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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 (CO₂); thus, carbon recycling is developing as a technology for reducing CO₂ 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 CO₂ must be developed to meet industrial demands while minimizing environmental impacts to

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

Eukaryotic microalgae and prokaryotic cyanobacteria drive the Calvin-Benson-Bassham (CBB) cycle by utilizing sunlight as an energy source to assimilate CO₂ (Fig. 1). Because of their more effective utilization of solar energy compared to terrestrial plants in generating biomass photosynthetically, microalgae and cyanobacteria are promising and versatile producers of biofuels (biodiesel, lipids, and ethanol), commodity chemicals, and value-added intrinsic compounds (e.g., phycocyanin, carotenoids, and polyunsaturated fatty acids) (Ho et al., 2014b; Knoot et al., 2018; Oliver et al., 2016). Biofuel and bio-based chemical production through molecular breeding technologies and development of mass cultivation methods has been attempted using various microalgae, including *Chlamydomonas* (Terashima et al., 2015) and *Chlorella* (Cao et al., 2017), and using cyanobacteria, including *Synechocystis* (Hidese et al., 2020) and *Synechococcus* (Hasunuma et al., 2019). However, the productivity of biofuels and commodity chemicals produced by microalgae and cyanobacteria are currently insufficient for cost-effective commercialization in most cases, and fail to meet social and industrial demands.

Multi-omics approaches, which combine omics analyses, such as genomics, transcriptomics, proteomics, lipidomics, and metabolomics, have been used to reveal the basic molecular mechanisms of cellular physiology, contributing to the identification of novel pathways and/or rate-limiting reactions in the biosynthetic pathway of target products (i.e., bottleneck steps) (Kim et al., 2012; Vavricka et al., 2020). Among these, metabolomics can comprehensively detect and quantify metabolites and therefore is highly useful for understanding comprehensive metabolic phenomena and their dynamic changes. Metabolomics can also determine the bottleneck step, which critically 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 CO₂ 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-NH₄OH 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 ¹H NMR spectra, including amino acids, sugars, organic acids, phosphagens, osmolytes, and nucleotides (Arora et al., 2018).

2.3. Flux analysis with stable isotopes

Static **metabolomics** can provide a comprehensive snapshot of metabolites at a single time point. However, time-course analysis is required to investigate the dynamic aspects of the metabolites. Stable isotope tracer experiments are useful for dynamic metabolic analysis in both MS- and NMR-based detection (Bacher et al., 2016). MS-based tracer detection enables the precise quantification of metabolic flux in combination with quantitative time-course metabolomics, referred to as dynamic metabolomics, in photosynthetic autotrophs, such as plants, microalgae, and cyanobacteria (Hasunuma et al., 2010; Hasunuma et al., 2013a). Feeding of $\text{NaH}^{13}\text{CO}_3$ enables the labeling of newly synthesized metabolites from CO_2 with ^{13}C , which contributed to the identification of a bottleneck step in all glycolysis and tricarboxylic acid (TCA) cycle metabolites as well as the component amino acids in the microalga *Chlamydomonas* sp. (Ho et al., 2017; Kato et al., 2019), and in the cyanobacteria *A. platensis* (Hasunuma et al., 2013a) and *Synechocystis* sp. (Hasunuma et al., 2016; Hidese et al., 2020). Other stable isotopes, including ^{15}N and ^{18}O , can also be used to trace metabolic flux (Kera et al., 2018; Zhang et al., 2018). In *Synechocystis* sp., ^{13}C and ^{15}N labeling experiments identified arginine dihydrolase as a key regulator of nitrogen metabolism (Zhang et al., 2018). By combining MS-based metabolic profiling with genomic analysis, stable isotope labeling, and non-target metabolomics revealed a novel biosynthetic pathway responsible for producing secondary metabolites (May et al., 2020).

Metabolic flux analysis (MFA) is a mathematical modeling approach that can determine the metabolic flux distribution based on integrated analysis of extracellular flux and intracellular isotope labeling in metabolic and isotopic steady states. In autotrophic metabolism, ^{13}C labeling in an isotopic steady state shows a uniform pattern 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 *Cryptothecodinium 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 al., 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 *bgall* mutant in *C. reinhardtii*

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

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

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 CO₂ 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 Moezelaar, 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 CO₂ 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

microbial contamination under growth-unsuitable conditions remains as a concern in astaxanthin production using *H. pluvialis*. A genetically engineered strain of *Synechococcus* sp. PCC 7002, a fast-growing marine cyanobacterium, harboring the β -carotene hydroxylase CrtZ and β -carotene ketolase CrtW derived from the marine bacterium *Brevundimonas* sp. SD212 showed an astaxanthin content of 3 mg/g dry cell weight and astaxanthin productivity of 3.35 mg/L/day (Hasunuma et al., 2019), which were comparable to the values obtained using *H. pluvialis*. Metabolic profiling revealed that the astaxanthin-producing cells highly accumulated 1-deoxy-D-xylulose 5-phosphate, which is the gateway metabolite in the MEP pathway. Dynamic metabolic profiling showed that the metabolic turnover of 3PGA, sedoheptulose 7-phosphate, F6P, and PEP was accelerated in astaxanthin-producing cells. Thus, activation of photosynthetic central metabolism in astaxanthin-producing cells appears to compensate for the low β -carotene availability as a light-harvesting pigment, likely via its conversion into astaxanthin (Hasunuma et al., 2019). Another study demonstrated enhanced photosynthesis and central metabolism, likely because of high astaxanthin flux, in the engineered *Synechocystis* sp. PCC 6803 strain (Diao et al., 2020). These findings may also be beneficial for producing other DMAPP-based chemicals, including zeaxanthin, ethioneone, and limonene (Pagels et al., 2021; Lin et al., 2021).

4.3. Re-routing assimilated CO₂ and improvement of the carbon fixation rate

Glycogen is a major carbon storage molecule in cyanobacteria and promising target for the redistribution of desired chemicals (Xu et al., 2013). INST-MFA of a glycogen synthase-deficient mutant (*glgA-I glgA-II*) of *Synechococcus* sp. PCC 7002 revealed that carbon resources derived from CO₂ fixation were partially diverted into

alternate storage molecules, such as glucosylglycerol and sucrose, by flexible partitioning of G1P between ADP-glucose and UDP-glucose without a major impact on the central metabolic pathway (Hendry et al., 2017). Disruption of *glgC*, which encodes G1P adenylyltransferase, causes impairment of glycogen synthesis, growth defects, and metabolite overflow, leading to excretion of several organic acids, including pyruvate, 2OG, and succinate (Carrieri et al., 2012; Cano et al., 2018). The growth defects in *glgC*-deficient mutants can be partially restored by isobutanol production (Li et al., 2014). According to $\text{NaH}^{14}\text{CO}_3$ incorporation analysis, 52% of the total fixed carbon was redirected into isobutanol biosynthesis in the *glgC*-deficient mutant, representing a rate 2.5-fold higher than that of the strain carrying *glgC*, indicating that isobutanol can be used as an alternative carbon sink to glycogen.

Metabolic engineering of the CBB cycle has also been attempted to improve the efficiency of carbon fixation. Fructose-1,6/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) and transketolase (TK) are key enzymes in the CBB cycle and represent a potential bottleneck step in carbon fixation. Fluxomic analysis by INST-MFA in combination with proteomics indicated a decrease in oxidative pentose phosphate pathway activity when either FBP/SBPase or TK was overexpressed in PCC 6803, resulting in increased carbon fixation efficiency, whereas TK overexpression enhanced the susceptibility of the strain to oxidative stress (Yu King Hing et al., 2019). A more recent study showed that overexpression of two CBB cycle enzymes among FBP/SBPase, TK, and aldolase contributed to enhanced ethanol production when compared to overexpression of a single CBB cycle enzyme (Roussou et al., 2021).

Metabolomic analyses of the glucose-tolerant strain *Synechocystis* sp. PCC 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 O₂ photoreduction in photosystem I, improved cell growth in PCC 6803 with glycogen accumulation by increasing O₂ 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 CO₂ 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 CO₂ 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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981

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-copalyl diphosphate; 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, butyraldehyde; 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-**bis**phosphate; 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

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

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

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 (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)

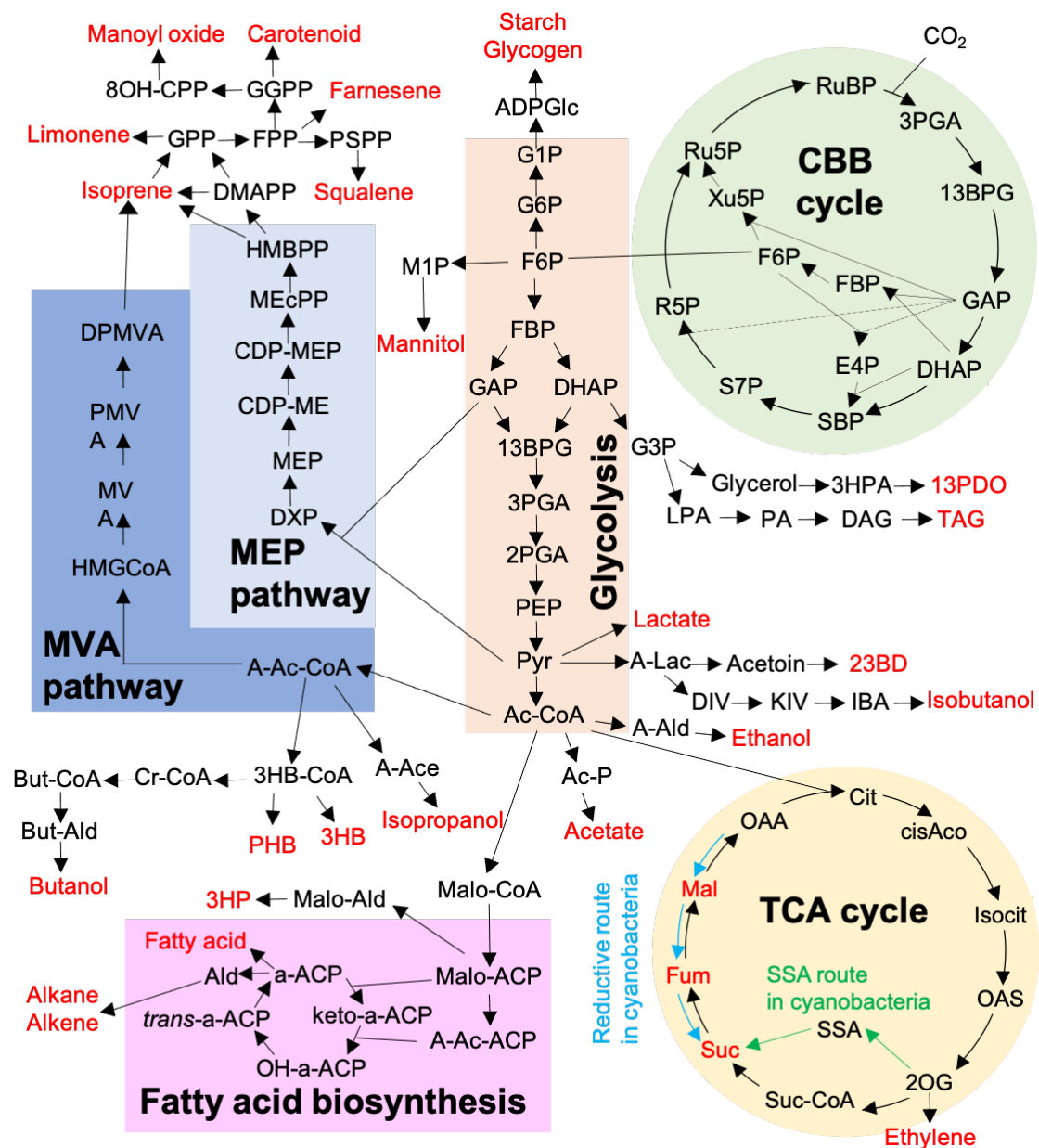
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Table 4. Metabolome-based strain construction in cyanobacteria

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> OE: <i>prk</i> , <i>rbcLXS</i> ,	>1.6	Cheah et al. (2020)
23BD	PCC 7942	<i>galP</i> , <i>zwf</i> , and <i>gnd</i> KO: <i>cp12</i>	5.0	Kanno et al. (2017)
D-Lactate	PCC 6803	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)

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

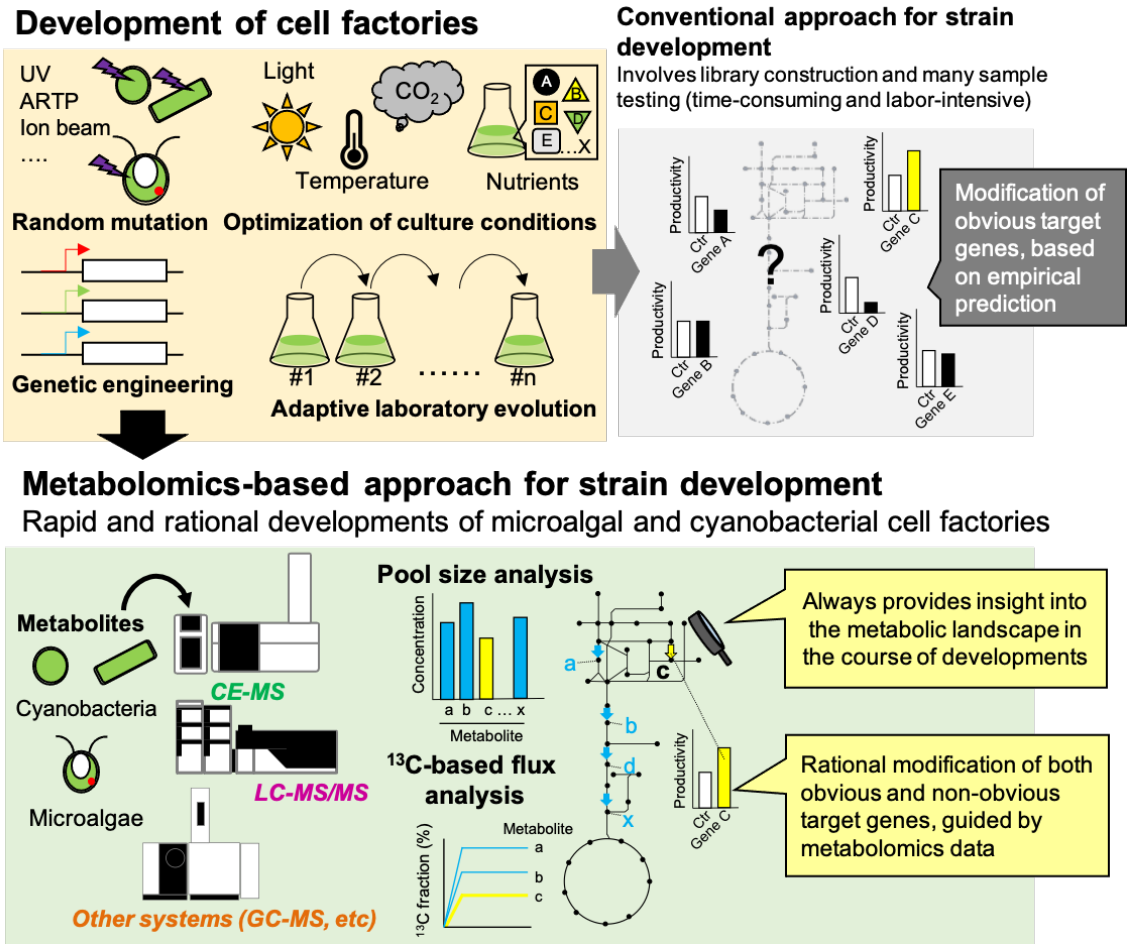
1032 **Figure 1.**



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1035 **Figure 2.**



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