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

3

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19

20 **Abstract**

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

34

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

37

38 1. Introduction

39 One of the major challenges in developing a sustainable global economy is
40 transitioning from conventional petroleum-based chemical manufacturing to bio-based
41 carbon neutral alternatives. Productivity and cost performance are the main
42 considerations for meeting the industrial requirements of bio-based commodity chemicals,
43 in addition to ensuring environmental sustainability and feedstock supplementation
44 (Keasling, 2012; Nielsen et al., 2014). Recent innovations in biotechnology based on a
45 design-build-test-learn (DBTL) biological engineering cycle (Carbonell et al., 2018;
46 Vavricka et al., 2020) have motivated research on the development of various chemicals
47 via plant biomass fermentation, leading to the establishment of global biofoundries
48 (Hillson et al., 2019). Numerous studies of biofuels and bio-based chemicals using
49 representative fermentative microorganisms, such as yeasts and *Escherichia coli*, have
50 been reported (Hasunuma et al., 2013b; Keasling, 2016; Keasling et al., 2021; Nielsen
51 and Liu et al., 2016). Utilization of plant biomass has been promoted as an alternative
52 feedstock to fossil resources, reflecting social infiltration of the bioeconomy concept that
53 produces biofuels and bio-based chemicals from atmospheric carbon dioxide (CO₂); thus,
54 carbon recycling is developing as a technology for reducing CO₂ emissions. However,
55 cropland competition with food production and the limited availability of freshwater are
56 important limitations to effectively utilizing plant biomass. Environmental problems,
57 such as ecosystem destruction due to excessive deforestation, are important concerns
58 related to the use of plant biomass as feedstock, even for non-edible plants. Therefore, in
59 addition to plant biomass fermentation, bioproduction processes using photosynthetic
60 microorganisms that produce valuable compounds directly from atmospheric CO₂ must
61 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 CO₂ (**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

86 optimal culture conditions (Hasunuma et al., 2013a; Hasunuma et al., 2014; Ho et al.,
87 2015). Focusing on the photosynthetic production of biofuels and bio-chemicals by using
88 microalgae and cyanobacteria, this review highlights recent advances in metabolomics
89 technologies and the applications of these methods for identifying key metabolic
90 processes, dynamics, and bottleneck steps that can be engineered to improve target
91 compound production.

92

93 **2. Recent technologies applicable to microalgal and cyanobacterial metabolomics**

94 *2.1. Metabolomics techniques*

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

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

115

116 2.2. Microalgal and cyanobacterial metabolomics

117 In photosynthetic organisms, such as microalgae and cyanobacteria, the
118 lighting condition is the critical environmental factor that immediately alters the
119 intracellular metabolic status (Maruyama et al., 2017; Kato et al., 2021). For accurate
120 metabolic profiling, it is necessary to strictly control photosynthesis-related
121 environmental factors, such as the light intensity and CO₂ concentration. In addition, to
122 obtain reliable metabolomic snapshots of biological systems, a suitable quenching
123 method is needed to immediately stop any cellular metabolic activities at a specific
124 sampling time (Chakdar et al, 2021; Ho et al., 2015). The conventional method of
125 extracting total lipids from tissue cells using a super-cooled mixture of chloroform,
126 methanol, and water (Folch et al, 1987) has more recently been applied for metabolite
127 extraction of microalgae, such as *Chlamydomonas* sp. (Ho et al., 2015; Kato et al., 2021)
128 and *Chlorella sorokiniana* (Chen et al., 2019; Chen et al., 2021). In addition, the cold
129 methanol quenching method has been used for metabolite extraction from microalgae,
130 such as *Haematococcus pluvialis* (Lv et al., 2016) and *Chlorella* sp. (Vello et al., 2018),
131 and from cyanobacteria (Hasunuma et al, 2013a; Hasunuma et al., 2016; Young et al.,
132 2011). A methanol-chloroform-NH₄OH system was developed to improve the extraction
133 of polar compounds from cyanobacteria, such as sugar phosphates and bisphosphates

134 (Prasannan et al., 2018). Other methods, including pressurized liquid extraction and
135 supercritical fluid extraction, are suitable for extracting carotenoids and have been applied
136 to *H. pluvialis* (Jaime et al., 2010) and *Scenedesmus almeriensis* (Mehariya et al., 2019).
137 Takenaka et al. (2021) reported an LC-MS/MS-based metabolomics system for
138 the identification and absolute quantification of approximately 150 primary metabolites,
139 including amino acids, sugars, organic acids, nucleotides, and vitamins. A similar number
140 of ionic metabolites can be identified and quantified using CE-coupled TOF-MS systems
141 in both cation and anion modes (Hasunuma et al., 2016). Yuan et al. (2018) identified
142 over 300 metabolites in another MS metabolomics study. Microalgal metabolic profiling
143 often focuses on lipids and fatty acids (known as lipidomic profiling) using a suitable MS
144 metabolomics system. For example, lipids with very long-chain fatty acids in
145 *Botryococcus braunii* held together by a lipid biofilm matrix were identified using non-
146 aqueous reversed-phase LC-MS/MS (Řezanka et al., 2018), a complex mixture of natural
147 triacylglycerol (TAG) of the snow alga *Chloromonas pichincha* (Řezanka et al., 2014)
148 was identified using ESI-MS and silver LC/atmospheric pressure chemical ionization-
149 MS, and fatty acids in the microalgal strain *Micractinium* sp. IC-76 were identified using
150 a GC-MS-based approach (Piligaev et al., 2018).

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

156

157 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** $\text{NaH}^{13}\text{CO}_3$ 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

182 the metabolic pathway (Shastri and Morgan, 2007). In addition, diurnal light-dark cycle
183 renders short-lived metabolic steady states in photosynthesis. Isotopically nonstationary
184 ¹³C MFA (INST-MFA) is another modeling approach that estimates autotrophic fluxes in
185 a metabolic steady state by measuring transient isotope incorporation prior to reaching an
186 isotopic steady state following ¹³C-labeling (Adebiyi et al., 2015; Cheah and Young,
187 2018; Jazmin et al. 2014; Young et al. 2011). Development of the carbon mapping model
188 imSyn617 and associated algorithmic updates effectively reduced the computation time
189 (Gopalakrishnan et al., 2018).

190

191 3. Metabolomics approaches for biofuel production in microalgae

192 3.1. General applications of metabolomics in microalgae

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

205 Metabolomics has been utilized in microalgae biofuel production for two

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

216

217 3.2. Elucidation of metabolic changes in microalgae under nutrient depletion

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

230 starvation has been suggested to trigger nitrogen assimilation from proteins and the
231 photosynthetic machinery in other *Chlorella* strains (Vello et al., 2018), and an increase
232 in GABA under nitrogen depletion was observed in *Scenedesmus quadricauda*
233 (Sulochana and Arumugam, 2020). In *Parachlorella kessleri*, metabolites of the TCA
234 cycle, such as citrate and 2-oxoglutarate (2OG), were suggested to play important roles
235 in diverting the flux toward acetyl-CoA, leading to the accumulation of neutral lipids
236 under nutrient-depleted conditions (Shaikh et al., 2019). An increase in citrate under low
237 nitrogen conditions was reported in *Nannochloropsis oceanica* (Xiao et al., 2013). In *N.*
238 *salina*, nitrogen availability was shown to affect glycerol consumption during
239 mixotrophic cultivation and utilization for lipid synthesis (Poddar et al., 2020). These
240 results led to a hypothesis common in many microalgae species: the degradation products
241 of BCAAs can be converted to acetyl-CoA, a precursor for fatty acid synthesis, via the
242 TCA cycle and contribute to lipid synthesis under nitrogen-depleted conditions (Allen et
243 al., 2011; Ge et al., 2014). A *C. reinhardtii* mutant defective in BCAA catabolism
244 accumulated less TAG than the parental strain during nitrogen starvation (Liang et al.,
245 2019). In *Dunaliella tertiolecta*, a TAG-rich mutant showed upregulation of genes
246 involved in BCAA degradation and an increased pool size of acetyl-CoA (Yao et al., 2017).
247 In addition, an imbalance in the C/N ratio due to lipid synthesis without corresponding
248 nitrogen intake can be recovered by upregulating the protein degradation process (Poddar
249 et al., 2020). In the diatom *Phaeodactylum tricornutum*, in which betaine lipids are
250 thought to be the major source of TAG synthesis, the levels of amino acids, including
251 valine, leucine, and isoleucine, and TCA cycle intermediates, including citrate, were
252 significantly decreased during nitrogen starvation (Popko et al., 2016), whereas increased
253 TAG accumulation by overexpression of the BCAA catabolic pathway enzyme 3-

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

257

258 3.3. Elucidation of metabolic mechanisms in mutant microalgae

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

302 *4.1. Advantages of cyanobacteria for chemical production*

303 Compared with terrestrial plants, cyanobacteria have important advantages,
304 **such as** faster growth under simple culture conditions, high solar energy capture efficiency,
305 and utilization of a bicarbonate intermediate as a carbon source for conversion to desired
306 products (Carroll et al., 2018; Srivastava and Shukla, 2021). Unicellular cyanobacteria,
307 including *Synechocystis* sp. PCC 6803, *S. elongatus* PCC 7942, and *Synechococcus* sp.
308 PCC 7002, are genetically tractable **and are easy to genetically manipulate because of**
309 recent advances in synthetic biology tools (Behle et al., 2020; Huang et al., 2013; Markley
310 et al., 2016; Sengupta et al. 2019; Sengupta et al., 2020; Yao et al., 2016). Accordingly,
311 cyanobacteria are attractive platforms for the successful production of various chemicals,
312 including alcohols (e.g., ethanol, 1-butanol, isopropanol, 2-methyl-1-butanol, 1,2-
313 propanediol, 1,3-propanediol, and isobutanol), organic acids (e.g., 3-hydroxypropionate,
314 3-hydroxybutyrate, D-lactate, succinate, malate, and fumarate), free fatty acids, isoprene,
315 ethylene, and carotenoids. In cyanobacteria, the main goals of metabolomics-guided
316 strain engineering approaches are (1) to redirect carbon flow from CO₂ toward production
317 of the desired chemical, and (2) to improve the carbon fixation rate. Examples of
318 metabolome-based strain **development** in cyanobacteria are summarized in **Table 4**.

319

320 *4.2. Rational metabolic engineering through identifying the bottleneck reaction*

321 *4.2.1. Alcohol and aldehyde production*

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

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

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

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

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

358

359 4.2.2. Organic acid production

360 Cyanobacteria have also been studied as promising producers of succinate and
361 D-lactate to develop environmentally friendly, biodegradable plastics (Katayama et al.,
362 2018). *Synechocystis* sp. PCC 6803 converts intracellular glycogen into organic acids,
363 including succinate, D-lactate, fumarate, and malate, by autofermentation under dark
364 anoxic conditions (Stal and Moezelaar, 1997). Strain PCC 6803 possesses a D-lactate
365 dehydrogenase (D-LDH) gene and can secrete a large amount of D-lactate through
366 autofermentation (Ito et al., 2017). However, increasing D-lactate production requires
367 supplementation with the D-LDH substrate pyruvate. Overexpression of ME, a malate
368 decarboxylase that converts malate to pyruvate, increased D-lactate production in PCC
369 6803 by more than 2-fold (Hidese et al., 2020). Dynamic metabolic profiling revealed
370 that overexpression of ME enhanced the metabolic turnover of malate and metabolites
371 involved in glycolysis. In contrast, an ME-deficient strain accumulated malate and
372 glycolysis metabolites, such as G6P, F6P, fructose-1,6-bisphosphate, 3-phosphoglycerate
373 (3PGA), and PEP. In an ME-overexpressing strain, metabolic turnover in glycolysis was

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

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

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

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

402

403 4.2.3. Carotenoid production

404 Carbon partitioning accompanying CO₂ incorporation is among the most
405 important factors for increasing target chemical production. Cyanobacteria utilize the 2-
406 C-methyl-D-erythritol 4-phosphate (MEP) pathway to synthesize carotenoids. Dimethyl
407 dimethylallyl diphosphate (DMAPP), the final product of the MEP pathway, serves as a
408 precursor for isoprene synthesis. Metabolomics of the isoprene-producing recombinant
409 strain *Synechocystis* sp. PCC 6803, which harbors plant-derived isoprene synthase,
410 revealed that the limitation in isoprene production is an insufficient DMAPP level (Pade
411 et al., 2016). Therefore, the mevalonic acid (MVA) pathway, another pathway for
412 DMAPP synthesis, was introduced into the isoprene-producing cells as a bypass of the
413 MEP pathway, resulting in increased isoprene levels. Isoprene production was also
414 improved by overexpression of the enzymes Ipi, Dxs, and IspD, whose catalytic reactions
415 were identified as bottleneck steps within the MEP pathway (Englund et al., 2018).

416 The carotenoid content of cyanobacteria is significantly lower than that of
417 eukaryotic microalgae and vascular plants (Pagels et al., 2021). Many approaches have
418 been reported for producing astaxanthin, a valuable carotenoid with superior
419 antioxidative properties. In the microalga *H. pluvialis*, astaxanthin accumulates under
420 nutrient-deficient and high-light conditions, leading to large-scale market application
421 (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 CO₂ 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 CO₂ 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 $\text{NaH}^{14}\text{CO}_3$ 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

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

475 **Improving** photosynthesis is another major challenge for constructing
476 cyanobacterial cell factories for large-scale applications (Hasunuma et al., 2014).
477 Dynamic metabolomics clearly revealed that overexpression of *flv3*, which encodes an
478 NAD(P)H:oxygen oxidoreductase involved in alternative electron flow associated with
479 NADPH-coupled O₂ photoreduction in photosystem I, improved cell growth in PCC 6803
480 with glycogen accumulation **by increasing** O₂ evolution, intracellular ATP levels, and
481 CBB cycle **turnover**.

482

483 **5. Advantages of photosynthetic metabolomics in cell factory development**

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

494 photosynthetic metabolomics can reveal the comprehensive metabolite pool size and its
495 dynamic changes in microalgae and cyanobacteria, critically contributing to the
496 **understanding** of the CO₂ fixation-derived carbon distribution and determination of a rate-
497 limiting step in the biosynthetic pathway. **These data are extremely useful for identifying**
498 **a target reaction that should be intensified or weakened to improve the production of**
499 **targeted compounds. Engineered strains constructed using this approach can be further**
500 **subjected to metabolomics. Therefore,** metabolomics is a powerful tool in synthetic
501 biology based on the DBTL platform (Carbonell et al., 2018; Vavricka et al., 2020).
502 **Unfortunately, metabolomics-based synthetic biology has not been realized in microalgae**
503 **biofuel production because of the limited availability of nuclear transformation by**
504 **homologous recombination in the major microalgae (Nelson and Lefebvre, 1995;**
505 **Sodeinde and Kindle, 1993). This limitation will be overcome in part by using recently**
506 **developed genome editing tools in several microalgae (Ng et al., 2020). In addition,**
507 **genetic engineering is applicable in microalgae, such as *Cyanidioschyzon merolae***
508 **(Pancha et al., 2021); therefore, these species are suited to metabolomics-based strain**
509 **improvement for biofuel production.**

510

511 **6. Conclusions**

512 The recent applications of metabolomics to photosynthetic production of
513 biofuels and bio-based chemicals in microalgae and cyanobacteria **were** reviewed. In
514 cyanobacteria, metabolomics has contributed to the rational design of metabolic pathways
515 for carbon flow redirection from assimilated CO₂ toward desired products, such as
516 alcohols, organic acids, and carotenoids. **In contrast, metabolomics-based synthetic**
517 **biology has not been achieved in microalgae biofuel production but will be realized by**

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

521

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533

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982 **Figure captions**

983

984 **Figure 1. Metabolic pathways and derived compounds.**

985 13BPG, 1,3-bisphosphoglycerate; 13PDO, 1,3-propanediol; 23BD, 2,3-butanediol;
986 2PGA, 2-phosphoglycerate; 2OG, 2-oxoglutarate; 3HB, 3-hydroxybutyrate; 3HB-CoA,
987 3-hydroxybutyryl-CoA; 3HP, 3-hydroxypropionate; **3HPA, 3-hydroxypropionaldehyde;**
988 3PGA, 3-phosphoglycerate; 8OH-CPP, 8-hydroxy-copalylidiphosphate; A-Ace,
989 acetoacetate; **A-Ac-ACP, acetoacetyl-ACP;** A-Ac-CoA, acetoacetyl-CoA; A-Ald,
990 acetaldehyde; **a-ACP, acyl-ACP; ACP, acyl carrier protein; ADP, Adenosinediphosphate;**
991 ADPGlc, ADP-glucose; Ac-CoA, acetyl-CoA; Ac-P, acetyl-phosphate; A-Lac,
992 acetolactate; Ald, aldehyde; But-Ald, butyrylaldehyde; But-CoA, butyryl-CoA; CBB
993 cycle, Calvin-Benson-Bassham cycle; CDP-ME, 4-diphosphocytidyl-2-C-
994 methylerythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate;
995 cisAco, *cis*-aconitate; Cit, citrate; CoA, coenzyme A; Cr-CoA, crotonyl-CoA; DAG,
996 diacylglycerol; DHAP, dihydroxyacetone phosphate; DIV, 2,3-dihydroxyisovalerate;
997 DMAPP, dimethylallyl diphosphate; DPMVA, diphosphomevalonate; DXP, 1-deoxy-D-
998 xylulose 5-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP,
999 fructose 1,6-bisphosphate; FPP, farnesyl diphosphate; Fum, fumarate; G1P, glucose 1-
1000 phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GGPP,
1001 geranylgeranyl diphosphate; GPP, geranyl diphosphate; G3P, glycerol 3-phosphate;
1002 HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMG-CoA, 3-hydroxy-3-
1003 methylglutaryl-CoA; IBA, isobutyraldehyde; Isocit, isocitrate; KIV, 2-ketoisovalerate;
1004 **keto-a-ACP, beta-keto-acyl-ACP;** LPA, lysophosphatidic acid; M1P, mannitol 1-
1005 phosphate; MEP, 2-C-methylerythritol 4-phosphate; MEcPP, 2-C-methyl-D-erythritol

1006 2,4-cyclodiphosphate; MVA, mevalonate; Mal, malate; **Malo-ACP, malonyl-ACP**; Malo-
1007 Ald, malonyl-aldehyde; Malo-CoA, malonyl-CoA; OAA, oxaloacetate; OAS,
1008 oxalosuccinate; OH-a-**ACP**, beta-hydroxy-acyl-ACP; PA, phosphatidic acid; PEP,
1009 phosphoenolpyruvate; PHB, polyhydroxybutyrate; PMVA, phosphomevalonate; PSPP,
1010 presqualene diphosphate; Pyr, pyruvate; R5P, ribose 5-phosphate; RuBP, ribulose 1,5-
1011 bisphosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SBP,
1012 sedoheptulose 1,7-bisphosphate; Suc, **succinate**; Suc-CoA, succinyl-CoA; SSA, succinic
1013 semialdehyde; TAG, triacylglycerol; TCA cycle, tricarboxylic acid cycle; *trans*-a-ACP,
1014 *trans*-delta²-enoyl-acyl-**ACP**; Xu5P, **xylulose 5-phosphate**.

1015

1016 **Figure 2. Metabolomics-based approach for developing cell factories.**

1017 Metabolomics-based approaches for **the rational and rapid development of** cell factories
1018 in microalgae and cyanobacteria compared with conventional approaches.

1019

1020 **Table 1. Comparison of major advantages and drawbacks of MS- and NMR-based**
 1021 **metabolomics.**

Analytical technology	Advantages	Drawbacks
Mass spectrometry (MS)	<ul style="list-style-type: none"> • Large number of detectable metabolites • High quantifiable • High reproducibility, sensitivity, and selectivity • High reproducibility 	<ul style="list-style-type: none"> • Need chromatography to increase selectivity; • Sample derivatization for GC-MS • Destructive nature
Nuclear magnetic resonance (NMR)	<ul style="list-style-type: none"> • Inherently nondestructive nature • Identification of novel compounds • High quantifiable, minimal sample preparation, and relatively fast measurement • High reproducibility 	<ul style="list-style-type: none"> • Low sensitivity and selectivity

1022

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

Microalgae	Methodology	Number of analyzed metabolites	Findings	Reference
<i>Chlamydomonas reinhardtii</i>	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)
<i>Chlorella</i>	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)
<i>Scenedesmus quadricauda</i>	LC-MS	12	GABA increases under nitrogen depletion.	Sulochana and Arumugam (2020)
<i>Parachlorella kessleri</i>	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)
<i>Nannochloropsis oceanica</i>	¹ H NMR	29	Citrate increases under low nitrogen conditions.	Xiao et al. (2013)
<i>Phaeodactylum tricornutum</i>	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
<i>Chlamydomonas reinhardtii</i> <i>sta6</i> (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)
<i>Chlorella sorokiniana</i> 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)
<i>Cryptocodinium cohnii</i> 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)
<i>Chlamydomonas reinhardtii</i> <i>bgall</i> (high starch and low lipid)	LC-MS	14	Lower levels of G6P, F6P, and acetyl-CoA upon nitrogen starvation.	Hung et al. (2016)
<i>Chlamydomonas</i> sp. KOR1 (phytglycogen 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)

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1027 **Table 4. Metabolome-based strain construction in cyanobacteria**

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

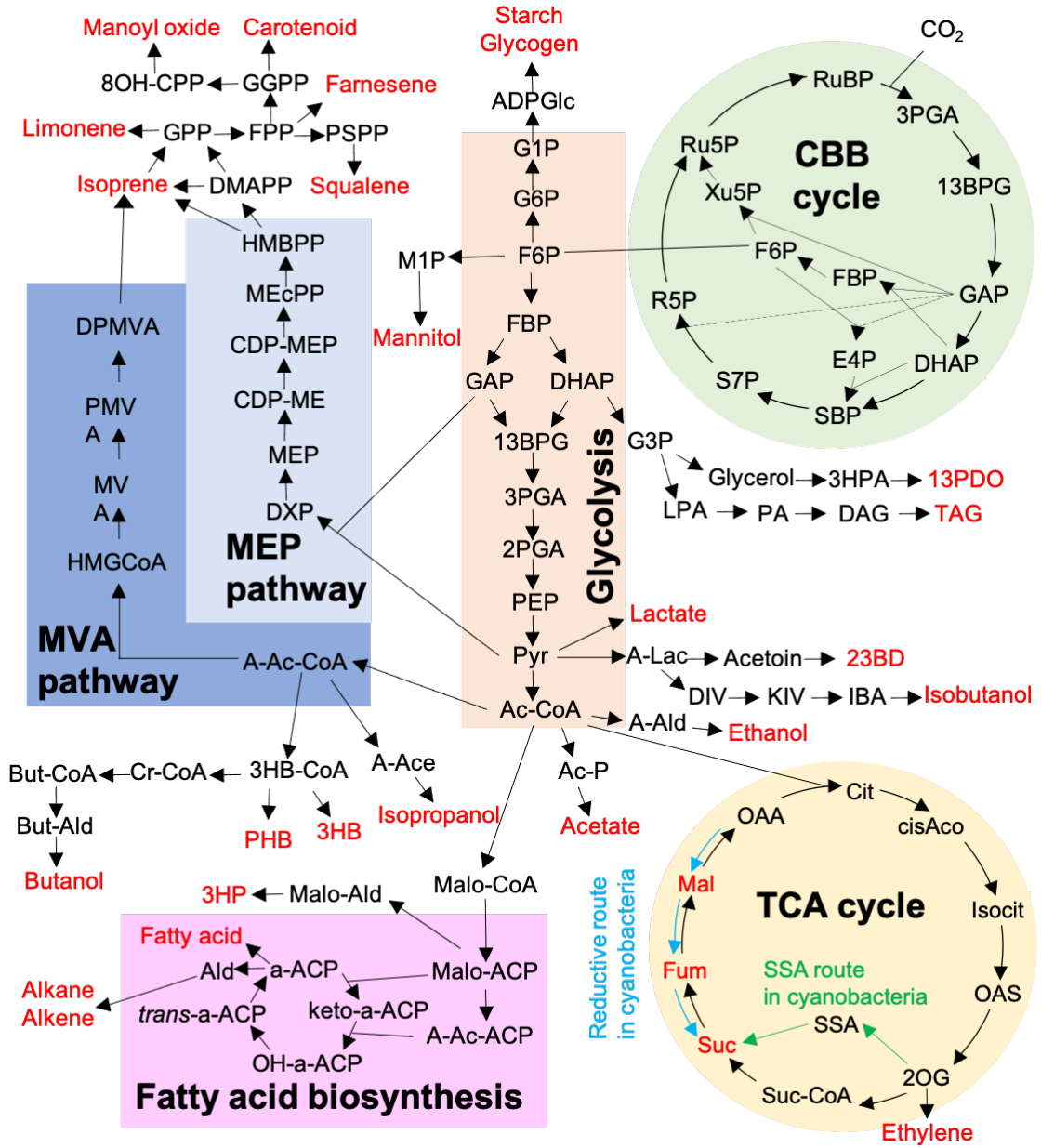
1028 PHB, polyhydroxybutyrate; IBA, isobutyraldehyde; 23BD, 2,3-butanediol; 13PDO, 1,3-

1029 propandiol; PCC 6803, *Synechocystis* sp. PCC 6803; PCC 7942, *Synechococcus*

1030 *elongatus* PCC 7942; OE, overexpression; KO, knockout

1031

1032 **Figure 1.**



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