harvesting and electron transport through a variety of possible pathways [47,48]. Furthermore, now that many BGCs can be effectively detected in cyanobacteria using genome mining [49,50] and bioinformatics programs (e.g., antiSMASH) [51], incorporating their corresponding biosynthetic reactions into metabolic models becomes an important task. The biosynthetic reactions for several clusters have been characterized, but the majority have not. More comprehensive information on secondary metabolite biosynthetic reactions would help to systematically evaluate the production capacity of secondary metabolites using metabolic models. GSMs will be more useful in metabolic engineering of secondary metabolism if they are expanded by incorporating more experimental meta-omics datasets and gap-filling model parameters. GSMs for modeling secondary metabolism should be further developed for rational engineering to enhance the production of secondary metabolites. In synthetic biology, GSM and MFA can be used to guide genome-scale engineering by providing insights into the metabolic pathways and enzymes that are critical for achieving a specific metabolic goal. MFA can be used to identify the metabolic pathways and enzymes that need to be modified or engineered in order to redirect the flux of metabolites towards a desired product or pathway. This information can be used to design and engineer synthetic metabolic pathways that can produce high-value metabolites. In addition, MFA can be used to identify the metabolic trade-offs and limitations that can arise from genome-scale engineering.

Metabolic Flux Analysis: ^{13}C-MFA and INST-MFA

MFA is another model-based analysis approach that can be used to calculate metabolic fluxes. In MFA, metabolic fluxes are estimated from experimentally measured rates, such as substrate uptake rate, oxygen uptake rate, growth rate and product secretion rates, subject to stoichiometric constraints (Figure 3). MFA differs from FBA in that there is no assumption regarding the optimal performance of the cell. As such, MFA can be used to quantify fluxes for cells grown under industrially relevant growth conditions, e.g., during nutrient limitation or in the presence of growth-inhibitory compounds,

which is currently beyond the scope of most FBA-based techniques. In MFA, the flow of metabolites through a network model is calculated by solving a least-squares regression problem to minimize the sum of squared residuals (SSR) between model-simulated and experimentally determined measurements. An important application of MFA includes determining the yields of key cellular cofactors such as ATP, NADH, and NADPH under different growth conditions [52]. This can provide valuable insights into the flow of energy and electrons. The main limitation of MFA is that it uses simplified and context-specific metabolic network models for analysis because external rate measurements generally don't provide enough constraints to estimate fluxes for all known intracellular pathways. For example, measuring oxygen uptake rate and carbon dioxide production rate during heterotrophic growth often doesn't provide independent constraints, since these two rates can be calculated from other measured rates through the electron balance and carbon balance, respectively [14]. Thus, in order to apply MFA in practice, some pathways must be neglected from the network model to ensure that there are enough measurements to precisely estimate the fluxes of the remaining selected pathways in the model.

An alternative to model simplification is to obtain additional measurements from stable isotope labeling experiments, which can provide detailed information on the path of carbon flow within intracellular networks. MFA based on stable isotope measurements typically relies on two possible approaches: isotopic stationary metabolic flux analysis, or ^{13}C-metabolic flux analysis (^{13}C-MFA) and isotopically nonstationary metabolic flux analysis (INST-MFA) (Figure 3). Both ^{13}C-MFA and INST-MFA offer a better understanding of cyanobacterial biosynthetic pathways and the metabolic flux changes that occur in response to their modulation, which can be used to guide further metabolic engineering efforts as reviewed previously [7]. Tracer substrates can be labeled with ^{2}H, ^{13}C, ^{15}N, ^{17}O, or ^{18}O, but so far the most widely used stable isotope is ^{13}C because every bioorganic molecule contains carbon atoms that are transferred and rearranged due to biochemical reactions [7]. The activity of most biochemical pathways can be assessed through monitoring the rates and patterns of isotope enrichment in downstream metabolites following supplementation of a ^{13}C-labeled substrate. This is the foundation of ^{13}C-MFA and is a powerful method for characterizing *in vivo* metabolism [53]. In ^{13}C-MFA, one or more tracer experiments are performed where live cell cultures are fed with ^{13}C-labeled substrates (e.g. [1,2-^{13}C] glucose) until the ^{13}C enrichment patterns of measured intracellular metabolites and macromolecules (e.g., proteins, RNA and glycogen) fully equilibrate. Labeling patterns and steady-state carbon mass isotopomer distributions (MIDs) are then

quantified for various cellular metabolites using analytical techniques such as mass spectrometry (MS) and/or nuclear magnetic resonance (NMR). The elementary metabolite unit (EMU) framework is the most widely used approach for modeling isotopic labeling in ^{13}C-MFA and is at the core of several major software packages [52,54].

^{13}C-MFA has been widely applied in heterotrophic or mixotrophic cyanobacterial cultures to identify the metabolic pathway dependencies involved in the production of specific biochemicals [7, 5]. In contrast, autotrophic cyanobacterial cultures assimilate carbon solely from CO_2 and therefore produce a uniform steady-state ^{13}C-labeling pattern in all metabolites when labeled with $^{13}CO_2$. These steady-state patterns do not depend on fluxes, making conventional steady-state ^{13}C-MFA ineffective for quantifying autotrophic metabolism. However, the transient patterns of $^{13}CO_2$ labeling are sensitive to fluxes and can be used to estimate flux values with INST-MFA, which does not rely on isotopic steady state assumptions. INST-MFA treats the metabolic network as a dynamic system and offers a number of unique advantages over ^{13}C-MFA. First, it can be applied to estimate fluxes in autotrophic systems, which consume only single-carbon substrates. Second, INST-MFA is suitable for systems that label slowly due to the presence of large intermediate pools or pathway bottlenecks. Last, it offers increased measurement sensitivity to estimate reversible exchange fluxes and metabolite pool sizes, which represents a potential framework for integrating metabolite analysis with MFA [52]. ^{13}C-MFA and INST-MFA are now established techniques that are routinely applied in metabolic engineering to quantify metabolic fluxes. Many procedures and protocols have been optimized and standardized for these techniques in the past decade. Recent papers have compiled comprehensive step-by-step protocols and troubleshooting guidelines for conducting high-resolution MFA studies [14,55]. Fluxes are estimated using software, and the most commonly used software tools for MFA have been reviewed previously [55,56].

In recent years, machine learning (ML) and artificial intelligence (AI) have been increasingly used in conjunction with metabolic analysis [57,58]. ML and AI algorithms can be used to analyze large and complex datasets generated from MFA and FBA experiments and to predict the metabolic fluxes and behaviors of cells under different conditions. For example, ML algorithms can be trained to analyze the expression levels of genes and enzymes in a metabolic network and predict the metabolic flux distribution based on this information. AI can also be used to optimize the predictions of FBA models by incorporating COBRA for more accurate and realistic predictions of metabolic fluxes in cells. Another application of ML and AI in MFA and FBA is the identification of key

metabolic pathways and enzymes that are critical for the growth and survival of cells under different conditions [59,60]. ML algorithms can be used to analyze large datasets of gene expression and enzyme activity and to identify the most important metabolic pathways and enzymes that are associated with specific cellular functions or phenotypes.

Analytical considerations for large-scale metabolomics and fluxomics

Metabolomics and fluxomics provide holistic information on cellular metabolism. Both approaches can be simultaneously applied to gain complementary information on complex metabolic pathway activities and how they are regulated *in vivo* [52,61]. Unlike metabolites, fluxes are not physical entities and thus cannot be measured directly but can be determined from other measurements. Metabolomics and fluxomics typically require the characterization and quantitative analysis of a variety of metabolites (e.g., amino acids, lipids, nucleotides, carbohydrates, organic acids, and secondary metabolites). Thus, many sample analysis workflows, measurement techniques, and software tools are shared between these two platforms. The physical and chemical properties of metabolites are diverse and are often distributed over a wide range of concentrations inside cells [14]. Metabolomics and fluxomics capabilities have advanced due to the continuous improvement of analytical tools for measuring primary and secondary metabolites. Typically, metabolite measurements for ^{13}C-MFA and INST-MFA comprise five basic steps that are summarized in Figure 4. Selection of appropriate sample harvesting, quenching (to preserve the *in vivo* metabolic state), extraction, derivatization, cleanup, as well as adaptation of suitable analytical (MS and NMR) instrumentation and data analysis tools, are critical for achieving precise quantitation of the abundance and isotope enrichment of intracellular metabolites [62]. Although these technologies are transferable across different organisms, adapting these protocols and selecting appropriate instruments tends to be organism- and hypothesis-specific [56]. The above steps are further complicated when applied to photoautotrophic suspension cultures because they require rapid separation of liquid culture media from cellular biomass prior to metabolite extraction, while avoiding possible artefacts that can arise due to unintended culture shading or incomplete sample quenching [62].

Concluding Remarks and Future Perspectives

Cyanobacteria are potential hosts for biotechnological and industrial applications. However, cyanobacteria have more complex energy generation and distribution processes because they are

photoautotrophic, and non-intuitive regulatory inputs such as redox state and circadian rhythms should be considered when manipulating their metabolic networks. Several systems metabolic engineering tools have already been established and successfully employed for engineering primary metabolism in cyanobacteria. FBA and MFA provide complementary tools for metabolic engineering of primary metabolic pathways, and these tools and strategies could be extended for engineering secondary metabolism. Engineering strains for production of secondary metabolites involves unique considerations because the BGCs of secondary pathways encode a complex and poorly characterized system in cyanobacteria, and therefore optimization of secondary metaboliteproduction requires systematic analysis before actual metabolic engineering can occur. For the optimal production and potential commercialization of secondary metabolites, researchers need to have a better understanding of the working mechanisms of BGCs and secondary metabolic pathways (see Box 2). The function, distribution, and regulation of enzymes in secondary pathways and their relationship to primary metabolism remain largely unknown. Quantifying fluxes in primary metabolic processes that produce secondary metaboliteprecursors would be highly advantageous. With the help of metabolomics-assisted MFA, the flux distribution at key metabolic nodes in intermediary metabolism can be examined, identifying fluxes that lead to futile or wasteful pathways and determining how competing pathways regulate flux in wild-type and engineered strains under various physiological conditions. Such information can aid in understanding how environmental and genetic factors impact primary metabolism and, in turn, the production of the building blocks for secondary metabolic pathways. INST-MFA has identified new strategies for the overproduction of terpenoid (limonene) products in *Synechococcus* PCC 7002 by revealing differential flux through pyruvate biosynthesis pathways. The results of INST-MFA imply that ATP:NADPH ratios are crucial for the formation of limonene since they demonstrate metabolite channeling in the amphibolic loop including PEP carboxylase, malate dehydrogenase, and malic enzyme [63].

Furthermore, there is a need to investigate, identify and develop non-model strains that exhibit unique capabilities for secondary metaboliteproduction. Processes of systems metabolic engineering need to be applied at an early phase of strain development (e.g., genome mining and host selection). This will facilitate the introduction of state-of-the-art synthetic biology tools to enhance secondary metabolite production, which were initially developed for model organisms. Secondary metaboliteproduction involves tight coordination between carbon (C) and nitrogen (N) metabolism; therefore, novel tracing experiments should be designed and applied to analyze both C/N metabolism

simultaneously by feeding both ^{13}C and ^{15}N tracers for qualitative determination and quantitative estimation of metabolic fluxes in secondary metabolic pathways. Here, we propose the combination of ^{13}C and ^{15}N-labeled tracers to interrogate carbon and nitrogen metabolism within a single experiment to gain better insight into the secondary metabolism of cyanobacteria. These flux estimations demand new analytical (MS and NMR) tools to characterize and estimate the labeling and abundance of secondary metabolites. Such stable isotope-based MFA studies allow quantitative estimation of C and N flows from feedstocks to the central metabolic pathways and further into the secondary pathways and desired end products. However, the diverse chemical nature of secondary metabolites is highly challenging for current analytical tools. Therefore, new extraction protocols and high-throughput MS and NMR methods should be developed for the accurate estimation of a diverse range of secondary metabolites.

Acknowledgements

This work is supported by a Ramanujan Fellowship (file no. RJF/2020/000043) awarded to P.K.B. by Science and Engineering Research Board (SERB), Department of Science and Technology (DST), New Delhi, India.

Declaration of interests

None are declared.

References

1 Wang, F. *et al.* (2021) Technologies and perspectives for achieving carbon neutrality. *Innov.* 2, 100180

2 Soergel, B. *et al.* (2021) A sustainable development pathway for climate action within the UN 2030 Agenda. *Nat. Clim. Chang. 2021 118* 11, 656–664

3 Lee, J.W. *et al.* (2012) Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nat. Chem. Biol. 2012 86* 8, 536–546

4 Choi, K.R. *et al.* Systems Metabolic Engineering Strategies: Integrating Systems and Synthetic Biology with Metabolic Engineering. , *Trends in Biotechnology*, 37. 01-Aug-(2019) , Elsevier Ltd, 817–837

5 Angermayr, S.A. *et al.* (2015) Metabolic engineering of cyanobacteria for the synthesis of

commodity products. *Trends Biotechnol.* 33, 352–361

6 Oliver, N.J. *et al.* (2016) Cyanobacterial metabolic engineering for biofuel and chemical production. *Curr. Opin. Chem. Biol.* 35, 43–50

7 Babele, P.K. and Young, J.D. (2019) Applications of stable isotope-based metabolomics and fluxomics toward synthetic biology of cyanobacteria. *Wiley Interdiscip. Rev. Syst. Biol. Med.* DOI: 10.1002/wsbm.1472

8 Schwarz, D. *et al.* (2013) Recent Applications of Metabolomics Toward Cyanobacteria. *Metabolites* 3, 72–100

9 Forchhammer, K. and Selim, K.A. (2020) Carbon/nitrogen homeostasis control in cyanobacteria. *FEMS Microbiol. Rev.* 44, 33–53

10 Adebiyi, A.O. *et al.* (2015) 13C flux analysis of cyanobacterial metabolism. *Photosynth. Res.* 126, 19–32

11 Wang, H. *et al.* (2021) From unusual suspect to serial killer: Cyanotoxins boosted by climate change may jeopardize megafauna. *Innov.* 2, 100092

12 Singh, A. and Babele, P.K. (2020) Dynamics of harmful cyanobacterial blooms and their toxins: environmental and human health perspectives and management strategies. In *Advances in Cyanobacterial Biology* pp. 301–317, Elsevier

13 Jones, M.R. *et al.* (2021) CyanoMetDB, a comprehensive public database of secondary metabolites from cyanobacteria. *Water Res.* 196, 117017

14 Antoniewicz, M.R. (2021) A guide to metabolic flux analysis in metabolic engineering: Methods, tools and applications. *Metab. Eng.* 63, 2–12

15 Carroll, A.L. *et al.* (2018) Metabolic engineering tools in model cyanobacteria. *Metab. Eng.* 50, 47–56

16 Jeong, Y. *et al.* (2020) Current Status and Future Strategies to Increase Secondary Metabolite Production from Cyanobacteria. *Microorg. 2020, Vol. 8, Page 1849* 8, 1849

17 Pade, N. *et al.* (2016) Insights into isoprene production using the cyanobacterium Synechocystis sp. PCC 6803. *Biotechnol. Biofuels* DOI: 10.1186/s13068-016-0503-4

18 Chaves, J.E. *et al.* (2017) Engineering Isoprene Synthase Expression and Activity in Cyanobacteria. DOI: 10.1021/acssynbio.7b00214

19 Lin, P.C. *et al.* (2021) Enhanced limonene production in a fast-growing cyanobacterium through combinatorial metabolic engineering. *Metab. Eng. Commun.* 12, e00164

20 Lee, H.J. *et al.* (2017) Direct Conversion of CO2 to α-Farnesene Using Metabolically Engineered Synechococcus elongatus PCC 7942. *J. Agric. Food Chem.* 65, 10424–10428

21 Choi, S.Y. *et al.* (2016) Photosynthetic conversion of CO2 to farnesyl diphosphate-derived phytochemicals (amorpha-4,11-diene and squalene) by engineered cyanobacteria. *Biotechnol. Biofuels* 9, 202

22 Diao, J. *et al.* (2020) Tailoring cyanobacteria as a new platform for highly efficient synthesis of astaxanthin. *Metab. Eng.* 61, 275–287

23 Yang, G. *et al.* (2018) Photosynthetic Production of Sunscreen Shinorine Using an Engineered Cyanobacterium. *ACS Synth. Biol.* 7, 664–671

24 Koch, M. *et al.* (2019) PHB is produced from Glycogen turn-over during nitrogen starvation in Synechocystis sp. PCC 6803. *Int. J. Mol. Sci.* 20,

25 Koch, M. *et al.* (2020) Maximizing PHB content in Synechocystis sp. PCC 6803: a new metabolic engineering strategy based on the regulator PirC. *Microb. Cell Fact.* 19, 1–12

26 McAtee, A.G. *et al.* (2015) Application of isotope labeling experiments and 13C flux analysis to enable rational pathway engineering. *Curr. Opin. Biotechnol.* 36, 50–56

27 Hasunuma, T. *et al.* (2019) Single-Stage Astaxanthin Production Enhances the Nonmevalonate Pathway and Photosynthetic Central Metabolism in Synechococcus sp. PCC 7002. *ACS Synth. Biol.* 8, 2701–2709

28 Gao, X. *et al.* (2016) Engineering the methylerythritol phosphate pathway in cyanobacteria for photosynthetic isoprene production from CO2. *Energy Environ. Sci.* 9, 1400–1411

29 Lin, P.-C. *et al.* (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium Synechocysti s sp. PCC 6803. *Sci. Rep.* 7, 17503

30 Nirati, Y. *et al.* (2022) Quantitative insight into the metabolism of isoprene-producing Synechocystis sp. PCC 6803 using steady state 13C-MFA. *Photosynth. Res.* 154, 195–206

31 Zhang, C.C. *et al.* (2018) Carbon/Nitrogen Metabolic Balance: Lessons from Cyanobacteria. *Trends Plant Sci.* 23, 1116–1130

32 Beversdorf, L.J. *et al.* (2015) Long-term monitoring reveals carbon-nitrogen metabolism key to microcystin production in eutrophic lakes. *Front. Microbiol.* 6, 456

33 Wagner, N.D. *et al.* (2019) Biological Stoichiometry Regulates Toxin Production in Microcystis aeruginosa (UTEX 2385). *Toxins 2019, Vol. 11, Page 601* 11, 601

34 Shih, M.L. and Morgan, J.A. Metabolic flux analysis of secondary metabolism in plants. , *Metabolic Engineering Communications*, 10. 01-Jun-(2020) , Elsevier B.V., e00123

35 Broddrick, J.T. *et al.* (2016) Unique attributes of cyanobacterial metabolism revealed by improved genome-scale metabolic modeling and essential gene analysis. *Proc. Natl. Acad. Sci. U. S. A.* 113, E8344–E8353

36 Gu, C. *et al.* Current status and applications of genome-scale metabolic models. , *Genome Biology*, 20. 13-Jun-(2019) , BioMed Central Ltd., 1–18

37 Qian, X. *et al.* (2017) Flux balance analysis of photoautotrophic metabolism: Uncovering new biological details of subsystems involved in cyanobacterial photosynthesis. *Biochim. Biophys. Acta - Bioenerg.* 1858, 276–287

38 Gu, C. *et al.* Current status and applications of genome-scale metabolic models. DOI: 10.1186/s13059-019-1730-3

39 Strutz, J. *et al.* (2019) Metabolic kinetic modeling provides insight into complex biologicalquestions, but hurdles remain. *Curr. Opin. Biotechnol.* 59, 24

40 Janasch, M. *et al.* (2019) Kinetic modeling of the Calvin cycle identifies flux control and stable metabolomes in Synechocystis carbon fixation. *J. Exp. Bot.* 70, 973

41 He, L. *et al.* (2015) Simulating cyanobacterial phenotypes by integrating flux balance analysis, kinetics, and a light distribution function. *Microb. Cell Fact.* 14, 1–11

42 Raman, K. and Chandra, N. (2009) Flux balance analysis of biological systems: applications

and challenges. *Brief. Bioinform.* 10, 435–449

43 Orth, J.D. *et al.* What is flux balance analysis? , *Nature Biotechnology*, 28. Mar-(2010) , 245–248

44 Santos-Merino, M. *et al.* (2019) New applications of synthetic biology tools for cyanobacterial metabolic engineering. *Front. Bioeng. Biotechnol.* 7, 33

45 Vijayakumar, S. and Angione, C. (2021) Protocol for hybrid flux balance, statistical, and machine learning analysis of multi-omic data from the cyanobacterium Synechococcus sp. PCC 7002. *STAR Protoc.* 2,

46 Fujisawa, T. *et al.* (2017) CyanoBase: a large-scale update on its 20th anniversary. *Nucleic Acids Res.* 45, D551–D554

47 Nogales, J. *et al.* (2012) Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. *Proc. Natl. Acad. Sci. U. S. A.* 109, 2678–2683

48 Toyoshima, M. *et al.* (2019) Flux balance analysis of cyanobacteria reveals selective use of photosynthetic electron transport components under different spectral light conditions. *Photosynth. Res. 2019 1431* 143, 31–43

49 Popin, R.V. *et al.* (2021) Mining of Cyanobacterial Genomes Indicates Natural Product Biosynthetic Gene Clusters Located in Conjugative Plasmids. *Front. Microbiol.* 12, 3353

50 Jin, X. *et al.* (2021) Identification of putative biosynthetic gene clusters for tolyporphins in multiple filamentous cyanobacteria. *Life* 11,

51 Blin, K. *et al.* (2019) Recent development of antiSMASH and other computational approaches to mine secondary metabolite biosynthetic gene clusters. *Brief. Bioinform.* 20, 1103–1113

52 Cheah, Y.E. and Young, J.D. (2018) Isotopically nonstationary metabolic flux analysis (INST-MFA): putting theory into practice. *Curr. Opin. Biotechnol.* 54, 80–87

53 Dai, Z. and Locasale, J.W. (2017) Understanding metabolism with flux analysis: From theory to application. *Metab. Eng.* 43, 94–102

54 Rahim, M. *et al.* (2022) INCA 2.0: A tool for integrated, dynamic modeling of NMR- and

MS-based isotopomer measurements and rigorous metabolic flux analysis. *Metab. Eng.* 69, 275–285

55 Long, C.P. and Antoniewicz, M.R. (2019) High-resolution 13C metabolic flux analysis. *Nat. Protoc. 2019 1410* 14, 2856–2877

56 Wang, Y. *et al.* (2020) Metabolic Flux Analysis—Linking Isotope Labeling and Metabolic Fluxes. *Metabolites* 10, 1–21

57 Vijayakumar, S. *et al.* (2020) A Hybrid Flux Balance Analysis and Machine Learning Pipeline Elucidates Metabolic Adaptation in Cyanobacteria. *iScience* 23, 101818

58 Konno, N. and Iwasaki, W. (2023) Machine learning enables prediction of metabolic system evolution in bacteria. *Sci. Adv.* 9,

59 A, S. *et al.* (2021) Advances in flux balance analysis by integrating machine learning and mechanism-based models. *Comput. Struct. Biotechnol. J.* 19, 4626–4640

60 Zampieriid, G. *et al.* (2019) Machine and deep learning meet genome-scale metabolic modeling. DOI: 10.1371/journal.pcbi.1007084

61 Jang, C. *et al.* (2018) Leading Edge Primer Metabolomics and Isotope Tracing. *Cell* 173, 822–837

62 Wang, B. and Young, J.D. (2022) 13C-Isotope-Assisted Assessment of Metabolic Quenching during Sample Collection from Suspension Cell Cultures. *Anal. Chem.* 94, 7787–7794

63 Newman, D.M. *et al.* (2022) Characterizing Photosynthetic Biofuel Production: Isotopically Non-Stationary 13C Metabolic Flux Analysis on Limonene Producing Synechococcus sp. PCC 7002. *Front. Energy Res.* 10, 926

64 Kultschar, B. and Llewellyn, C. (2018) Secondary Metabolites in Cyanobacteria. *Second. Metab. - Sources Appl.* DOI: 10.5772/INTECHOPEN.75648

65 Kim, H.U. *et al.* (2016) Metabolic engineering with systems biology tools to optimize production of prokaryotic secondary metabolites. *Nat. Prod. Rep.* 33, 933–941

66 Yoon, V. and Nodwell, J.R. (2014) Activating secondary metabolism with stress and chemicals. *J. Ind. Microbiol. Biotechnol.* 41, 415–424

67 Wu, C. and van der Donk, W.A. (2021) Engineering of new-to-nature ribosomally synthesized and post-translationally modified peptide natural products. *Curr. Opin. Biotechnol.* 69, 221–231

68 Khalifa, S.A.M. *et al.* (2021) Cyanobacteria-From the Oceans to the Potential Biotechnological and Biomedical Applications. *Mar. Drugs* 19,

69 Kageyama, H. and Waditee-Sirisattha, R. (2018) Mycosporine-Like Amino Acids as Multifunctional Secondary Metabolites in Cyanobacteria: From Biochemical to Application Aspects. *Stud. Nat. Prod. Chem.* 59, 153–194

70 Santos-Merino, M. *et al.* (2018) Engineering the fatty acid synthesis pathway in Synechococcus elongatus PCC 7942 improves omega-3 fatty acid production. *Biotechnol. Biofuels* 11, 239

71 Santos-Merino, M. *et al.* (2022) Synechococcus elongatus PCC 7942 as a Platform for Bioproduction of Omega-3 Fatty Acids. *Life* 12, 810

72 Troschl, C. *et al.* (2017) Cyanobacterial PHA Production-Review of Recent Advances and a Summary of Three Years' Working Experience Running a Pilot Plant. *Bioeng. (Basel, Switzerland)* 4,

73 Carpine, R. *et al.* (2020) Industrial Production of Poly-β-hydroxybutyrate from CO2: Can Cyanobacteria Meet this Challenge? *Process. 2020, Vol. 8, Page 323* 8, 323

Box 1. Secondary metabolism and metabolites in cyanobacteria

Secondary or specialized metabolism involves biochemical pathways that fulfill a multitude of functions for the growth and survival of cyanobacteria in nature. Typically, secondary metabolic pathways are fueled by primary metabolism (glycolysis, TCA cycle, shikimate pathway, etc.) and often produce precursors for the synthesis of diverse secondary metabolites (Figure 1) [13,64]. secondary metabolites are low-molecular-mass organic molecules, usually produced during the late stationary phase (idiophase), which are not essential for growth, development, or reproduction (in contrast to primary metabolites such as lipids, amino acids, carbohydrates, and nucleic acids) [65]. secondary metabolites are frequently produced in response to stress conditions and provide adaptive benefits to organisms by giving them a competitive advantage in their natural environment [65,66]. Many secondary metabolites are **ribosomally synthesized and post-translationally modified**

563 **peptides (RiPPs)**, which are encoded by specific **biosynthetic gene clusters (BGCs)** [65,67]. BGCs
564 encode core biosynthetic enzymes (e.g., polyketide synthase and non-ribosomal peptide synthetase)
565 responsible for creating and modifying intermediate metabolites, as well as regulatory transcription
566 factors and transporters that control the trafficking of these metabolites and necessary precursors [49].
567 Cyanobacterial genomes are naturally rich in BGCs, enabling cyanobacteria to produce a wide
568 diversity of natural products such as terpenes, alkaloids, polyketides, and **non-ribosomal peptides**
569 **(NRPs)** (Figure 1) that have bioactive properties with various commercial and pharmaceutical uses
570 such as antibacterial, antifungal, anticancer, antituberculosis, immunosuppressive, anti-
571 inflammatory, and antioxidant treatments [13,68].

572 A number of cyanobacterial species also produce **cyanotoxins** for defense [12] or as
573 protectants, and **MAAs** for mitigating photodamage and oxidative stress [68,69]. Many studies have
574 shown that cyanobacteria produce omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and
575 docosahexaenoic acid (DHA), which are known to prevent inflammatory cardiovascular diseases and
576 are used as dietary supplements [70][71]. The use of marine cyanobacteria in cosmetics,
577 cosmeceutical formulations and thalassotherapy provides many benefits, including the maintenance
578 of skin structure and function, due to the presence of bioactive components. These same compounds
579 confer protection to cyanobacterial cells against external environmental conditions.
580 **Polyhydroxyalkanoates (PHAs)** are polyesters produced by many cyanobacterial strains, that can
581 be used as a substitute for non-biodegradable plastics [72,73]. Although the valuable properties of
582 secondary metabolites have long been recognized, knowledge about their potential human and
583 ecotoxicological risks is still at an early stage. In addition, upscaling secondary metabolite production
584 requires a deeper understanding of cyanobacterial metabolism in order to develop efficient host
585 strains and cultivation systems.

586 **Box 2. Important questions related to control of secondary metabolism in cyanobacteria**

587 1. What are the genetic and metabolic components that impede carbon fixation and
588 diversion of metabolic flux into high-value secondary metabolites?
589 2. What processes control the shift from primary to secondary metabolism that occurs when
590 growth rate slows?
591 3. How is the trade-off between primary and secondary metabolism altered by the C/N
592 balance, and how does the C/N ratio affect the formation of cyanobacterial blooms and
593 cyanotoxins production in natural water reservoirs?

4. What are the critical branch points that control distribution of flux into desired secondary metabolites and away from undesired products, and how are these flux ratios regulated?

Glossary

Biosynthetic gene clusters: A physically clustered group of two or more genes in a particular genome that together encode a biosynthetic pathway for the production of a specialized metabolite (including its chemical variants).

Cyanotoxins: A diverse group of toxic compounds (e.g., microcystins) produced by harmful cyanobacterial blooms, which include liver toxins, nerve toxins, and skin toxins with human and animal health hazards.

Constraint-based reconstruction and analysis (COBRA): Systems biology approach used to build and simulate metabolic networks using mathematical representations of biochemical reaction, gene-protein reaction association, and physiological and biochemical constraints.

Flux balance analysis (FBA): Powerful approach for the constraint-based analyses of (genome-scale) metabolic networks, to identify optimal steady-state flux distributions and metabolic capabilities of biochemical networks.

Genome-scale metabolic model (GSM): A mathematical model that represents all known metabolic reactions of a biological system and computationally describes gene-protein-reaction associations for all metabolic enzymes in an organism, and can be simulated to predict metabolic fluxes for various systems-level metabolic studies.

Isotopically nonstationary Metabolic Flux Analysis (INST-MFA): Provides an important new platform for mapping carbon fluxes that is especially applicable to autotrophic organisms, industrial bioprocesses, high-throughput experiments, and other systems that are not amenable to steady-state ^{13}C MFA experiments.

Metabolic flux: The rate at which molecules (metabolites) move through a specific metabolic pathway at steady state.

Mycosporine-like amino acids (MAAs): A large family of small (<400 Da), water-soluble, colorless multipurpose secondary metabolites with high molar extinction coefficient, which serve as UV-B sunscreens..

Non-ribosomal peptides: Therapeutically important and biologically active secondary metabolites synthesized via multidomain mega-enzymes named nonribosomal peptide synthetases (NRPSs) without cell ribosomal machinery or messenger RNA.

Polyhydroxyalkanoates (PHAs): A family of biodegradable polyesters that are produced by an extensive variety of microorganisms synthesized under unbalanced growth for intracellular carbon and energy storage purposes, and as a survival mechanism.

Ribosomally synthesized and post-translationally modified peptides (RiPPs): A major class of natural products with a high degree of structural diversity, and an associated wide range of biological activities from antimicrobial to antiallodynic.

Systems metabolic engineering (SME): A multidisciplinary approach that combines traditional metabolic engineering with systems biology, synthetic biology, and evolutionary engineering.

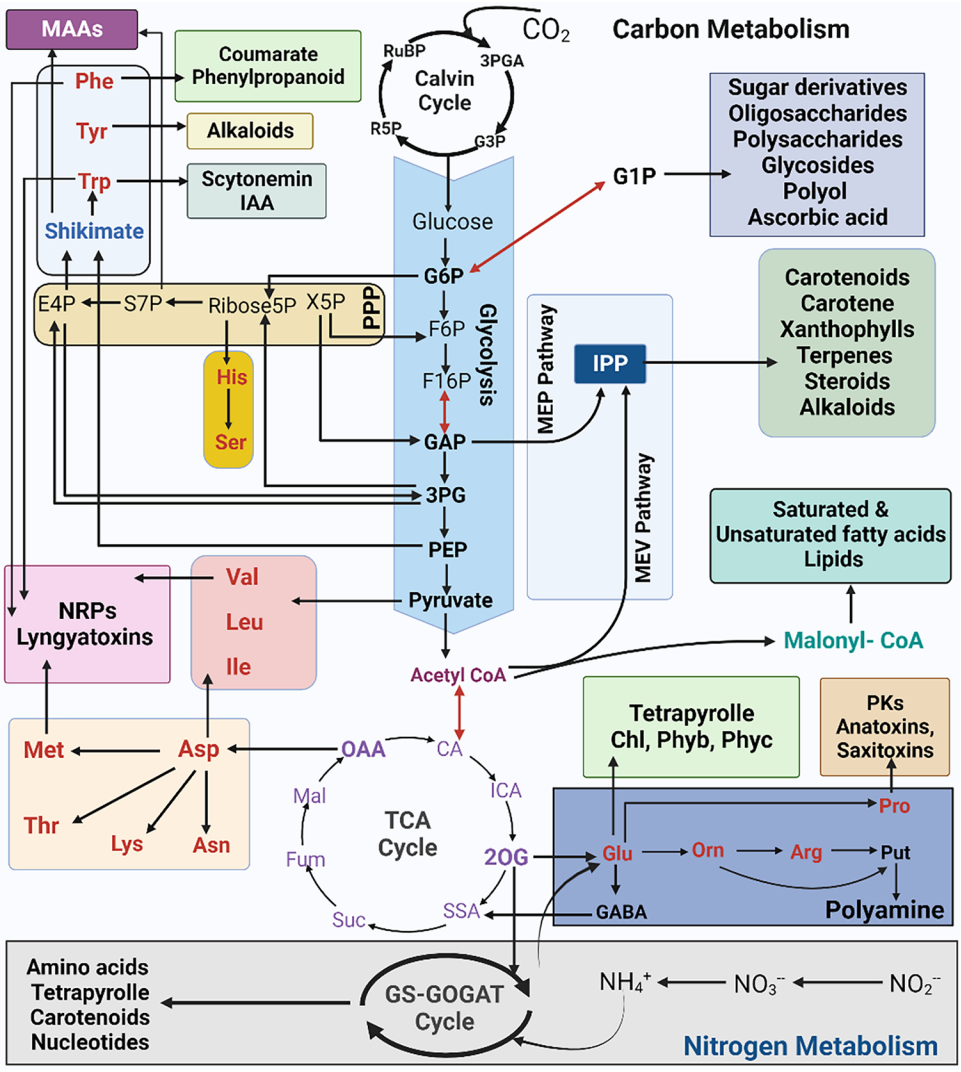
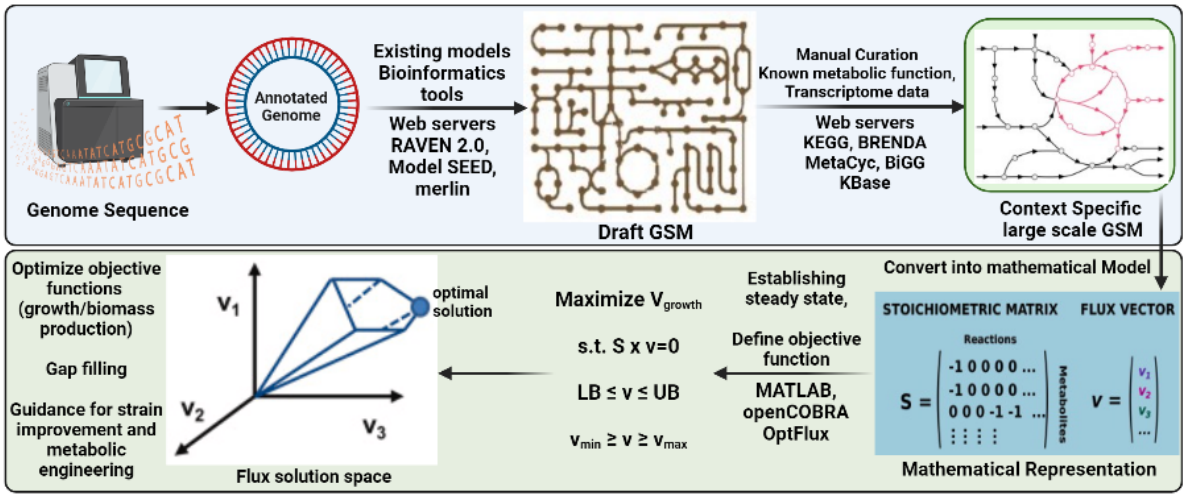
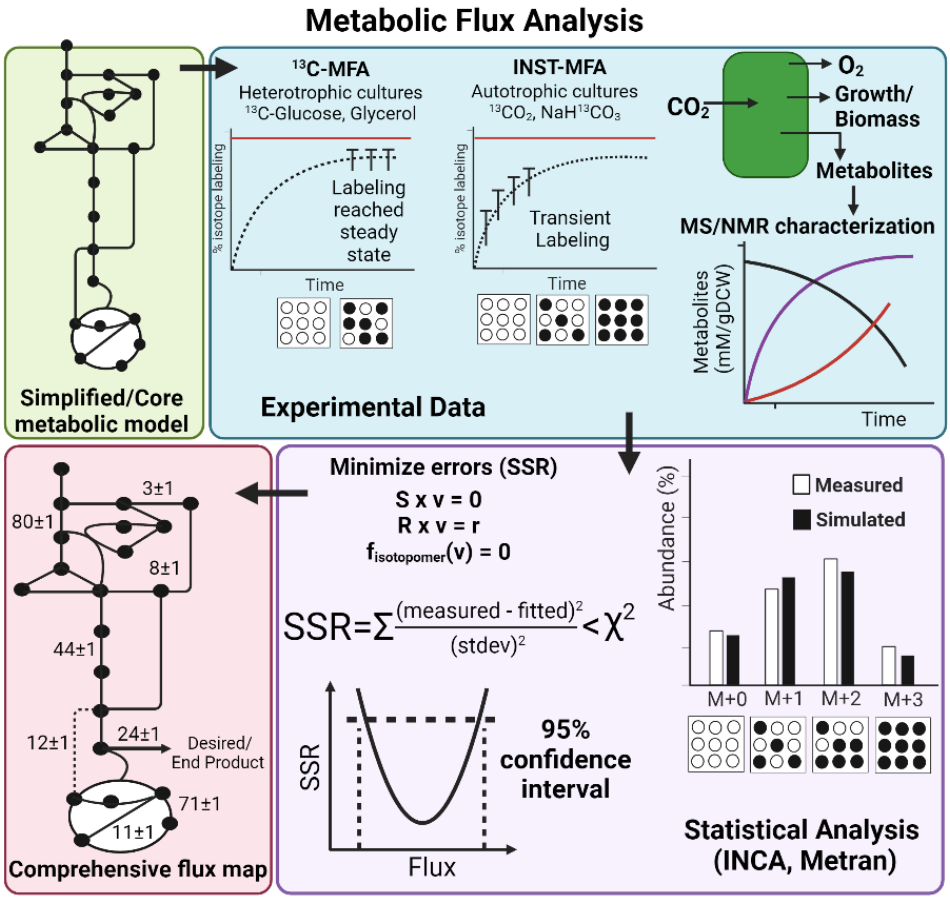


Figure 1. Primary and secondary metabolic pathways in cyanobacteria. Cyanobacteria fix carbon through the Calvin-Benson-Bassham (CBB) cycle and fuel the glycolysis. Together with glycolysis and the oxidative pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle is one of the three most important pathways of central carbohydrate metabolism which produces precursors and intermediates for a variety of secondary metabolites (alkaloids, polyamines, cyanotoxins, tetrapyrrole pigments etc.) through several unusual i.e., shikimate, acetate, MEP pathways. Cyanobacteria balance their carbon and nitrogen for normal growth, development, and reproduction.Different forms of inorganic nitrogen can be used by cyanobacteria and are assimilated in the form of ammonium (NH_4^+) through the glutamine synthetase-glutamine oxoglutarate aminotransferase/glutamate synthase (GS-GOGAT) cycle using 2-oxoglutarate (2-OG) as a carbon skeleton. Glutamate (Glu) and glutamine (Gln), the two amino acids produced from the GS-GOGAT cycle, are also important nitrogen donors for the synthesis of a variety of nitrogen-containing secondary metabolites. **Abbreviations**: 3PG: 3-phosphoglycerate, Arg: arginine, Asp: aspartate, Asn: asparagine, CA: citrate, Chl: chlorophyll, E4P: erythrose-4-phosphate, F6P: fructose-6-phosphate, F16P: fructose-1,6-bisphosphate, Fum: fumarate, GABA: gamma-aminobutyric acid; G1P: glucose-1-phosphate, G6P: glucose-6-phosphate, GAP: glyceraldehyde-3-phosphate, His: histidine, Ile: isoleucine, IPP: isopentenyl pyrophosphate; ICA: isocitrate, IAA: indole acetic acid, MAAs: mycosporine-like amino acids, Leu: leucine, Lys: lysine, Mal: malate, MEP: methylerythritol-phosphate, Met: methionine, , NRPs: nonribosomal peptides, OAA: oxaloacetate, Orn: ornithine, PEP: phosphoenolpyruvate, Phyb: phycobilin, Phyc: phycocyanin, Phe: phenylalanine, PKs: polyketides, , Pro: proline, Put: putrescine, R5P: ribulose-5-phosphate, RuBP: ribulose-1,5-bisphosphate, S7P: sedoheptulose-7-phosphate, Ser: serine, SSA: succinic semialdehyde, Suc: succinate, Thr: threonine, Trp: tryptophan, Tyr: tyrosine, Val: valine, X5P: xylulose-5-phosphate.



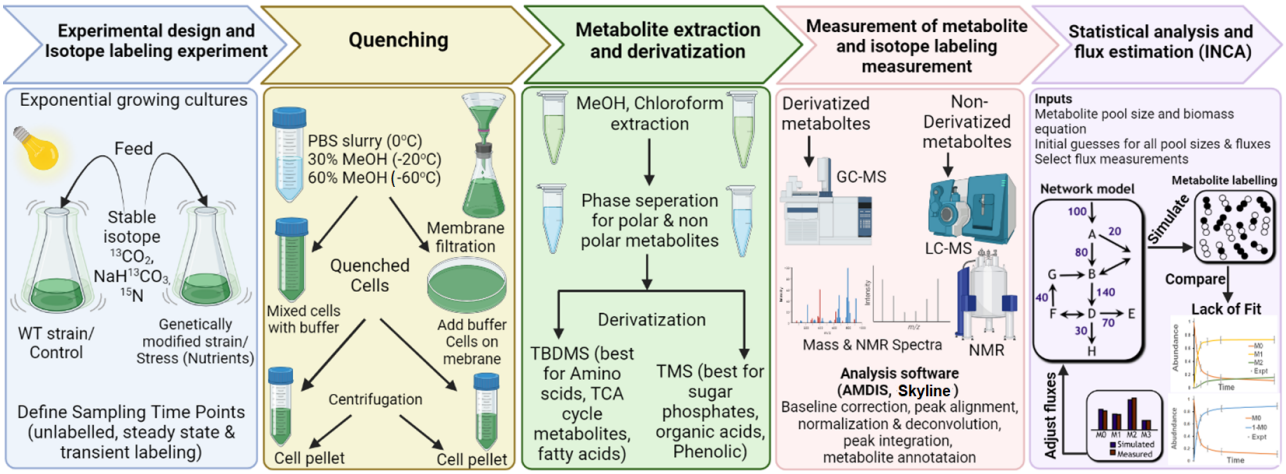
657

658 **Figure 2. Illustration of the species- and context-specific genome-scale metabolic model (GSM)**
659 **curation and reconstruction process**. The reconstruction approach combines a draft ortholog-GSM
660 derived from the existing template models using RAVEN, Model SEED, or Merlin packages. To
661 curate a species- and context-specific GSM, the metabolic networks are extracted from KEGG,
662 BRENDA, or BiGG databases and can be further refined with existing transcriptome data. A
663 schematic workflow of FBA applied to large-scale metabolic network model involves (i) converting
664 the GSM into mathematical equations, (ii) estimating metabolic fluxes based on a hypothesized
665 metabolic objective function, and (iii) interpreting the solution to identify metabolic pathways for
666 metabolic engineering.



667

668 **Figure 3. The overall framework of metabolic flux analysis (MFA) in cyanobacteria.** MFA of
669 heterotrophic and/or autotrophic metabolism in cyanobacteria is classified as ^{13}C-MFA (steady state)
670 or INST-MFA (nonstationary/transient state) isotope-labeling approaches. Flux estimation is based
671 on fitting experimental data, consisting of external rates and isotope labeling patterns, to a core or
672 simplified metabolic network model. Statistical analysis is performed by automated software tools
673 (e.g., INCA, Metran), which perform MFA calculations by minimizing the sum-of-squared residuals
674 (SSR) between simulated and experimental measurements and automatically provide several
675 statistical metrics that can be used to assess goodness-of-fit and quantify accurate 95% confidence
676 intervals for the estimated fluxes.



677

678 **Figure 4. Comprehensive step-by-step protocols for conducting high-resolution ^{13}C-MFA and**
679 **INST-MFA studies.** Exponentially growing wild-type (WT) and/or genetically modified (mutant)
680 cells are fed stable isotopes (^{13}C and/or ^{15}N tracers) in a culture flask or bioreactor. Cell cultures are
681 sampled at multiple time points and immediately mixed with a pre-chilled quenching solution, such
682 as ice-cold phosphate buffered saline (PBS) or −20°C methanol (MeOH), and instantly placed in an
683 ice bath. Subsequently, cells are harvested by centrifugation, flash frozen in liquid nitrogen, and
684 stored at −80°C until metabolite extraction. Metabolites are extracted with a suitable solvent mixture
685 (i.e., chloroform/methanol) and phase separated for polar and nonpolar primary and secondary
686 metabolites. Extracted metabolites are thoroughly dried or vacuum evaporated to remove the
687 extraction solvent. For gas chromatography-mass spectrometry (GC-MS) analysis, dried metabolites
688 are converted to either tert-butyldimethylsilyl (TBDMS) or trimethylsilyl (TMS) derivatives, while
689 for liquid chromatography-mass spectrometry (LC-MS) or nuclear magnetic resonance (NMR),
690 metabolites are dissolved in a compatible solvent prior to analysis, identification, and quantification.
691 Isotope-labeled metabolite measurements are incorporated into mathematical analysis tools (e.g.,
692 INCA) for data modeling and flux estimation.

693