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Metabolomics-based engineering for biofuel and bio-based chemical production in microalgae and cyanobacteria: A review

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Abstract

Metabolomics, an essential tool in modern synthetic biology based on the design-build-test-learn platform, is useful for obtaining a detailed understanding of cellular metabolic mechanisms through comprehensive analyses of the metabolite pool size and its dynamic changes. Metabolomics is critical to the design of a rational metabolic engineering strategy by determining the rate-limiting reaction and assimilated carbon distribution in a biosynthetic pathway of interest. Microalgae and cyanobacteria are promising photosynthetic producers of biofuels and bio-based chemicals, with high potential for developing a bioeconomic society through bio-based carbon neutral manufacturing. Metabolomics technologies optimized for photosynthetic organisms have been developed and utilized in various microalgal and cyanobacterial species. This review provides a concise overview of recent achievements in photosynthetic metabolomics, emphasizing the importance of microalgal and cyanobacterial cell factories that satisfy industrial requirements.

Keywords: photosynthetic metabolomics, microalgae, cyanobacteria, biofuel, bio-based chemicals

1. Introduction

One of the major challenges in developing a sustainable global economy is transitioning from conventional petroleum-based chemical manufacturing to bio-based carbon neutral alternatives. Productivity and cost performance are the main considerations for meeting the industrial requirements of bio-based commodity chemicals, in addition to ensuring environmental sustainability and feedstock supplementation (Keasling, 2012; Nielsen et al., 2014). Recent innovations in biotechnology based on a design-build-test-learn (DBTL) biological engineering cycle (Carbonell et al., 2018; Vavricka et al., 2020) have motivated research on the development of various chemicals via plant biomass fermentation, leading to the establishment of global biofoundries (Hillson et al., 2019). Numerous studies of biofuels and bio-based chemicals using representative fermentative microorganisms, such as yeasts and *Escherichia coli*, have been reported (Hasunuma et al., 2013b; Keasling, 2016; Keasling et al., 2021; Nielsen and Liu et al., 2016). Utilization of plant biomass has been promoted as an alternative feedstock to fossil resources, reflecting social infiltration of the bioeconomy concept that produces biofuels and bio-based chemicals from atmospheric carbon dioxide (CO_2); thus, carbon recycling is developing as a technology for reducing CO_2 emissions. However, cropland competition with food production and the limited availability of freshwater are important limitations to effectively utilizing plant biomass. Environmental problems, such as ecosystem destruction due to excessive deforestation, are important concerns related to the use of plant biomass as feedstock, even for non-edible plants. Therefore, in addition to plant biomass fermentation, bioproduction processes using photosynthetic microorganisms that produce valuable compounds directly from atmospheric CO_2 must be developed to meet industrial demands while minimizing environmental impacts to

62 establish a bioeconomic society (Leong et al., 2021).

63 Eukaryotic microalgae and prokaryotic cyanobacteria drive the Calvin-Benson-
64 Bassham (CBB) cycle by utilizing sunlight as an energy source to assimilate CO_2 (**Fig.**
65 **1**). Because of their more effective utilization of solar energy compared to terrestrial
66 plants in generating biomass photosynthetically, microalgae and cyanobacteria are
67 promising and versatile producers of biofuels (biodiesel, lipids, and ethanol), commodity
68 chemicals, and value-added intrinsic compounds (e.g., phycocyanin, carotenoids, and
69 polyunsaturated fatty acids) (Ho et al., 2014b; Knoot et al., 2018; Oliver et al., 2016).
70 Biofuel and bio-based chemical production through molecular breeding technologies and
71 development of mass cultivation methods has been attempted using various microalgae,
72 including *Chlamydomonas* (Terashima et al., 2015) and *Chlorella* (Cao et al., 2017), and
73 using cyanobacteria, including *Synechocystis* (Hidese et al., 2020) and *Synechococcus*
74 (Hasunuma et al., 2019). However, the productivity of biofuels and commodity chemicals
75 produced by microalgae and cyanobacteria are currently insufficient for cost-effective
76 commercialization in most cases, and fail to meet social and industrial demands.

77 Multi-omics approaches, which combine omics analyses, such as genomics,
78 transcriptomics, proteomics, lipidomics, and metabolomics, have been used to reveal the
79 basic molecular mechanisms of cellular physiology, contributing to the identification of
80 novel pathways and/or rate-limiting reactions in the biosynthetic pathway of target
81 products (i.e., bottleneck steps) (Kim et al., 2012; Vavricka et al., 2020). Among these,
82 metabolomics can comprehensively detect and quantify metabolites and therefore is
83 highly useful for understanding comprehensive metabolic phenomena and their dynamic
84 changes. Metabolomics can also determine the bottleneck step, which critically
85 contributes to the design of rational genetic engineering strategies and determination of

optimal culture conditions (Hasunuma et al., 2013a; Hasunuma et al., 2014; Ho et al., 2015). Focusing on the photosynthetic production of biofuels and bio-chemicals by using microalgae and cyanobacteria, this review highlights recent advances in metabolomics technologies and the applications of these methods for identifying key metabolic processes, dynamics, and bottleneck steps that can be engineered to improve target compound production.

2. Recent technologies applicable to microalgal and cyanobacterial metabolomics

2.1. Metabolomics techniques

Metabolomics involves static and dynamic metabolic analyses using a targeted or non-targeted approach. Dynamic metabolic analysis determines the rate of intracellular metabolism. Non-targeted metabolomics involves global profiling of both known and unknown metabolites, whereas targeted metabolomics can precisely quantify a few metabolites of interest (Fukusaki, 2014; Schwarz et al., 2013). Metabolomics employs either mass spectrometry (MS)-based techniques for comprehensively identifying and absolutely quantifying targeted metabolites with high sensitivity and selectivity or nuclear magnetic resonance (NMR)-based techniques for analyzing isotope-labeled metabolites (Babele et al., 2020; Dieterle et al., 2011) (**Table 1**). Chromatography-coupled MS is widely used for the global profiling of microbial metabolomes (Hollywood et al., 2018). MS-based metabolomics in combination with multiple reaction monitoring, a common feature of triple-quadrupole instruments, enables quantitative analysis of targeted metabolites with high precision (Zhou and Yin, 2016). Common mass analyzers for metabolomics include time-of-flight (TOF) systems, quadrupole systems, and ion-trap systems that are compatible with suitable ionization methods, such as matrix-assisted

laser desorption/ionization and electrospray ionization (ESI). Liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), and ion-mobility spectrometry are additional techniques that can be paired with MS systems to increase coverage and resolution by separating metabolites depending on their physical and/or chemical properties (Dettmer et al., 2007).

2.2. Microalgal and cyanobacterial metabolomics

In photosynthetic organisms, such as microalgae and cyanobacteria, the lighting condition is the critical environmental factor that immediately alters the intracellular metabolic status (Maruyama et al., 2017; Kato et al., 2021). For accurate metabolic profiling, it is necessary to strictly control photosynthesis-related environmental factors, such as the light intensity and CO_2 concentration. In addition, to obtain reliable metabolomic snapshots of biological systems, a suitable quenching method is needed to immediately stop any cellular metabolic activities at a specific sampling time (Chakdar et al, 2021; Ho et al., 2015). The conventional method of extracting total lipids from tissue cells using a super-cooled mixture of chloroform, methanol, and water (Folch et al, 1987) has more recently been applied for metabolite extraction of microalgae, such as *Chlamydomonas* sp. (Ho et al., 2015; Kato et al., 2021) and *Chlorella sorokiniana* (Chen et al., 2019; Chen et al., 2021). In addition, the cold methanol quenching method has been used for metabolite extraction from microalgae, such as *Haematococcus pluvialis* (Lv et al., 2016) and *Chlorella* sp. (Vello et al., 2018), and from cyanobacteria (Hasunuma et al, 2013a; Hasunuma et al., 2016; Young et al., 2011). A methanol-chloroform-NH_4OH system was developed to improve the extraction of polar compounds from cyanobacteria, such as sugar phosphates and bisphosphates

(Prasannan et al., 2018). Other methods, including pressurized liquid extraction and supercritical fluid extraction, are suitable for extracting carotenoids and have been applied to *H. pluvialis* (Jaime et al., 2010) and *Scenedesmus almeriensis* (Mehariya et al., 2019).

Takenaka et al. (2021) reported an LC-MS/MS-based metabolomics system for the identification and absolute quantification of approximately 150 primary metabolites, including amino acids, sugars, organic acids, nucleotides, and vitamins. A similar number of ionic metabolites can be identified and quantified using CE-coupled TOF-MS systems in both cation and anion modes (Hasunuma et al., 2016). Yuan et al. (2018) identified over 300 metabolites in another MS metabolomics study. Microalgal metabolic profiling often focuses on lipids and fatty acids (known as lipidomic profiling) using a suitable MS metabolomics system. For example, lipids with very long-chain fatty acids in *Botryococcus braunii* held together by a lipid biofilm matrix were identified using non-aqueous reversed-phase LC-MS/MS (Řezanka et al., 2018), a complex mixture of natural triacylglycerol (TAG) of the snow alga *Chloromonas pichinchae* (Řezanka et al., 2014) was identified using ESI-MS and silver LC/atmospheric pressure chemical ionization-MS, and fatty acids in the microalgal strain *Micractinium* sp. IC-76 were identified using a GC-MS-based approach (Piligaev et al., 2018).

The NMR-based metabolomics technique involves non-destructive and non-biased detection of a large number of metabolites. In the microalga *Scenedesmus* sp., NMR metabolomics identified ~45 metabolites through analysis of cumulative one-dimensional ^{1}H NMR spectra, including amino acids, sugars, organic acids, phosphagens, osmolytes, and nucleotides (Arora et al., 2018).

2.3. Flux analysis with stable isotopes

158 Static metabolomics can provide a comprehensive snapshot of metabolites at a
159 single time point. However, time-course analysis is required to investigate the dynamic
160 aspects of the metabolites. Stable isotope tracer experiments are useful for dynamic
161 metabolic analysis in both MS- and NMR-based detection (Bacher et al., 2016). MS-
162 based tracer detection enables the precise quantification of metabolic flux in combination
163 with quantitative time-course metabolomics, referred to as dynamic metabolomics, in
164 photosynthetic autotrophs, such as plants, microalgae, and cyanobacteria (Hasunuma et
165 al., 2010; Hasunuma et al., 2013a). Feeding of $NaH^{13}CO_3$ enables the labeling of newly
166 synthesized metabolites from CO_2 with ^{13}C, which contributed to the identification of a
167 bottleneck step in all glycolysis and tricarboxylic acid (TCA) cycle metabolites as well
168 as the component amino acids in the microalga *Chlamydomonas* sp. (Ho et al., 2017; Kato
169 et al., 2019), and in the cyanobacteria *A. platensis* (Hasunuma et al., 2013a) and
170 *Synechocystis* sp. (Hasunuma et al., 2016; Hidese et al., 2020). Other stable isotopes,
171 including ^{15}N and ^{18}O, can also be used to trace metabolic flux (Kera et al., 2018; Zhang
172 et al., 2018). In *Synechocystis* sp., ^{13}C and ^{15}N labeling experiments identified arginine
173 dihydrolase as a key regulator of nitrogen metabolism (Zhang et al., 2018). By combining
174 MS-based metabolic profiling with genomic analysis, stable isotope labeling, and non-
175 target metabolomics revealed a novel biosynthetic pathway responsible for producing
176 secondary metabolites (May et al., 2020).
177 Metabolic flux analysis (MFA) is a mathematical modeling approach that can
178 determine the metabolic flux distribution based on integrated analysis of extracellular
179 flux and intracellular isotope labeling in metabolic and isotopic steady states. In
180 autotrophic metabolism, ^{13}C labeling in an isotopic steady state shows a uniform pattern
181 in all downstream metabolites; hence, it does not reflect carbon atom rearrangements in

the metabolic pathway (Shastri and Morgan, 2007). In addition, diurnal light-dark cycle renders short-lived metabolic steady states in photosynthesis. Isotopically nonstationary ^{13}C MFA (INST-MFA) is another modeling approach that estimates autotrophic fluxes in a metabolic steady state by measuring transient isotope incorporation prior to reaching an isotopic steady state following ^{13}C-labeling (Adebiyi et al., 2015; Cheah and Young, 2018; Jazmin et al. 2014; Young et al. 2011). Development of the carbon mapping model imSyn617 and associated algorithmic updates effectively reduced the computation time (Gopalakrishnan et al., 2018).

3. Metabolomics approaches for biofuel production in microalgae

3.1. General applications of metabolomics in microalgae

Biofuels have attracted global attention because of increased social concerns regarding global warming and fossil fuel depletion. Several eukaryotic microalgae, such as *Chlamydomonas* (Ho et al., 2017), *Chlorella* (Feng et al., 2011), and *Dunaliella* (Yuan et al., 2019), show potential for generating biofuel because they can photosynthetically produce lipids, such as TAGs, which serve as biofuel feedstocks. Unfortunately, the availability of nuclear transformation by homologous recombination is limited in major microalgae, including *Chlamydomonas reinhardtii* (Nelson and Lefebvre, 1995; Sodeinde and Kindle, 1993); therefore, valuable strains for biofuel production have mainly been obtained using random mutagenesis-based approaches. Recent innovative technologies of fluorescence-activated and image-activated cell sorting further enabled the screening of desired cells from a vast number of mutated cells in a high-throughput and high-accuracy manner (Nitta et al., 2020).

Metabolomics has been utilized in microalgae biofuel production for two

primary purposes: (1) to evaluate metabolic changes under lipid-producing conditions, such as nutrient depletion, and (2) to evaluate the metabolic mechanism that alters lipid accumulation in mutants. Recently, metabolic analyses were performed in various microalgae, including *Coccomyxa melkonianii* (Fais et al., 2021), *Isochrysis galbana* (Aguilera-Sáez et al., 2019), and *Micractinium* sp. (Piligaev et al., 2018). This analysis is also useful for guiding the production of value-added long-chain polyunsaturated fatty acids for dietary supplements. For example, metabolomics revealed the metabolic phenomena underlying production of docosahexaenoic acid (DHA) in *Crypthecodinium cohnii* (Liu et al., 2020), DHA and odd-chain fatty acids in *Schizochytrium* sp. (Wang et al., 2019), and arachidonic acid in *Lobosphaera incisa* (Kokabi et al., 2019).

3.2. Elucidation of metabolic changes in microalgae under nutrient depletion

Nutrient depletion, particularly nitrogen depletion, generally enhances lipid production in microalgae. Recent metabolomic findings related to microalgal lipid production under nitrogen deletion are summarized in **Table 2**. In *C. reinhardtii*, highly distinctive metabolite profiles were obtained under nitrogen-, phosphorus-, sulfur-, or iron-depleted conditions (Bölling and Fiehn, 2005). Further investigation of the metabolic response to nitrogen starvation suggested a link between cell growth and nitrogen metabolism via branched-chain amino acids (BCAAs) (Valledor et al., 2014). Comparative metabolic profiling of *Chlorella* species suggested that proteins and BCAAs are degraded and converted into intermediate molecules, such as proline, alanine, arginine, and succinate, and the non-proteinogenic amino acid gamma-aminobutyric acid (GABA), leading to C/N disequilibrium; the excess carbon is diverted into lipid synthesis via the GABA pathway, glycolysis, and TCA cycle (Chen et al., 2017). Similarly, nitrogen

starvation has been suggested to trigger nitrogen assimilation from proteins and the photosynthetic machinery in other *Chlorella* strains (Vello et al., 2018), and an increase in GABA under nitrogen depletion was observed in *Scenedesmus quadricauda* (Sulochana and Arumugam, 2020). In *Parachlorella kessleri*, metabolites of the TCA cycle, such as citrate and 2-oxoglutarate (2OG), were suggested to play important roles in diverting the flux toward acetyl-CoA, leading to the accumulation of neutral lipids under nutrient-depleted conditions (Shaikh et al., 2019). An increase in citrate under low nitrogen conditions was reported in *Nannochloropsis oceanica* (Xiao et al., 2013). In *N. salina*, nitrogen availability was shown to affect glycerol consumption during mixotrophic cultivation and utilization for lipid synthesis (Poddar et al., 2020). These results led to a hypothesis common in many microalgae species: the degradation products of BCAAs can be converted to acetyl-CoA, a precursor for fatty acid synthesis, via the TCA cycle and contribute to lipid synthesis under nitrogen-depleted conditions (Allen et al., 2011; Ge et al., 2014). A *C. reinhardtii* mutant defective in BCAA catabolism accumulated less TAG than the parental strain during nitrogen starvation (Liang et al., 2019). In *Dunaliella tertiolecta*, a TAG-rich mutant showed upregulation of genes involved in BCAA degradation and an increased pool size of acetyl-CoA (Yao et al., 2017). In addition, an imbalance in the C/N ratio due to lipid synthesis without corresponding nitrogen intake can be recovered by upregulating the protein degradation process (Poddar et al., 2020). In the diatom *Phaeodactylum tricornutum*, in which betaine lipids are thought to be the major source of TAG synthesis, the levels of amino acids, including valine, leucine, and isoleucine, and TCA cycle intermediates, including citrate, were significantly decreased during nitrogen starvation (Popko et al., 2016), whereas increased TAG accumulation by overexpression of the BCAA catabolic pathway enzyme 3-

hydroxyisobutyryl-CoA hydrolase was also reported (Pan et al., 2017). Thus, recent metabolomics studies suggest that in addition to carbon, nitrogen metabolism is important for lipid synthesis in many microalgae species.

3.3. Elucidation of metabolic mechanisms in mutant microalgae

Metabolomics is also useful for examining metabolic mechanisms in engineered microalgal strains. Particularly, the lipid accumulation mechanism in strains engineered to have an altered starch-related pathway has been extensively investigated (**Table 3**). A *C. reinhardtii sta6* mutant lacking the ADP-glucose pyrophosphorylase gene is unable to convert glucose-1-phosphate (G1P) to ADP-glucose, resulting in a starchless strain with high lipid accumulation (Krishnan et al., 2015). Metabolomics revealed that under high light conditions, the *sta6* mutant accumulated precursors for the biosynthesis of lipids, amino acids, and starch. Similarly, a starchless mutant of *C. sorokiniana* SLM2 showed enhanced lipid accumulation under high light and nutrient limitation conditions (Wu et al., 2019). In this mutant, the oxidative pentose phosphate pathway was enhanced and glycolysis pathway (providing precursors for both fatty acids and glycerol backbone synthesis) was augmented. In addition, the GABA shunt, which is a bypass of the TCA cycle, was induced in the mutant SLM2 strain, suggesting its contribution to enhancing lipid accumulation. A 16D mutant strain of *C. cohnii* was developed to have decreased starch and extracellular polysaccharide contents, and increased lipid and DHA contents (Lv et at., 2020). Metabolomics of 16D suggested that the central carbon metabolic pathways, such as the Embden-Meyerhof pathway, Krebs cycle, and pentose phosphate pathway, were strengthened, and the starch and extracellular polysaccharide biosynthetic pathways were attenuated. In contrast to these mutants, a *bgal1* mutant in *C. reinhardtii*

278 overaccumulates starch accompanied by decreased TAG accumulation (Hung et al., 2016).
279 Metabolomics revealed that under nitrogen starvation, the levels of glucose 6-phosphate
280 (G6P), fructose 6-phosphate (F6P), and acetyl-CoA in the *bgal1* mutant were lower than
281 those in the wild-type strain, suggesting a defect in the conversion of starch into lipids in
282 the mutant.

283 The green microalga *Chlamydomonas* sp. JSC4 is another promising strain for
284 biofuel production (Ho et al., 2014a; Ho et al., 2017; Kato et al., 2017). However, high
285 accumulation of starch, rather than of lipids, under light/dark cycling inevitable in outdoor
286 cultivation, remains a challenge limiting its production capacity (Kato et al., 2019). To
287 improve lipid production under light/dark cycling, JSC4 was randomly mutagenized,
288 resulting in the selection of a lipid-rich mutant KOR1 (Kato et al., 2021). KOR1 harbors
289 mutations in the starch debranching enzyme (DBE) gene, which causes structural changes
290 in intracellular carbohydrates from water-insoluble starch into highly branched and water-
291 soluble phytoglycogens (Dauvillée et al. 2001). Metabolomics revealed significantly
292 increased levels of F6P, G6P, phosphoenolpyruvate (PEP), pyruvate, acetyl-CoA, and
293 glycerol 3-phosphate (G3P) derived from phytoglycogen degradation in KOR1 cells.
294 Thus, metabolomic analysis revealed the lipid accumulation mechanism in DBE-deficient
295 microalgae in which a structural change of carbohydrates into degradable phytoglycogen
296 ultimately enhanced carbon repartitioning into lipid synthesis (Kato et al., 2021). Thus,
297 metabolomics provided insight into a distinctive phenomenon in microalgae lipid
298 production, such as the contribution of nitrogen metabolism and enhancement of carbon
299 repartitioning into lipid synthesis in starch-related mutants.
300

301 **4. Photosynthetic metabolomics in cyanobacteria**

4.1. Advantages of cyanobacteria for chemical production

Compared with terrestrial plants, cyanobacteria have important advantages, such as faster growth under simple culture conditions, high solar energy capture efficiency, and utilization of a bicarbonate intermediate as a carbon source for conversion to desired products (Carroll et al., 2018; Srivastava and Shukla, 2021). Unicellular cyanobacteria, including *Synechocystis* sp. PCC 6803, *S. elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002, are genetically tractable and are easy to genetically manipulate because of recent advances in synthetic biology tools (Behle et al., 2020; Huang et al., 2013; Markley et al., 2016; Sengupta et al. 2019; Sengupta et al., 2020; Yao et al., 2016). Accordingly, cyanobacteria are attractive platforms for the successful production of various chemicals, including alcohols (e.g., ethanol, 1-butanol, isopropanol, 2-methyl-1-butanol, 1,2-propanediol, 1,3-propanediol, and isobutanol), organic acids (e.g., 3-hydroxypropionate, 3-hydroxybutyrate, D-lactate, succinate, malate, and fumarate), free fatty acids, isoprene, ethylene, and carotenoids. In cyanobacteria, the main goals of metabolomics-guided strain engineering approaches are (1) to redirect carbon flow from CO_2 toward production of the desired chemical, and (2) to improve the carbon fixation rate. Examples of metabolome-based strain development in cyanobacteria are summarized in **Table 4**.

4.2. Rational metabolic engineering through identifying the bottleneck reaction

4.2.1. Alcohol and aldehyde production

Photosynthesis-based bioproduction of alcohol as an alternative to petrochemical production has recently attracted attention. Pioneering research demonstrated that ethanol could be produced (450 nmol/L in 7 days) by a recombinant *S. elongatus* PCC 7942 strain that converts pyruvate to ethanol via heterologous pyruvate

decarboxylase and alcohol dehydrogenase enzymes (Deng and Coleman, 1999). More recently, Kopka et al. (2017) applied a metabolomics approach and found that intracellular carbon allocation caused the accumulation of soluble sugars and glycogen during long-term ethanol production in another ethanol-producing recombinant strain of *Synechococcus* sp. PCC 7002, suggesting that lowering the ratio of ATP and NADPH cofactors is advantageous for ethanol production.

The iterative cycle of widely targeted metabolomics was shown to be successful for increasing 1-butanol production (Fathima et al., 2018). For example, 1-butanol was produced from *S. elongatus* PCC 7942 via seven enzymatic reaction steps from acetyl-CoA by heterologous expression of the corresponding enzymes, demonstrating that the balance of metabolic flux is important for improving the target chemical titer (Noguchi et al., 2016). First, the reduction step converting butanoyl-CoA to butanal was identified as the bottleneck step. Improvement of heterologous CoA-acylating propionaldehyde dehydrogenase, which is responsible for this bottleneck step, increased the target compound and free CoA regeneration, in turn leading to increased acetyl-CoA synthesis. Second, the newly discovered bottleneck step was improved by overexpressing heterologous acetyl-CoA carboxylase, resulting in increased levels of 1-butanol.

To systematically remove bottlenecks in cyanobacterial metabolic pathways, INST-MFA was applied for photosynthetic production of isobutyraldehyde (IBA), an alternative biofuel, in recombinant strains of *S. elongatus* PCC 7942, in which pyruvate was converted to IBA via four-step reactions by heterologous acetolactate synthase, dihydroxy-acid dehydratase, acetohydroxy acid isomeroreductase, and α-ketoisovalerate decarboxylase (Atsumi et al., 2009). Metabolomic analysis identified the pyruvate kinase (PK) reaction step and bypass pathway involving the enzymes PEP carboxylase (PEPC),

malate dehydrogenase (MDH), and malic enzyme (ME) as potential bottlenecks. Single overexpression of the PK gene and overexpression of both MDH and ME led to significant improvements in the specific productivity of IBA by increasing pyruvate availability (Jazmin et al. 2017). Furthermore, downregulation of pyruvate dehydrogenase and overexpression of PEP carboxykinase improved IBA productivity by redirecting the metabolic flux toward pyruvate synthesis (Cheah et al., 2020). These findings provide further guidance for rational metabolic engineering to produce other pyruvate-based chemicals, such as lactate, 2,3-butanediol, and ethanol.

4.2.2. Organic acid production

Cyanobacteria have also been studied as promising producers of succinate and D-lactate to develop environmentally friendly, biodegradable plastics (Katayama et al., 2018). *Synechocystis* sp. PCC 6803 converts intracellular glycogen into organic acids, including succinate, D-lactate, fumarate, and malate, by autofermentation under dark anoxic conditions (Stal and Moezalaar, 1997). Strain PCC 6803 possesses a D-lactate dehydrogenase (D-LDH) gene and can secrete a large amount of D-lactate through autofermentation (Ito et al., 2017). However, increasing D-lactate production requires supplementation with the D-LDH substrate pyruvate. Overexpression of ME, a malate decarboxylase that converts malate to pyruvate, increased D-lactate production in PCC 6803 by more than 2-fold (Hidese et al., 2020). Dynamic metabolic profiling revealed that overexpression of ME enhanced the metabolic turnover of malate and metabolites involved in glycolysis. In contrast, an ME-deficient strain accumulated malate and glycolysis metabolites, such as G6P, F6P, fructose-1,6-bisphosphate, 3-phosphoglycerate (3PGA), and PEP. In an ME-overexpressing strain, metabolic turnover in glycolysis was

assumed to be improved because of the decreased allosteric inhibitory effect of malate on the activity of PK, which catalyzes the conversion of PEP into pyruvate. Production of D-lactate in the ME-overexpressing strain was further improved by overexpressing the D-LDH gene and knocking out the acetate kinase gene *ackA*, which is responsible for generating acetate from acetyl phosphate (Osanai et al., 2015).

Succinate could be synthesized in PCC 6803 via the TCA cycle under dark anoxic conditions; however, it was unclear whether this was achieved via an oxidative or reductive route. Dynamic metabolic profiling of PCC 6803 revealed that succinate is synthesized via glycolysis, the anaplerotic pathway, and the reductive route of the TCA cycle (Hasunuma et al., 2016). In addition, the condensation reaction of PEP and bicarbonate into oxaloacetate, which is catalyzed by PEPC, was identified as the bottleneck step in succinate production. Accordingly, overexpression of PEPC and supplementation with bicarbonate improved succinate production in PCC 6803. Succinate production and glycogen consumption were further enhanced by elevating the autofermentation temperature from 30 °C to 37 °C (Hasunuma et al., 2018). The levels of intracellular metabolites involved in glycolysis (G6P, F6P, G3P) and the TCA cycle (succinate, fumarate, malate) also increased with increasing temperature, along with increased metabolic turnover of glycolysis, the reductive TCA cycle, and PEPC enzyme activity. Thus, photosynthetic metabolomics revealed the metabolic mechanism underlying increased succinate production, and the bottleneck step catalyzed by PEPC could be simply enhanced by elevating the autofermentation temperature.

Succinate production was also enhanced in a mutant lacking *ackA* and overexpressing *sigE* via increased glycogen catabolism and organic acid biosynthesis, with the production level reaching 5-fold that in the wild-type. Based on these findings,

the reductive route of the TCA cycle was intensified by overexpressing the malate dehydrogenase gene *citH* (Iijima et al., 2021). An extremely high succinate titer of 4.2 g/L was achieved in PCC 6803 by autofermentation under dark anoxic conditions with product stripping by medium exchange.

4.2.3. Carotenoid production

Carbon partitioning accompanying CO_2 incorporation is among the most important factors for increasing target chemical production. Cyanobacteria utilize the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway to synthesize carotenoids. Dimethyl dimethylallyl diphosphate (DMAPP), the final product of the MEP pathway, serves as a precursor for isoprene synthesis. Metabolomics of the isoprene-producing recombinant strain *Synechocystis* sp. PCC 6803, which harbors plant-derived isoprene synthase, revealed that the limitation in isoprene production is an insufficient DMAPP level (Pade et al., 2016). Therefore, the mevalonic acid (MVA) pathway, another pathway for DMAPP synthesis, was introduced into the isoprene-producing cells as a bypass of the MEP pathway, resulting in increased isoprene levels. Isoprene production was also improved by overexpression of the enzymes Ipi, Dxs, and IspD, whose catalytic reactions were identified as bottleneck steps within the MEP pathway (Englund et al., 2018).

The carotenoid content of cyanobacteria is significantly lower than that of eukaryotic microalgae and vascular plants (Pagels et al., 2021). Many approaches have been reported for producing astaxanthin, a valuable carotenoid with superior antioxidative properties. In the microalga *H. pluvialis*, astaxanthin accumulates under nutrient-deficient and high-light conditions, leading to large-scale market application (Ahirwar et al., 2021); however, long-term cultivation is required, and the sensitivity of

422 microbial contamination under growth-unsuitable conditions remains as a concern in
423 astaxanthin production using *H. pluvialis*. A genetically engineered strain of
424 *Synechococcus* sp. PCC 7002, a fast-growing marine cyanobacterium, harboring the β-
425 carotene hydroxylase CrtZ and β-carotene ketolase CrtW derived from the marine
426 bacterium *Brevundimonas* sp. SD212 showed an astaxanthin content of 3 mg/g dry cell
427 weight and astaxanthin productivity of 3.35 mg/L/day (Hasunuma et al., 2019), which
428 were comparable to the values obtained using *H. pluvialis*. Metabolic profiling revealed
429 that the astaxanthin-producing cells highly accumulated 1-deoxy-D-xylulose 5-phosphate,
430 which is the gateway metabolite in the MEP pathway. Dynamic metabolic profiling
431 showed that the metabolic turnover of 3PGA, sedoheptulose 7-phosphate, F6P, and PEP
432 was accelerated in astaxanthin-producing cells. Thus, activation of photosynthetic central
433 metabolism in astaxanthin-producing cells appears to compensate for the low β-carotene
434 availability as a light-harvesting pigment, likely via its conversion into astaxanthin
435 (Hasunuma et al., 2019). Another study demonstrated enhanced photosynthesis and
436 central metabolism, likely because of high astaxanthin flux, in the engineered
437 *Synechocystis* sp. PCC 6803 strain (Diao et al., 2020). These findings may also be
438 beneficial for producing other DMAPP-based chemicals, including zeaxanthin, ethineone,
439 and limonene (Pagels et al., 2021; Lin et al., 2021).
440
441 *4.3. Re-routing assimilated CO_2 and improvement of the carbon fixation rate*
442 Glycogen is a major carbon storage molecule in cyanobacteria and promising
443 target for the redistribution of desired chemicals (Xu et al., 2013). INST-MFA of a
444 glycogen synthase-deficient mutant (*glgA-I glgA-II*) of *Synechococcus* sp. PCC 7002
445 revealed that carbon resources derived from CO_2 fixation were partially diverted into

446 alternate storage molecules, such as glucosylglycerol and sucrose, by flexible partitioning
447 of G1P between ADP-glucose and UDP-glucose without a major impact on the central
448 metabolic pathway (Hendry et al., 2017). Disruption of *glgC*, which encodes G1P
449 adenylyltransferase, causes impairment of glycogen synthesis, growth defects, and
450 metabolite overflow, leading to excretion of several organic acids, including pyruvate,
451 2OG, and succinate (Carrieri et al., 2012; Cano et al., 2018). The growth defects in *glgC*-
452 deficient mutants can be partially restored by isobutanol production (Li et al., 2014).
453 According to $NaH^{14}CO_3$ incorporation analysis, 52% of the total fixed carbon was
454 redirected into isobutanol biosynthesis in the *glgC*-deficient mutant, representing a rate
455 2.5-fold higher than that of the strain carrying *glgC*, indicating that isobutanol can be used
456 as an alternative carbon sink to glycogen.
457 Metabolic engineering of the CBB cycle has also been attempted to improve
458 the efficiency of carbon fixation. Fructose-1,6/sedoheptulose-1,7-bisphosphatase
459 (FBP/SBPase) and transketolase (TK) are key enzymes in the CBB cycle and represent a
460 potential bottleneck step in carbon fixation. Fluxomic analysis by INST-MFA in
461 combination with proteomics indicated a decrease in oxidative pentose phosphate
462 pathway activity when either FBP/SBPase or TK was overexpressed in PCC 6803,
463 resulting in increased carbon fixation efficiency, whereas TK overexpression enhanced
464 the susceptibility of the strain to oxidative stress (Yu King Hing et al., 2019). A more
465 recent study showed that overexpression of two CBB cycle enzymes among FBP/SBPase,
466 TK, and aldolase contributed to enhanced ethanol production when compared to
467 overexpression of a single CBB cycle enzyme (Roussou et al., 2021).
468 Metabolomic analyses of the glucose-tolerant strain *Synechocystis* sp. PCC
469 6803 showed that both the oxidative pentose phosphate pathway and glycolysis are

activated under mixotrophic conditions rather than under autotrophic conditions (Yoshikawa et al., 2013). In addition, nitrogen limitation or dark conditions induced major shifts in metabolites, such as sugar accumulation (Qian et al., 2018; Wan et al, 2017). These findings emphasize that condition-dependent changes in metabolites are useful for rerouting metabolism into desired products.

Improving photosynthesis is another major challenge for constructing cyanobacterial cell factories for large-scale applications (Hasunuma et al., 2014). Dynamic metabolomics clearly revealed that overexpression of *flv3*, which encodes an NAD(P)H:oxygen oxidoreductase involved in alternative electron flow associated with NADPH-coupled O_2 photoreduction in photosystem I, improved cell growth in PCC 6803 with glycogen accumulation by increasing O_2 evolution, intracellular ATP levels, and CBB cycle turnover.

5. Advantages of photosynthetic metabolomics in cell factory development

To develop microalgal and cyanobacterial cell factories, the current level of productivity of biofuels and bio-chemicals must be further improved through strain improvement and/or optimization of culture conditions (**Fig. 2**). Strain improvement has conventionally been conducted via trial-and-error experiments based on speculation from previous reports. This process is time-consuming and labor-intensive because a large number of genetically engineered or randomly mutagenized strains is constructed and evaluated without a landscape of the metabolic pathway. In contrast, in the metabolomics-based approach, a strain with desired properties can be rapidly and rationally designed based on metabolic information, including bottleneck steps in a biosynthetic pathway and wasteful byproduct accumulation (Teoh et al., 2015). As summarized in this review,

photosynthetic metabolomics can reveal the comprehensive metabolite pool size and its dynamic changes in microalgae and cyanobacteria, critically contributing to the understanding of the CO_2 fixation-derived carbon distribution and determination of a rate-limiting step in the biosynthetic pathway. These data are extremely useful for identifying a target reaction that should be intensified or weakened to improve the production of targeted compounds. Engineered strains constructed using this approach can be further subjected to metabolomics. Therefore, metabolomics is a powerful tool in synthetic biology based on the DBTL platform (Carbonell et al., 2018; Vavricka et al., 2020). Unfortunately, metabolomics-based synthetic biology has not been realized in microalgae biofuel production because of the limited availability of nuclear transformation by homologous recombination in the major microalgae (Nelson and Lefebvre, 1995; Sodeinde and Kindle, 1993). This limitation will be overcome in part by using recently developed genome editing tools in several microalgae (Ng et al., 2020). In addition, genetic engineering is applicable in microalgae, such as *Cyanidioschyzon merolae* (Pancha et al., 2021); therefore, these species are suited to metabolomics-based strain improvement for biofuel production.

6. Conclusions

The recent applications of metabolomics to photosynthetic production of biofuels and bio-based chemicals in microalgae and cyanobacteria were reviewed. In cyanobacteria, metabolomics has contributed to the rational design of metabolic pathways for carbon flow redirection from assimilated CO_2 toward desired products, such as alcohols, organic acids, and carotenoids. In contrast, metabolomics-based synthetic biology has not been achieved in microalgae biofuel production but will be realized by

using microalgae that can be transformed via homologous recombination. Improving the throughput and sensitivity of metabolic analysis will further accelerate the development of microalgal and cyanobacterial cell factories.

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References

1. Adebiyi, A.O., Jazmin, L.J., Young, J.D., 2015. ^{13}C flux analysis of cyanobacterial metabolism. Photosynth. Res. 126 (1), 19–32.
2. Aguilera-Sáez, L.M., Abreu, A.C., Camacho-Rodríguez, J., González-López, C.V., Del Carmen Cerón-García, M., Fernández I., 2019. NMR Metabolomics as an Effective Tool To Unravel the Effect of Light Intensity and Temperature on the Composition of the Marine Microalgae *Isochrysis galbana*. J. Agric. Food Chem. 67 (14), 3879–3889.

3. Ahirwar, A., Meignen, G., Jahir Khan, M., Sirotiya, V., Harish, Scarsini, M., Roux, S., Marchand, J., Schoefs, B., Vinayak, V., 2021. "Light modulates transcriptomic dynamics upregulating astaxanthin accumulation in *Haematococcus*: A review". Bioresour. Technol. 340, 125707.

4. Allen, A.E., Dupont, C.L., Oborník, M., Horák, A., Nunes-Nesi, A., McCrow, J.P., Zheng, H., Johnson, D.A., Hu, H., Fernie, A.R., Bowler, C., 2011. Evolution and metabolic significance of the urea cycle in photosynthetic diatoms. Nature 473 (7346), 203–207.

5. Arora, N., Dubey, D., Sharma, M., Patel, A., Guleria, A., Pruthi, P.A., Kumar, D., Pruthi, V., Poluri, K.M., 2018. NMR-Based Metabolomic Approach To Elucidate the Differential Cellular Responses during Mitigation of Arsenic(III, V) in a Green Microalga. ACS Omega 3 (9), 11847–11856.

6. Atsumi, S., Higashide, W., Liao, J.C., 2009. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat. Biotechnol., 27 (12), 1177–1180.

7. Bacher, A., Chen, F., Eisenreich, W. 2016. Decoding Biosynthetic Pathways in Plants by Pulse-Chase Strategies Using $^{13}CO_2$ as a Universal Tracer. Metabolites, 6 (3), 21.

8. Babele, P.K., Young, J.D., 2020. Applications of stable isotope-based metabolomics and fluxomics toward synthetic biology of cyanobacteria. Wiley Interdiscip. Rev. Syst. Biol. Med. 12 (3), e1472.

9. Behle, A., Saake, P., Germann, A.T., Dienst, D., Axmann, I.M., 2020. Comparative Dose-Response Analysis of Inducible Promoters in Cyanobacteria. ACS Synth. Biol. 9 (4), 843–855.

565 10. Bölling, C., Fiehn, O., 2005. Metabolite profiling of *Chlamydomonas reinhardtii*
566 under nutrient deprivation. Plant Physiol. 139 (4), 1995 –2005.
567 11. Cano, M., Holland, S.C., Artier, J., Burnap, R.L., Ghirardi, M., Morgan, J.A., Yu, J.,
568 2018. Glycogen Synthesis and Metabolite Overflow Contribute to Energy Balancing
569 in Cyanobacteria. Cell Rep. 23 (3), 667–672.
570 12. Cao, S., Zhou, X., Jin, W., Wang, F., Tu, R., Han, S., Chen, H., Chen, C., Xie, G.J.,
571 Ma, F., 2017. Improving of lipid productivity of the oleaginous microalgae *Chlorella*
572 *pyrenoidosa* via atmospheric and room temperature plasma (ARTP). Bioresour.
573 Technol. 244 (Pt 2), 1400–1406.
574 13. Carbonell, P., Jervis, A.J., Robinson, C.J., Yan, C., Dunstan, M., Swainston, N.,
575 Vinaixa, M., Hollywood, K.A., Currin, A., Rattray, N.J.W., Taylor, S., Spiess, R.,
576 Sung, R., Williams, A.R., Fellows, D., Stanford, N.J., Mulherin, P., Le Feuvre, R.,
577 Barran, P., Goodacre, R., Turner, N.J., Goble, C., Chen, G.G., Kell, D.B., Micklefield,
578 J., Breitling, R., Takano, E., Faulon, J.L., Scrutton, N.S., 2018. An automated
579 Design-Build-Test-Learn pipeline for enhanced microbial production of fine
580 chemicals. Commun. Biol. 1, 66.
581 14. Carrieri, D., Paddock, T., Maness, P-C., Seibert, M., Yu, J., 2012. Photo-catalytic
582 conversion of carbon dioxide to organic acids by a recombinant cyanobacterium
583 incapable of glycogen storage. Energy Environ. Sci. 5, 9457–9461.
584 15. Carroll, A.L., Case, A.E., Zhang, A., Atsumi, S., 2018. Metabolic engineering tools
585 in model cyanobacteria. Metab. Eng. 50, 47–56.
586 16. Chakdar, H., Hasan, M., Pabbi, S., Nevalainen, H., Shukla, P., 2021. High-
587 throughput proteomics and metabolomic studies guide re-engineering of metabolic
588 pathways in eukaryotic microalgae: A review. Bioresour. Technol. 321, 124495.

17. Cheah, Y.E., Young, J.D., 2018. Isotopically nonstationary metabolic flux analysis (INST-MFA): putting theory into practice. Curr. Opin. Biotechnol. 54, 80–87.
18. Cheah, Y.E., Xu, Y., Sacco, S.A., Babele, P.K., Zheng, A.O., Johnson, C.H., Young, J.D., 2020. Systematic identification and elimination of flux bottlenecks in the aldehyde production pathway of *Synechococcus elongatus* PCC 7942. Metab. Eng. 60, 56–65.
19. Chen, H., Zheng, Y., Zhan, J., He, C., Wang, Q., 2017. Comparative metabolic profiling of the lipid-producing green microalga *Chlorella* reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism. Biotechnol. Biofuels 10, 153.
20. Chen, J.H., Kato, Y., Matsuda, M., Chen, C.Y., Nagarajan, D., Hasunuma, T., Kondo, A., Dong, C.D., Lee, D.J., Chang, J.S., 2019. A novel process for the mixotrophic production of lutein with *Chlorella sorokiniana* MB-1-M12 using aquaculture wastewater. Bioresour. Technol. 290, 121786.
21. Chen, J.H., Kato, Y., Matsuda, M., Chen, C.Y., Nagarajan, D., Hasunuma, T., Kondo, A., Chang, J.S., 2021. Lutein production with *Chlorella sorokiniana* MB-1-M12 using novel two-stage cultivation strategies - metabolic analysis and process improvement. Bioresour. Technol. 334, 125200.
22. Dauvillée, D., Colleoni, C., Mouille, G., Buléon, A., Gallant, D.J., Bouchet, B., Morell M.K., d'Hulst, C., Myers, A.M., Ball, S.G., 2001. Two loci control phytoglycogen production in the monocellular green alga *Chlamydomonas reinhardtii*. Plant Physiol. 125 (4), 1710–1722.
23. Deng, M.D., Coleman, J.R., 1999. Ethanol synthesis by genetic engineering in cyanobacteria. Appl. Environ. Microbiol. 65 (2), 523–528.

24. Dettmer, K., Aronov, P.A., Hammock, B.D., 2007. Mass spectrometry-based metabolomics. Mass Spectrom. Rev. 26 (1), 51–78.
25. Diao, J., Song, X., Zhang, L., Cui, J., Chen, L., Zhang, W., 2020. Tailoring cyanobacteria as a new platform for highly efficient synthesis of astaxanthin. Metab. Eng. 61, 275–287.
26. Dieterle, F., Riefke, B., Schlotterbeck, G., Ross, A., Senn, H., Amberg, A., 2011. NMR and MS methods for metabonomics. Methods Mol. Biol. 691, 385–415.
27. Englund, E., Shabestary, K., Hudson, E.P., Lindberg, P., 2018. Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. Metab. Eng. 49, 164–177.
28. Fais, G., Malavasi, V., Scano, P., Soru, S., Caboni, P., Cao, G., 2021. Metabolomics and lipid profile analysis of *Coccomyxa melkonianii* SCCA 048. Extremophiles 25 (4), 357–368.
29. Fathima, A.M., Chuang, D., Laviña, W.A., Liao, J., Putri, S.P., Fukusaki, E., 2018. Iterative cycle of widely targeted metabolic profiling for the improvement of 1-butanol titer and productivity in *Synechococcus elongatus*. Biotechnol. Biofuels. 11, 188.
30. Feng, P., Deng, Z., Hu, Z., Fan, L., 2011. Lipid accumulation and growth of *Chlorella zofingiensis* in flat plate photobioreactors outdoors. Bioresour. Technol. 102 (22), 10577–10584.
31. Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226 (1), 497–509.

636 32. Fukusaki, E., 2014. Application of Metabolomics for High Resolution Phenotype
637 Analysis. Mass Spectrom. (Tokyo). 3 (Spec Iss 3), S0045.
638 33. Ge, F., Huang, W., Chen, Z., Zhang, C., Xiong, Q., Bowler, C., Yang, J., Xu, J., Hu,
639 H., 2014. Methylcrotonyl-CoA Carboxylase Regulates Triacylglycerol
640 Accumulation in the Model Diatom *Phaeodactylum tricornutum*. Plant Cell 26 (4),
641 1681–1697.
642 34. Gopalakrishnan, S., Pakrasi, H.B., Maranas, C.D., 2018. Elucidation of
643 photoautotrophic carbon flux topology in *Synechocystis* PCC 6803 using genome-
644 scale carbon mapping models. Metab. Eng. 47, 190–199.
645 35. Hasunuma, T., Harada, K., Miyazawa, S., Kondo, A., Fukusaki, E., Miyake, C. 2010.
646 Metabolic turnover analysis by a combination of *in vivo* ^{13}C-labelling from $^{13}CO_2$
647 and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the C_3
648 photosynthetic pathway in *Nicotiana tabacum* leaves. J. Exp. Bot. 61 (4), 1041–1051.
649 36. Hasunuma, T., Kikuyama, F., Matsuda, M., Aikawa, S., Izumi. Y., Kondo, A., 2013a.
650 Dynamic metabolic profiling of cyanobacterial glycogen biosynthesis under
651 conditions of nitrate depletion. J. Exp. Bot. 64 (10), 2943–2954.
652 37. Hasunuma, T., Okazaki, F., Okai, N., Hara, K.Y., Ishii, J., Kondo, A., 2013b. A
653 review of enzymes and microbes for lignocellulosic biorefinery and the possibility
654 of their application to consolidated bioprocessing technology. Bioresour. Technol.
655 135, 513–522.
656 38. Hasunuma, T., Matsuda, M., Senga, Y., Aikawa, S., Toyoshima, M., Shimakawa, G.,
657 Miyake, C., Kondo, A., 2014. Overexpression of *flv3* improves photosynthesis in the
658 cyanobacterium *Synechocystis* sp. PCC6803 by enhancement of alternative electron
659 flow. Biotechnol. Biofuels. 7 (1), 493.

660 39. Hasunuma, T., Matsuda, M., Kondo, A., 2016. Improved sugar-free succinate
661 production by *Synechocystis* sp. PCC 6803 following identification of the limiting
662 steps in glycogen catabolism. Metab. Eng. Commun. 3, 130–141.
663 40. Hasunuma, T., Matsuda, M., Kato, Y., Vavricka, C.J., Kondo, A., 2018. Temperature
664 enhanced succinate production concurrent with increased central metabolism
665 turnover in the cyanobacterium *Synechocystis* sp. PCC 6803. Metab. Eng. 48, 109–
666 120.
667 41. Hasunuma, T., Takaki, A., Matsuda, M., Kato, Y., Vavricka, C.J., Kondo, A., 2019.
668 Single-Stage Astaxanthin Production Enhances the Nonmevalonate Pathway and
669 Photosynthetic Central Metabolism in *Synechococcus* sp. PCC 7002. ACS Synth.
670 Biol. 8 (12), 2701–2709.
671 42. Hendry, J.I., Prasannan, C., Ma, F., Möllers, K.B., Jaiswal, D., Digmurti, M., Allen,
672 D.K., Frigaard, N.U., Dasgupta, S., Wangikar, P.P., 2017. Rerouting of carbon flux
673 in a glycogen mutant of cyanobacteria assessed via isotopically non-stationary ^{13}C
674 metabolic flux analysis. Biotechnol. Bioeng. 114 (10), 2298–2308.
675 43. Hidese, R., Matsuda, M., Osanai, T., Hasunuma, T., Kondo, A., 2020. Malic Enzyme
676 Facilitates D-Lactate Production through Increased Pyruvate Supply during Anoxic
677 Dark Fermentation in *Synechocystis* sp. PCC 6803. ACS Synth. Biol. 9 (2), 260–268.
678 44. Hillson, N., Caddick, M., Cai, Y., Carrasco, J.A., Chang, M.W., Curach, N.C., Bell,
679 D.J., Le Feuvre, R., Friedman, D.C., Fu, X., Gold, N.D., Herrgård, M.J., Holowko,
680 M.B., Johnson, J.R., Johnson, R.A., Keasling, J.D., Kitney, R.I., Kondo, A., Liu, C.,
681 Martin, V.J.J., Menolascina, F., Ogino, C., Patron, N.J., Pavan, M., Poh, C.L.,
682 Pretorius, I.S., Rosser, S.J., Scrutton, N.S., Storch, M., Tekotte, H., Travnik, E.,

683 Vickers, C.E., Yew, W.S., Yuan, Y., Zhao, H., Freemont, P.S., 2019. Building a
684 global alliance of biofoundries. Nat. Commun. 10 (1), 2040.
685 45. Hirokawa, Y., Matsuo, S., Hamada, H., Matsuda, F., Hanai, T., 2017. Metabolic
686 engineering of *Synechococcus elongatus* PCC 7942 for improvement of 1,3-
687 propanediol and glycerol production based on in silico simulation of metabolic flux
688 distribution. Microb. Cell Fact. 16 (1), 212.
689 46. Ho, S.H., Nakanishi, A., Ye, X., Chang, J.S., Hara, K., Hasunuma, T., Kondo, A.,
690 2014a. Optimizing biodiesel production in marine *Chlamydomonas* sp. JSC4 through
691 metabolic profiling and an innovative salinity-gradient strategy. Biotechnol. Biofuels.
692 7, 97.
693 47. Ho, S.H., Ye, X., Hasunuma, T., Chang, J.S., Kondo, A., 2014b. Perspectives on
694 engineering strategies for improving biofuel production from microalgae--a critical
695 review. Biotechnol. Adv. 32 (8), 1448–1459.
696 48. Ho, S.H., Nakanishi, A., Ye, X., Chang, J.S., Chen, C.Y., Hasunuma, T., Kondo, A.,
697 2015. Dynamic metabolic profiling of the marine microalga *Chlamydomonas* sp.
698 JSC4 and enhancing its oil production by optimizing light intensity. Biotechnol.
699 Biofuels. 8, 48.
700 49. Ho, S.H., Nakanishi, A., Kato, Y., Yamasaki, H., Chang, J.S., Misawa, N., Hirose,
701 Y., Minagawa, J., Hasunuma, T., Kondo, A., 2017. Dynamic metabolic profiling
702 together with transcription analysis reveals salinity-induced starch-to-lipid
703 biosynthesis in alga *Chlamydomonas* sp. JSC4. Sci. Rep. 7, 45471.
704 50. Hollywood, K.A., Schmidt, K., Takano, E., Breitling, R., 2018. Metabolomics tools
705 for the synthetic biology of natural products. Curr. Opin. Biotechnol. 54, 114–120.

706 51. Huang, H.H., Lindblad, P., 2013. Wide-dynamic-range promoters engineered for
707 cyanobacteria. J. Biol. Eng. 7 (1), 10.
708 52. Hung, C.H., Kanehara, K., Nakamura, Y., 2016. Isolation and characterization of a
709 mutant defective in triacylglycerol accumulation in nitrogen-starved
710 *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta. 1861 (9 Pt B), 1282–1293.
711 53. Iijima, H., Watanabe, A., Sukigara, H., Iwazumi, K., Shirai, T., Kondo, A., Osanai,
712 T., 2021. Four-carbon dicarboxylic acid production through the reductive branch of
713 the open cyanobacterial tricarboxylic acid cycle in *Synechocystis* sp. PCC 6803.
714 Metab. Eng. 65, 88–98.
715 54. Ito, S., Takeya, M., and Osanai, T., 2017. Substrate Specificity and Allosteric
716 Regulation of a D-Lactate Dehydrogenase from a Unicellular Cyanobacterium are
717 Altered by an Amino Acid Substitution. Sci. Rep. 7 (1), 15052.
718 55. Jaime, L., Rodríguez-Meizoso, I., Cifuentes, A., Santoyo, S., Suarez, S., Ibáñez, E.,
719 Señorans, F.J., 2010. Pressurized liquids as an alternative process to antioxidant
720 carotenoids' extraction from *Haematococcus pluvialis* microalgae. LWT - Food Sci.
721 and Technol. 43 (1) 105–112.
722 56. Jazmin, L.J., O'Grady, J.P., Ma, F., Allen, D.K., Morgan, J.A., Young, J.D., 2014.
723 Isotopically nonstationary MFA (INST-MFA) of autotrophic metabolism. Methods
724 Mol. Biol. 1090, 181–210.
725 57. Jazmin, L.J., Xu, Y., Cheah, Y.E., Adebiyi, A.O., Johnson, C.H., Young, J.D., 2017.
726 Isotopically nonstationary ^{13}C flux analysis of cyanobacterial isobutyraldehyde
727 production. Metab. Eng. 42, 9–18.
728 58. Kanno, M., Carroll, A.L., Atsumi, S., 2017. Global metabolic rewiring for improved
729 CO_2 fixation and chemical production in cyanobacteria. Nat. Commun. 8, 14724.

59. Katayama, N., Iijima, H., Osanai, T., 2018. Production of Bioplastic Compounds by Genetically Manipulated and Metabolic Engineered Cyanobacteria. Adv. Exp. Med. Biol. 1080, 155–169.

60. Kato, Y., Ho, S.H., Vavricka, C.J., Chang, J.S., Hasunuma, H., Kondo, A., 2017. Evolutionary engineering of salt-resistant *Chlamydomonas* sp. strains reveals salinity stress-activated starch-to-lipid biosynthesis switching. Bioresour. Technol. 245 (Pt B), 1484–1490.

61. Kato, Y., Fujihara, Y., Vavricka, C.J., Chang, J.S., Hasunuma, T., Kondo, A., 2019. Light/dark cycling causes delayed lipid accumulation and increased photoperiod-based biomass yield by altering metabolic flux in oleaginous *Chlamydomonas* sp.. Biotechnol. Biofuels 12, 39.

62. Kato, Y., Oyama, T., Inokuma, K., Vavricka, C.J., Matsuda, M., Hidese, R., Satoh, K., Oono, Y., Chang, J.S., Hasunuma, T., Kondo, A., 2021. Enhancing carbohydrate repartitioning into lipid and carotenoid by disruption of microalgae starch debranching enzyme. Commun. Biol. 4 (1), 450.

63. Kera, K., Fine, D.D., Wherritt, D.J., Nagashima, Y., Shimada, N., Ara, T., Ogata, Y., Sumner, L.W., Suzuki, H., 2018. Pathway-specific metabolome analysis with $^{18}O_2$-labeled *Medicago truncatula* via a mass spectrometry-based approach. Metabolomics 14 (5), 71.

64. Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. Metab Eng. 14 (3), 189–195.

65. Keasling, J., Garcia Martin, H., Lee, T.S., Mukhopadhyay, A., Singer, S.W., Sundstrom, E., 2021. Microbial production of advanced biofuels. Nat. Rev. Microbiol. doi: 10.1038/s41579-021-00577-w.

754 66. Kim, I.K., Roldão, A., Siewers, V., Nielsen, J., 2012. A systems-level approach for
755 metabolic engineering of yeast cell factories. FEMS Yeast Res. 12 (2), 228–248.
756 67. Knoot, C.J., Ungerer, J., Wangikar, P.P., Pakrasi, H.B., 2018. Cyanobacteria:
757 Promising biocatalysts for sustainable chemical production. J. Biol. Chem. 293 (14),
758 5044–5052.
759 68. Kokabi, K., Gorelova, O., Ismagulova, T., Itkin, M., Malitsky, S., Boussiba, S.,
760 Solovchenko, A., Khozin-Goldberg, I., 2019. Metabolomic foundation for
761 differential responses of lipid metabolism to nitrogen and phosphorus deprivation in
762 an arachidonic acid-producing green microalga. Plant Sci. 283, 95–115.
763 69. Kopka, J., Schmidt, S., Dethloff, F., Pade, N., Berendt, S., Schottkowski, M., Martin,
764 N., Dühring, U., Kuchmina, E., Enke, H., Kramer, D., Wilde, A., Hagemann, M.,
765 Friedrich, A., 2017. Systems analysis of ethanol production in the genetically
766 engineered cyanobacterium *Synechococcus* sp. PCC 7002. Biotechnol. Biofuels. 10,
767 56.
768 70. Krishnan, A., Kumaraswamy, G.K., Vinyard, D.J., Gu, H., Ananyev, G., Posewitz,
769 M.C., Dismukes, G.C., 2015. Metabolic and photosynthetic consequences of
770 blocking starch biosynthesis in the green alga *Chlamydomonas reinhardtii sta6*
771 mutant. Plant J. 81 (6), 947–960.
772 71. Leong, Y.K., Chew, K.W., Chen, W.H., Chang, J.S., Show, P.L., 2021. Reuniting
773 the Biogeochemistry of Algae for a Low-Carbon Circular Bioeconomy. Trends Plant
774 Sci. 26 (7), 729–740.
775 72. Li, X., Shen, C.R., Liao, J.C., 2014. Isobutanol production as an alternative metabolic
776 sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus*
777 *elongatus* PCC 7942. Photosynth. Res. 120 (3), 301–310.

778 73. Liang, Y., Kong, F., Torres-Romero, I., Burlacot, A., Cuine, S., Légeret, B., Billon,
779 E., Brotman, Y., Alseekh, S., Fernie, A.R., Beisson, F., Peltier, G., Li-Beisson, Y.,
780 2019. Branched-Chain Amino Acid Catabolism Impacts Triacylglycerol
781 Homeostasis in *Chlamydomonas reinhardtii*. Plant Physiol. 179 (4), 1502–1514.
782 74. Lin, P.C., Zhang, F., Pakrasi, H.B., 2021. Enhanced limonene production in a fast-
783 growing cyanobacterium through combinatorial metabolic engineering. Metab. Eng.
784 Commun. 12, e00164.
785 75. Liu, L., Wang, F., Pei, G., Cui, J., Diao, J., Lv, M., Chen, L., Zhang, W., 2020.
786 Repeated fed-batch strategy and metabolomic analysis to achieve high
787 docosahexaenoic acid productivity in *Crypthecodinium cohnii*. Microb. Cell Fact. 19
788 (1), 91.
789 76. Liu, Z., Ho, S.H., Hasunuma, T., Chang, J.S., Ren, N.Q., Kondo, A., 2016. Recent
790 advances in yeast cell-surface display technologies for waste biorefineries. Bioresour.
791 Technol. 215, 324–333.
792 77. Lv, H., Xia, F., Liu, M., Cui, X., Wahid, F., Jia, S., 2016. Metabolomic profiling of
793 the astaxanthin accumulation process induced by high light in *Haematococcus*
794 *pluvialis*. Algal Res. 20, 35–43.
795 78. Lv, M., Wang, F., Zeng, L., Bi, Y., Cui, J., Liu, L., Bi, Y., Chen, L., Zhang, W., 2020.
796 Identification and metabolomic analysis of a starch-deficient *Crypthecodinium*
797 *cohnii* mutant reveals multiple mechanisms relevant to enhanced growth and lipid
798 accumulation. Algal Res. 50, 102001
799 79. Markley, A.L., Begemann, M.B., Clarke, R.E., Gordon, G.C., Pfleger, B.F., 2016.
800 Synthetic biology toolbox for controlling gene expression in the cyanobacterium
801 *Synechococcus* sp. strain PCC 7002. ACS Synth. Biol. 4 (5), 595–603.

80. Maruyama, M., Nishiguchi, H., Toyoshima, M., Okahashi, N., Matsuda, F., Shimizu, H., 2019. Time-resolved analysis of short term metabolic adaptation at dark transition in *Synechocystis* sp. PCC 6803. J. Biosci. Bioeng. 128 (4), 424–428.

81. May, D.S., Crnkovic, C.M., Krunic, A., Wilson, T.A., Fuchs, J.R., Orjala, J.E., 2020. ^{15}N Stable Isotope Labeling and Comparative Metabolomics Facilitates Genome Mining in Cultured Cyanobacteria. ACS Chem. Biol. 15 (3), 758–765.

82. Mehariya, S., Iovine, A., Di Sanzo, G., Larocca, V., Martino, M., Leone, G.P., Casella, P., Karatza, D., Marino, T., Musmarra, D., Molino, A., 2019. Supercritical fluid extraction of lutein from *Scenedesmus almeriensis*. Molecules 24 (7), 1324.

83. Nelson, J.A., Lefebvre, P.A., 1995. Targeted disruption of the *NIT8* gene in *Chlamydomonas reinhardtii*. Mol. Cell. Biol. 15 (10), 5762–5769.

84. Ng, I.S., Keskin, B.B., Tan, S.I., 2020. A Critical Review of Genome Editing and Synthetic Biology Applications in Metabolic Engineering of Microalgae and Cyanobacteria. Biotechnol. J. 15 (8), e1900228.

85. Nielsen, J., Fussenegger, M., Keasling, J., Lee, S.Y., Liao, J.C., Prather, K., Palsson, B., 2014. Engineering synergy in biotechnology. Nat. Chem. Biol. 10 (5), 319–322.

86. Nielsen, J., Keasling, J.D., 2016. Engineering Cellular Metabolism. Cell 164 (6), 1185–1197.

87. Nitta, N., Iino, T., Isozaki, A., Yamagishi, M., Kitahama, Y., Sakuma, S., Suzuki, Y., Tezuka, H., Oikawa, M., Arai, F., Asai, T., Deng, D., Fukuzawa, H., Hase, M., Hasunuma, T., Hayakawa, T., Hiraki, K., Hiramatsu, K., Hoshino, Y., Inaba, M., Inoue, Y., Ito, T., Kajikawa, M., Karakawa, H., Kasai, Y., Kato, Y., Kobayashi, H., Lei, C., Matsusaka, S., Mikami, H., Nakagawa, A., Numata, K., Ota, T., Sekiya, T., Shiba, K., Shirasaki, Y., Suzuki, N., Tanaka, S., Ueno, S., Watarai, H., Yamano, T.,

826 Yazawa, M., Yonamine, Y., Di Carlo, D., Hosokawa, Y., Uemura, S., Sugimura, T.,
827 Ozeki, Y., Goda, K., 2020. Raman image-activated cell sorting. Nat. Commun. 11
828 (1), 3452.
829 88. Noguchi, S., Putri, S.P., Lan, E.I., Laviña, W.A., Dempo, Y., Bamba, T., Liao, J.C.,
830 Fukusaki, E., 2016. Quantitative target analysis and kinetic profiling of acyl-CoAs
831 reveal the rate-limiting step in cyanobacterial 1-butanol production. Metabolomics.
832 12, 26.
833 89. Oliver, N.J., Rabinovitch-Deere, C.A., Carroll, A.L., Nozzi, N.E., Case, A.E., Atsumi,
834 S., 2016. Cyanobacterial metabolic engineering for biofuel and chemical production.
835 Curr. Opin. Chem. Biol. 35, 43–50.
836 90. Osanai, T., Numata, K., Oikawa, A., Kuwahara, A., Iijima, H., Doi, Y., Tanaka, K.,
837 Saito, K., Hirai, M.Y., 2013. Increased bioplastic production with an RNA
838 polymerase sigma factor SigE during nitrogen starvation in *Synechocystis* sp. PCC
839 6803. DNA Res. 20 (6), 525–535.
840 91. Osanai, T., Shirai, T., Iijima, H., Nakaya, Y., Okamoto, M., Kondo, A., Hirai, M.Y.,
841 2015. Genetic manipulation of a metabolic enzyme and a transcriptional regulator
842 increasing succinate excretion from unicellular cyanobacterium. Front. Microbiol. 6,
843 1064.
844 92. Pade, N., Erdmann, S., Enke, H., Dethloff, F., Dühring, U., Georg, J., Wambutt, J.,
845 Kopka, J., Hess, W.R., Zimmermann, R., Kramer, D., Hagemann, M., 2016. Insights
846 into isoprene production using the cyanobacterium *Synechocystis* sp. PCC 6803.
847 Biotechnol. Biofuels. 9, 89.
848 93. Pagels, F., Vasconcelos, V., Guedes, A.C., 2021. Carotenoids from Cyanobacteria:
849 Biotechnological Potential and Optimization Strategies. Biomolecules 11 (5), 735.

850 94. Pan, Y., Yang, J., Gong, Y., Li, X., Hu, H., 2017. 3-Hydroxyisobutyryl-CoA
851 hydrolase involved in isoleucine catabolism regulates triacylglycerol accumulation
852 in *Phaeodactylum tricornutum*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 372 (1728),
853 20160409.
854 95. Pancha, I., Takaya, K., Tanaka, K., Imamura, S., 2021. The Unicellular Red Alga
855 *Cyanidioschyzon merolae*, an Excellent Model Organism for Elucidating
856 Fundamental Molecular Mechanisms and Their Applications in Biofuel Production.
857 Plants (Basel). 10 (6), 1218.
858 96. Piligaev, A.V., Sorokina, K.N., Shashkov, M.V., Parmon, V.N., 2018. Screening and
859 comparative metabolic profiling of high lipid content microalgae strains for
860 application in wastewater treatment. Bioresour. Technol. 250, 538–547.
861 97. Poddar, N., Elahee Doomun, S.N., Callahan, D.L., Kowalski, G.M., Martin, G.J.O.,
862 2020. The assimilation of glycerol into lipid acyl chains and associated carbon
863 backbones of *Nannochloropsis salina* varies under nitrogen replete and deplete
864 conditions. Biotechnol. Bioeng. 117 (11), 3299–3309.
865 98. Popko, J., Herrfurth, C., Feussner, K., Ischebeck, T., Iven, T., Haslam, R., Hamilton,
866 M., Sayanova, O., Napier, J., Khozin-Goldberg, I., Feussner, I., 2016. Metabolome
867 Analysis Reveals Betaine Lipids as Major Source for Triglyceride Formation, and
868 the Accumulation of Sedoheptulose during Nitrogen-Starvation of *Phaeodactylum*
869 *tricornutum*. PLoS One 11 (10), e0164673.
870 99. Prasannan, C.B., Jaiswal, D., Davis, R., Wangikar, P.P., 2018. An improved method
871 for extraction of polar and charged metabolites from cyanobacteria. PLoS One. 13
872 (10), e0204273.

100. Qian, X., Zhang, Y., Lun, D.S., Dismukes, G.C., 2018. Rerouting of Metabolism into Desired Cellular Products by Nutrient Stress: Fluxes Reveal the Selected Pathways in Cyanobacterial Photosynthesis. ACS Synth. Biol. 7 (5), 1465–1476.
101. Řezanka, T., Nedbalová, L., Procházková, L., Sigler, K., 2014. Lipidomic profiling of snow algae by ESI-MS and silver-LC/APCI-MS. Phytochemistry 100, 34–42.
102. Řezanka, T., Lukavský, J., Vítová, M., Nedbalová, L., Sigler, K., 2018. Lipidomic analysis of Botryococcus (Trebouxiophyceae, Chlorophyta) - Identification of lipid classes containing very long chain fatty acids by offline two-dimensional LC-tandem MS. Phytochemistry 148, 29–38.
103. Roussou, S., Albergati, A., Liang, F., Lindblad, P., 2021. Engineered cyanobacteria with additional overexpression of selected Calvin-Benson-Bassham enzymes show further increased ethanol production. Metab. Eng. Commun. 12, e00161.
104. Sawa, N., Tatsuke, T., Ogawa, A., Hirokawa, Y., Osanai, T., Hanai, T., 2019. Modification of carbon metabolism in *Synechococcus elongatus* PCC 7942 by cyanophage-derived sigma factors for bioproduction improvement. J. Biosci. Bioeng. 127 (2), 256–264.
105. Schwarz, D., Orf, I., Kopka, J., Hagemann, H., 2013. Recent applications of metabolomics toward cyanobacteria. Metabolites 3 (1), 72–100.
106. Sengupta, A., Madhu, S., Wangikar, P.P., 2020. A Library of Tunable, Portable, and Inducer-Free Promoters Derived from Cyanobacteria. ACS Synth. Biol. 9 (7), 1790–1801.
107. Sengupta, A., Sunder, A.V., Sohoni, S.V., Wangikar, P.P., 2019. Fine-Tuning Native Promoters of *Synechococcus elongatus* PCC 7942 To Develop a Synthetic Toolbox for Heterologous Protein Expression. ACS Synth. Biol. 8 (5), 1219–1223.

897 108.Shaikh, K.M., Nesamma, A.A., Abdin, M.Z., Jutur, P.P., 2019. Molecular profiling
898 of an oleaginous trebouxiophycean alga *Parachlorella kessleri* subjected to nutrient
899 deprivation for enhanced biofuel production. Biotechnol. Biofuels. 12, 182.
900 109.Shastri, A.A., Morgan, J.A., 2007. A transient isotopic labeling methodology for ^{13}C
901 metabolic flux analysis of photoautotrophic microorganisms. Phytochemistry 68 (16-
902 18), 2302–2312.
903 110.Sodeinde, O.A., Kindle, K.L., 1993. Homologous recombination in the nuclear
904 genome of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. U.S.A. 90 (19), 9199–
905 9203.
906 111.Srivastava, A., Shukla, P., 2021. Emerging tools and strategies in cyanobacterial
907 omics. Trends Biotechnol. S0167-7799 (21), 00130-X.
908 112.Stal, L.J., and Moezalaar, R., 1997. Fermentation in cyanobacteria. FEMS Microbiol.
909 Rev. 21 (2), 179−211.
910 113.Sulochana, S.B., Arumugam, M., 2020. Targeted Metabolomic and Biochemical
911 Changes During Nitrogen Stress Mediated Lipid Accumulation in *Scenedesmus*
912 *quadricauda* CASA CC202. Front. Bioeng. Biotechnol. 8, 585632.
913 114.Takenaka, M., Yoshida, T., Hori, Y., Bamba, T., Mochizuki, M., Vavricka, C.J.,
914 Hattori, T., Hayakawa, Y., Hasunuma, T., Kondo, A., 2021. An ion-pair free LC-
915 MS/MS method for quantitative metabolite profiling of microbial bioproduction
916 systems. Talanta 222, 121625.
917 115.Teoh, S.T., Putri, S., Mukai, Y., Bamba, T., Fukusaki, E., 2015. A metabolomics-
918 based strategy for identification of gene targets for phenotype improvement and its
919 application to 1-butanol tolerance in *Saccharomyces cerevisiae*. Biotechnol. Biofuels.
920 8, 144.

921 116. Terashima, M., Freeman, E.S., Jinkerson, R.E., Jonikas, M.C., 2015. A fluorescence-
922 activated cell sorting-based strategy for rapid isolation of high-lipid *Chlamydomonas*
923 mutants. Plant J. 81 (1), 147–159.
924 117. Valledor, L., Furuhashi, T., Recuenco-Muñoz, L., Wienkoop, S., Weckwerth, W.,
925 2014. System-level network analysis of nitrogen starvation and recovery in
926 *Chlamydomonas reinhardtii* reveals potential new targets for increased lipid
927 accumulation. Biotechnol. Biofuels. 7, 171.
928 118. Vavricka, C.J., Hasunuma, T., Kondo, A., 2020. Dynamic Metabolomics for
929 Engineering Biology: Accelerating Learning Cycles for Bioproduction. Trends
930 Biotechnol. 38 (1), 68–82.
931 119. Vello, V., Chu, W.L., Lim P.E., Abdul Majid, N., Phang, S.M., 2018. Metabolomic
932 profiles of tropical *Chlorella* species in response to physiological changes during
933 nitrogen deprivation. J. Appl. Phycol. 30, 3131–3151.
934 120. Yao, L., Cengic, I., Anfelt, J., Hudson, E.P., 2016. Multiple Gene Repression in
935 Cyanobacteria Using CRISPRi. ACS Synth. Biol. 5 (3), 207–212.
936 121. Wan, N., DeLorenzo, D.M., He, L., You, L., Immethun, C.M., Wang, G., Baidoo,
937 E.E.K., Hollinshead, W., Keasling, J.D., Moon, T.S., Tang, Y.J., 2017.
938 Cyanobacterial carbon metabolism: Fluxome plasticity and oxygen dependence.
939 Biotechnol. Bioeng. 114 (7), 1593–1602.
940 122. Wang, F., Bi, Y., Diao, J., Lv, M., Cui, J., Chen, L., Zhang, W., 2019. Metabolic
941 engineering to enhance biosynthesis of both docosahexaenoic acid and odd-chain
942 fatty acids in *Schizochytrium* sp. S31. Biotechnol. Biofuels. 12, 141.
943 123. Wu, M., Zhang, H., Sun, W., Li, Y., Hu, Q., Zhou, H., Han, D., 2019. Metabolic
944 plasticity of the starchless mutant of *Chlorella sorokiniana* and mechanisms

945 underlying its enhanced lipid production revealed by comparative metabolomics
946 analysis. Algal Res. 42, 101587.
947 124.Xiao, Y., Zhang, J., Cui, J., Feng, Y., Cui, Q., 2013. Metabolic profiles of
948 *Nannochloropsis oceanica* IMET1 under nitrogen-deficiency stress. Bioresour.
949 Technol. 130, 731–738.
950 125.Xu, Y., Guerra, L.T., Li, Z., Ludwig, M., Dismukes, G.C., Bryant, D.A., 2013.
951 Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp.
952 strain PCC 7002: Cell factories for soluble sugars. Metab. Eng. 16, 56–67.
953 126.Yao, L., Shen, H., Wang, N., Tatlay, J., Li, L., Tan, T.W., Lee, Y.K., 2017.Elevated
954 acetyl-CoA by amino acid recycling fuels microalgal neutral lipid accumulation in
955 exponential growth phase for biofuel production. Plant Biotechnol. J. 15 (4), 497–
956 509.
957 127.Yoshikawa, K., Hirasawa, T., Ogawa, K., Hidaka, Y., Nakajima, T., Furusawa, C.,
958 Shimizu, H., 2013. Integrated transcriptomic and metabolomic analysis of the central
959 metabolism of *Synechocystis* sp. PCC 6803 under different trophic conditions.
960 Biotechnol. J. 8 (5), 571–580.
961 128.Young, J.D., Shastri, A.A., Stephanopoulos, G., Morgan, J.A., 2011. Mapping
962 photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis.
963 Metab. Eng. 13 (6), 656–665.
964 129.Yu King Hing, N., Liang, F., Lindblad, P., Morgan, J.A., 2019. Combining
965 isotopically non-stationary metabolic flux analysis with proteomics to unravel the
966 regulation of the Calvin-Benson-Bassham cycle in *Synechocystis* sp. PCC 6803.
967 Metab. Eng. 56, 77–84.

130. Yuan, B.F., Zhu, Q.F., Guo, N., Zheng, S.J., Wang, Y.L., Wang, J., Xu, J., Liu, S.J., He, K., Hu, T., Zheng, Y.W., Xu, F.Q., Feng, Y.Q., 2018. Comprehensive Profiling of Fecal Metabolome of Mice by Integrated Chemical Isotope Labeling-Mass Spectrometry Analysis. Anal. Chem. 90 (5), 3512–3520.

131. Yuan, Y., Li, X., Zhao, Q., 2019. Enhancing growth and lipid productivity in *Dunaliella salina* under high light intensity and nitrogen limited conditions. Bioresour. Technol. Rep. 7, 100211.

132. Zhang, H., Liu, Y., Nie, X., Liu, L., Hua, Q., Zhao, G.P., Yang, C., 2018. The cyanobacterial ornithine-ammonia cycle involves an arginine dihydrolase. Nat. Chem. Biol. 14 (6), 575–581.

133. Zhou, J., Yin, Y., 2016. Strategies for large-scale targeted metabolomics quantification by liquid chromatography-mass spectrometry. Analyst 141 (23), 6362–6373.

Figure captions

Figure 1. Metabolic pathways and derived compounds.

13BPG, 1,3-bisphosphoglycerate; 13PDO, 1,3-propanediol; 23BD, 2,3-butanediol; 2PGA, 2-phosphoglycerate; 2OG, 2-oxoglutarate; 3HB, 3-hydroxybutyrate; 3HB-CoA, 3-hydroxybutyryl-CoA; 3HP, 3-hydroxypropionate; 3HPA, 3-hydroxypropionaldehyde; 3PGA, 3-phosphoglycerate; 8OH-CPP, 8-hydroxy-copalyldiphosphate; A-Ace, acetoacetate; A-Ac-ACP, acetoacetyl-ACP; A-Ac-CoA, acetoacetyl-CoA; A-Ald, acetaldehyde; a-ACP, acyl-ACP; ACP, acyl carrier protein; ADP, Adenosinediphosphate; ADPGlc, ADP-glucose; Ac-CoA, acetyl-CoA; Ac-P, acetyl-phosphate; A-Lac, acetolactate; Ald, aldehyde; But-Ald, butyrylaldehyde; But-CoA, butyryl-CoA; CBB cycle, Calvin-Benson-Bassham cycle; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; cisAco, *cis*-aconitate; Cit, citrate; CoA, coenzyme A; Cr-CoA, crotonyl-CoA; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; DIV, 2,3-dihydroxyisovalerate; DMAPP, dimethylallyl diphosphate; DPMVA, diphosphomevalonate; DXP, 1-deoxy-D-xylulose 5-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; FPP, farnesyl diphosphate; Fum, fumarate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; G3P, glycerol 3-phosphate; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IBA, isobutyraldehyde; Isocit, isocitrate; KIV, 2-ketoisovalerate; keto-a-ACP, beta-keto-acyl-ACP; LPA, lysophosphatidic acid; M1P, mannitol 1-phosphate; MEP, 2-C-methylerythritol 4-phosphate; MEcPP, 2-C-methyl-D-erythritol

2,4-cyclodiphosphate; MVA, mevalonate; Mal, malate; Malo-ACP, malonyl-ACP; Malo-Ald, malonyl-aldehyde; Malo-CoA, malonyl-CoA; OAA, oxaloacetate; OAS, oxalosuccinate; OH-a-ACP, beta-hydroxy-acyl-ACP; PA, phosphatidic acid; PEP, phosphoenolpyruvate; PHB, polyhydroxybutyrate; PMVA, phosphomevalonate; PSPP, presqualene diphosphate; Pyr, pyruvate; R5P, ribose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; Suc, succinate; Suc-CoA, succinyl-CoA; SSA, succinic semialdehyde; TAG, triacylglycerol; TCA cycle, tricarboxylic acid cycle; *trans*-a-ACP, *trans*-delta^2-enoyl-acyl-ACP; Xu5P, xylulose 5-phosphate.

Figure 2. Metabolomics-based approach for developing cell factories.

Metabolomics-based approaches for the rational and rapid development of cell factories in microalgae and cyanobacteria compared with conventional approaches.

Table 1. Comparison of major advantages and drawbacks of MS- and NMR-based metabolomics.

Analytical technology	Advantages	Drawbacks
Mass spectrometry (MS)	• Large number of detectable metabolites • High quantifiable • High reproducibility, sensitivity, and selectivity • High reproducibility	• Need chromatography to increase selectivity; Sample derivatization for GC-MS • Destructive nature
Nuclear magnetic resonance (NMR)	• Inherently nondestructive nature • Identification of novel compounds • High quantifiable, minimal sample preparation, and relatively fast measurement • High reproducibility	• Low sensitivity and selectivity

1023 **Table 2.** Findings of microalgae lipid synthesis under nitrogen depletion

Microalgae	Methodology	Number of analyzed metabolites	Findings	Reference
Chlamydomonas reinhardtii	GC-MS	77	Highly distinctive metabolite profiles under nitrogen-, phosphorus-, sulfur-, or iron-depleted conditions.	Bölling and Fiehn (2005)
	GC-MS	52	Functional network perspective in which cell growth and nitrogen metabolism are linked by BCAAs.	Valledor et al. (2014)
Chlorella	GC-MS, LC-MS/MS	40	Degradation of proteins/BCAAs and their conversion into certain amino acids/intermediate molecules cause excess carbon diversion into lipid synthesis under nitrogen depletion.	Chen et al. (2017)
	LC-MS/MS	122	Nitrogen assimilation from proteins and photosynthetic machinery for lipid synthesis in response to nitrogen depletion.	Vello et al. (2018)
Scenedesmus quadricauda	LC-MS	12	GABA increases under nitrogen depletion.	Sulochana and Arumugam (2020)
Parachlorella kessleri	GC-MS/MS	62	TCA cycle metabolites, such as citrate and 2OG, play a significant role in diverting flux toward lipid synthesis under nutrient-depleted conditions.	Shaikh et al. (2019)
Nannochloropsis oceanica	^{1}H NMR	29	Citrate increases under low nitrogen conditions.	Xiao et al. (2013)
Phaeodactylum tricornutum	LC-MS	50	Amino acids, including valine, leucine, and isoleucine, and TCA cycle intermediates, including citrate, decreased during nitrogen starvation.	Popko et al. (2016)

1024

1025 **Table 3.** Findings of microalgae with mutations in starch-related processes

Microalgae	Methodology	Number of analyzed metabolites	Finding	Reference
Chlamydomonas reinhardtii sta6 (starchless and high lipid)	LC-MS/MS	22	Accumulates precursors for biosynthesis of lipids, amino acids, and starch under high light conditions.	Krishnan et al. (2015)
Chlorella sorokiniana SLM2 (starchless and high lipid)	LC-MS/MS	316	Oxidative pentose phosphate pathway and glycolysis pathway are enhanced; GABA shunt is induced.	Wu et al. (2019)
Crypthecodinium cohnii 16D (low starch and high lipid)	LC-MS, GC-MS	94	Central carbon metabolic pathways, such as the Embden-Meyerhof, Krebs cycle, and pentose phosphate pathways, are enhanced, and starch and extracellular polysaccharide biosynthetic pathways are attenuated.	Lv et al. (2020)
Chlamydomonas reinhardtii bgal1 (high starch and low lipid)	LC-MS	14	Lower levels of G6P, F6P, and acetyl-CoA upon nitrogen starvation.	Hung et al. (2016)
Chlamydomonas sp. KOR1 (phytoglycogen accumulation instead of starch and high lipid)	CE-MS	14	Increased levels of F6P, G6P, PEP, pyruvate, acetyl-CoA, and G3P.	Kato et al. (2021)

1026

Table 4. Metabolome-based strain construction in cyanobacteria

Target	Strain	Genetic modification	Fold-increase	Reference
Acetate	PCC 7942	OE: S-CBS3-rpsD4 and *pta*	2.2	Sawa et al. (2019)
PHB	PCC 6803	OE: *sigE*	2.3	Osanai et al. (2013)
IBA	PCC 7942	OE: *pyk* or *mdh*/*me*	1.6 or 1.7	Jazmin et al. (2017)
	PCC 7942	OE: *pyk* and *pck*	>1.6	Cheah et al. (2020)
23BD	PCC 7942	OE: *prk*, *rbcLXS*, *galP*, *zwf*, and *gnd* KO: cp12	5.0	Kanno et al. (2017)
D-Lactate	PCC 6803	KO: *ackA*, OE: *ddh*	15.6	Hidese et al. (2020)
Succinate	PCC 6803	OE: *ppc*	>10	Hasunuma et al. (2018)
13PDO	PCC 7942	KO: *ndhF1* or *ndhD1*	1.3 or 1.2	Hirokawa et al. (2017)

PHB, polyhydroxybutyrate; IBA, isobutyraldehyde; 23BD, 2,3-butanediol; 13PDO, 1,3-propandiol; PCC 6803, *Synechocystis* sp. PCC 6803; PCC 7942, *Synechococcus elongatus* PCC 7942; OE, overexpression; KO, knockout

1032 **Figure 1.**

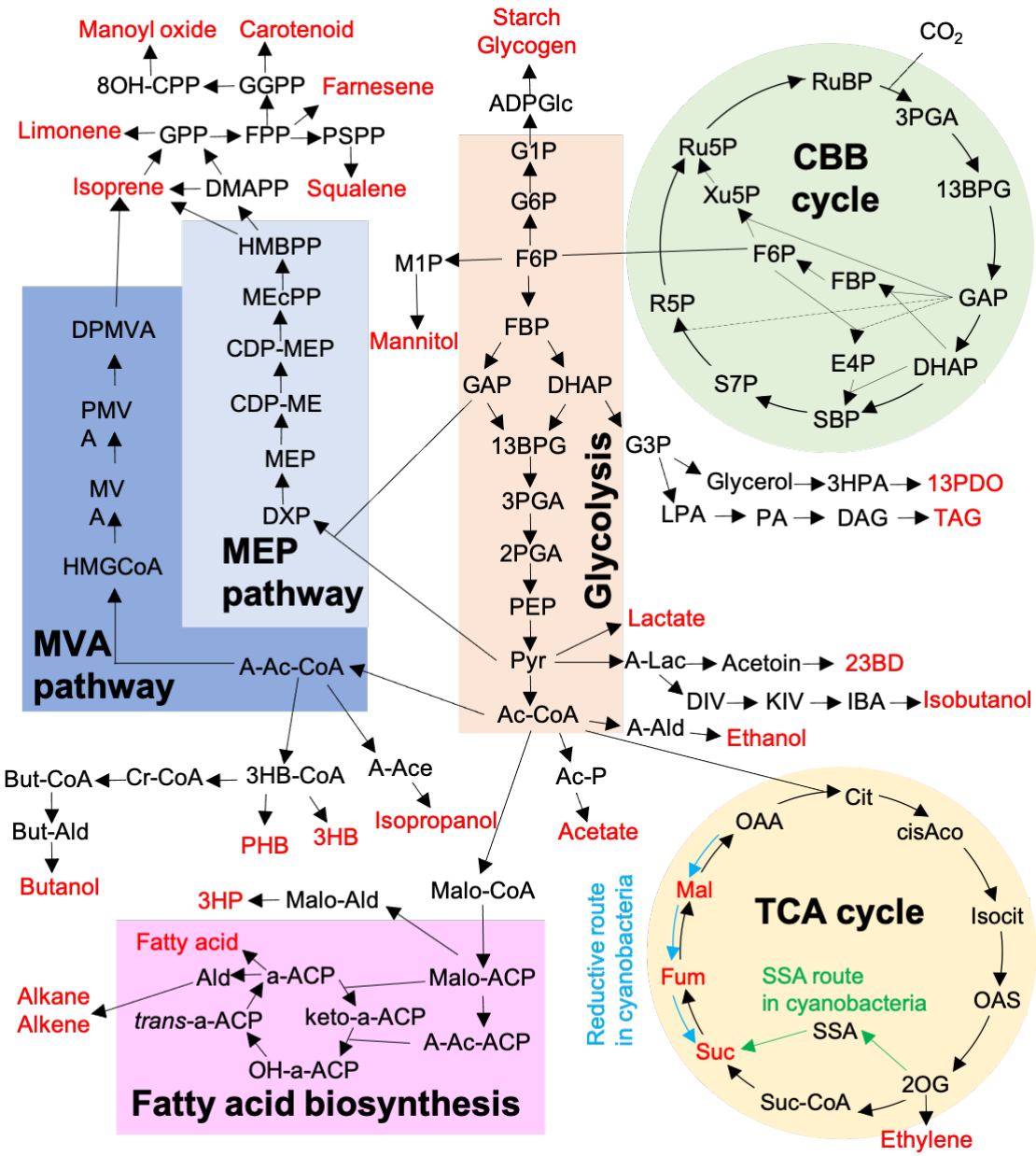

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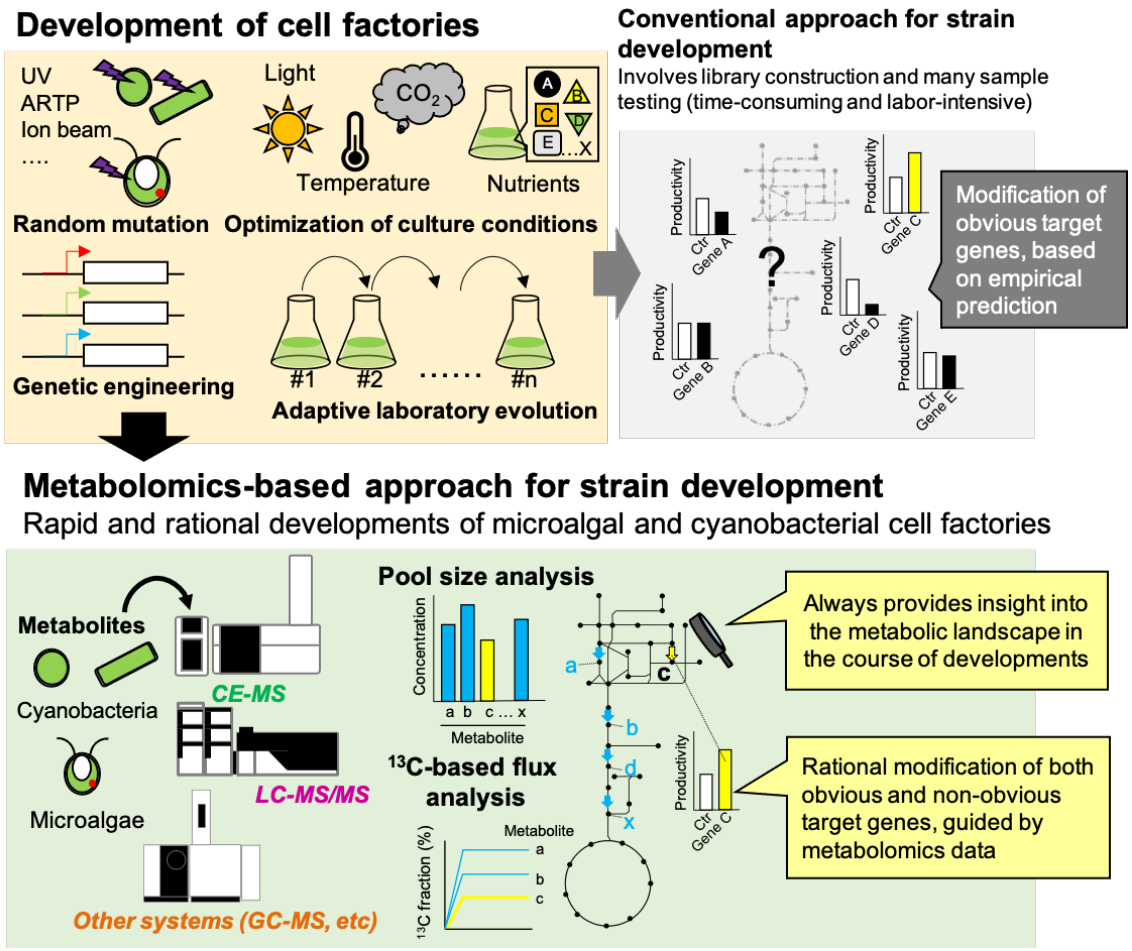
Figure 2.


Development of cell factories
UV
ARTP
Ion beam
....
Random mutation
Light
Temperature
Nutrients
Optimization of culture conditions
Genetic engineering
#1
#2
#n
Adaptive laboratory evolution
Conventional approach for strain development
Involves library construction and many sample testing (time-consuming and labor-intensive)
Productivity
Ctr
Gene A
Gene B
Gene C
Gene D
Gene E
Modification of obvious target genes, based on empirical prediction
Metabolomics-based approach for strain development
Rapid and rational developments of microalgal and cyanobacterial cell factories
Metabolites
Cyanobacteria
Microalgae
CE-MS
LC-MS/MS
Other systems (GC-MS, etc)
Pool size analysis
Concentration
Metabolite
13C-based flux analysis
13C fraction (%)
Always provides insight into the metabolic landscape in the course of developments
Rational modification of both obvious and non-obvious target genes, guided by metabolomics data