Synthetic Biology Approaches To Enhance Microalgal Productivity

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The major bottleneck in commercializing biofuels and other commodities produced by microalgae is the high cost associated with phototrophic cultivation. Improving microalgal productivities could be a solution to this problem. Synthetic biology methods have recently been used to engineer the downstream production pathways in several microalgal strains. However, engineering upstream photosynthetic and carbon fixation metabolism to enhance growth, productivity, and yield has barely been explored in microalgae. We describe strategies to improve the generation of reducing power from light, as well as to improve the assimilation of CO₂ by either the native Calvin cycle or synthetic alternatives. Overall, we are optimistic that recent technological advances will prompt long-awaited breakthroughs in microalgal research.

Towards Improving Upstream Photosynthetic and CO₂ Fixation Metabolism

Research on microalgae as a platform for biofuel production dates back to the 1980s [1]. Despite decades of research, the cost of microalgal biofuel is still considerably higher than the biofuel produced from traditional agricultural crops. The expensive cultivation of microalgae in large-scale photobioreactors has been a bottleneck for economically feasible production of low-value commodities. Hence, microalgae are currently only used to produce high-value compounds such as pigments and fatty acids, focusing on niche markets with limited competition [2,3]. However, the massive deforestation and other environmental consequences associated with the production of biofuels and biomass from traditional crops urges alternative sustainable production methods. Hence, research to realize the full potential of microalgae for sustainable production of biomass and products remains an urgent topic.

Current genetic engineering strategies to improve microalgal productivity of lipids, which are the precursors for biofuel production, have often resulted in reduced biomass production and/or growth rate. At least to some extent this is because the supply of carbon or energy is insufficient to meet the increased demands of engineered strains (Figure 1) [4–6]. Thus, to overcome carbon or energy shortage, further major improvements in microalgal productivities will require extensive rewiring of the upstream metabolic pathways such as carbon fixation, as well as the generation of ATP and reducing power by photosynthesis. Some of these strategies have been recently proposed and partly implemented in plants (reviewed in [7–9]). These strategies require varying levels of genetic engineering interventions ranging from up- or downregulation or knockout of one or a few native genes to the introduction of complete, complex non-native systems such as synthetic photosystems or synthetic carbon fixation pathways. Recent improvements in the synthetic biology toolbox for microalgae, for example CRISPR/Cas (see Glossary) technology (Box 1) [10], now allow similar explorations in microalgae, potentially leading to large improvements in their productivity.
In this review we elaborate on synthetic biology-based approaches for enhancing the light-driven generation of reducing power and CO₂ fixation to improve overall microalgal productivity. Moreover, we discuss potential ‘mixotrophy’ strategies to utilize inorganic and organic molecules, as a complement or alternative to light and CO₂, as energy and carbon sources to enhance microalgal productivities.

**Improving the Generation of Reducing Power from Light**

Limiting the losses in the conversion of solar energy during photosynthesis could provide additional energy for host metabolism. Some of the solar energy absorbed by the photosystems is lost via a process termed non-photochemical quenching (NPQ), which is influenced by the lag in the adaptation of photosystem antenna sizes to the light conditions (Box 2). For these reasons, it has been proposed to reduce the antenna size by genetic engineering as a strategy to reduce the NPQ and improve photosynthetic efficiency (PE). Truncation of light-harvesting antenna and disruption of the chloroplast signal recognition particle (CpSRP) protein have been the primary targets for reducing antenna size. CpSRP is responsible for translocating light-harvesting proteins to the thylakoid membrane of the chloroplast. Truncation of antennas led to improved PE and biomass productivity in several microalgal species (Table S2 in the supplemental information online). Contrasting observations were also made in some cases where reduction in antenna size did not make a difference or adversely affected the PE [11,12]. A more refined strategy to optimize light capture and PE would be to fine-tune the content of chlorophyll pigments (Chl). However, determining the optimal values of pigment content, especially for cultures grown under fluctuating light conditions, is impractical and could detrimentally affect engineered strains with adapted pigment and antennae composition in some conditions [13].

An alternative strategy to improve PE in microalgae would be to introduce heterologous photosystems that have a potentially higher light-harvesting efficiency or a wider absorption spectrum. Plant and microalgal photosystems only absorb light in the visible range from 400 nm to 700 nm, whereas ~50% of the available solar radiation falls outside that range [14]. Some of the non-absorbed light is emitted in the more energetic part of the spectrum (UV, <400 nm), whereas most of the non-captured light is in the IR spectrum (>700 nm). Although photons >700 nm are less energetic, and are insufficiently energetic to perform water-splitting, photons up to 1100 nm are still sufficiently energetic to drive the generation of a typical proton motive force of 200 mV and generate ATP [15]. Several bacterial photosystems found in nature have higher light-harvesting efficiencies and broader absorption spectra. Components of these bacterial photosystems could potentially be harnessed to improve PE. Complete rewiring of eukaryotic oxygenic photosynthesis to improve its efficiency has been proposed before [14,16]; however, rebuilding the highly complex multi-subunit photosystems may be highly ambitious. Nevertheless, replacement of some parts of native photosystems by heterologous counterparts has already been demonstrated in microalgae.

A study along these lines replaced the D1 subunit in photosystem II (PSII) of *Chlamydomonas reinhardtii* by its counterpart from the cyanobacterium *Synechococcus elongatus*. The mutants showed that the photochemistry of the PSII system could be improved for some variants under particular conditions (low or high light) [17]. Other studies showed that heterologous expression of PSII subunits D1 and PsbH from plants in the cyanobacterium *Synechocystis* did not improve the PE of the engineered strains [18–22]. To date, swapping of PSII subunits has not improved PE in cyanobacteria and microalgae under photobioreactor conditions. One reason could be that replacement of single proteins within the native photosystem complexes is not sufficient because efficient photosystem operation requires specific interactions between multiple proteins in the photosystem. In another study, a larger set of six core proteins of PSII was replaced by the
complete set of PSII core proteins from the microalgae Scenedesmus obliquus or Volvox carteri, or from the same strain of C. reinhardtii as a control. Complementation of the deleted PSII proteins successfully reconstituted 85%, 55%, and 53% of the photosynthetic activity upon expression of the core proteins from C. reinhardtii, S. obliquus, and V. carteri, respectively [23]. Although functional heterologous replacement of PSII was demonstrated, improved PE in microalgae has not yet been realized by PSII subunit engineering, and this will likely require more extensive rewiring and optimization of photosystems.

Other than introducing heterologous photosystem complexes, introducing the biosynthetic pathway for non-native pigments may increase the absorption range and PE. In plants, heterologous expression of photosynthetic proteins and pigment biosynthesis pathways that were lost during evolution resulted in improved photosynthesis, growth, and stress tolerance [24]. A similar strategy was recently followed for the improvement of PE in the microalga Nannochloropsis salina. The chlorophyllide a oxygenase (CrCAO) gene from C. reinhardtii was introduced to produce chlorophyll b (Chl b), which is not native to N. salina. This strategy resulted in a 26% higher cell number, 31% improved cell dry weight, and an 8% increase in total lipid content of N. salina under medium-light conditions after 12 days [25,26]. The production of Chl b in N. salina indicates the potential of producing non-native pigments in microalgae.

Following this approach, the chlorophyll f synthase (ChlF) recently discovered in some cyanobacteria, that produces the far-red light-absorbing chlorophyll f (Chl f), could be an interesting candidate for expression in microalgae [27]. This can lead to an expansion of the light absorption spectrum into the IR wavelengths. Recent insights have revealed that ChlF, which is highly homologous to the D1 subunit of PSII, probably forms a heterodimer with the

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**Figure 1. Schematic Representation of Algal Carbon Partitioning.** (A) CO2 fixed via photosynthesis is the sole source of carbon in photautotrophs, and downstream metabolism distributes the carbon into biomass production, and the accumulation of products such as polyunsaturated fatty acids (PUFAs) and lipids. (B) Conventional metabolic engineering approaches to enhance the production of metabolites focus on overexpressing the genes for the production pathway of these metabolites, mostly resulting in an improvement in metabolite production but a reduction in microalgal growth, that limits further increases in productivity. (C) To further enhance the productivity and the growth of engineered microalgae, the generation of reducing power and carbon fixation via photosynthesis could be improved. Abbreviations: CBB, Calvin–Benson–Bassham; LHC, light-harvesting complex.
D2 subunit of PSII to synthesize Chl f [28]. Remarkably, it was shown that, by engineering chimeric D1/ChlF proteins in *Synechocystis* sp. PCC 6803, which natively does not harbor Chl f pigments, the engineered strain could synthesize Chl f pigments [28]. The chimera strategy could potentially be interesting to enable Chl f synthesis in microalgae. However, laborious modification and design of these proteins for each target site and frequent off-target events have limited the use of these systems for gene editing [122].

In addition, UV-absorbing pigments found in Antarctic plants could further broaden the light spectrum accessible by microalgae upon heterologous expression, and possibly improve...
biodiversity or product yield [29]. However, the genes involved in the biosynthesis of these pigments remain to be elucidated.

Apart from improving the photosynthetic machinery, potential improvements could be achieved in the machinery that channels the photosynthetically excited electrons via various redox reactions to generate ATP: the electron transport chain (ETC) (Box 2). To improve ETC reactions, the rate-limiting protein complexes in the ETC should be identified for the species of interest, and the entire subunit could be replaced with a faster variant to improve electron flow and reduce NPQ [30]. Characterization of the ETC complexes in fast-growing microalgal and cyanobacterial strains could potentially reveal catalytically superior variants of these complexes. Taken together, inspired by the aforementioned studies, the synthetic biology tools could be exploited for stepwise improvement of the entire light reactions of photosynthesis, which could potentially dramatically enhance the microalgal productivity.

Improving Carbon Fixation
Overcoming the Limitations of Natural Carbon Fixation Systems in Microalgae
Microalgae assimilate CO₂ in the Calvin–Benson–Bassham (CBB) cycle via the carboxylating enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase-oxygenase) that has some shortcomings (Box 2), resulting in a relatively low rate and efficiency of carbon fixation. Multiple genetic engineering strategies have been implemented in various microalgal strains to partly overcome these shortcomings [9]. One strategy is to simply increase the levels of active RuBisCO enzyme to increase the overall carboxylation activity, assuming that the levels of functional protein are limiting growth. This strategy was reported in the microalgal strain *Nannochloropsis oceanica* where overexpression of native RuBisCO activase improved both growth rate (32%) and biomass accumulation (46%) [31]. In the cyanobacterium *Synechocystis* PCC 6803, overexpression of native RuBisCO improved growth rate and biomass production by ~20% [31,32].

Alternatively, native RuBisCO enzymes could be replaced by either superior natural or engineered RuBisCO variants. To select variants, it is important to consider their catalytic activity and their CO₂/O₂ specificity because RuBisCO variants with improved catalytic activity often have reduced CO₂ specificity, and vice versa [33]. This ‘trade-off’ is typically observed among natural RuBisCO variants [34]; in addition, directed engineering of RuBisCO to improve its performance only led to limited overall carboxylation improvements under ambient conditions [35–37]. Therefore, especially when microalgae are cultivated in atmospheric CO₂ conditions, this trade-off needs to be taken into account. However, under elevated CO₂ concentrations that are often applied in photobioreactors, the use of RuBisCO variants with increased catalytic rates (but with lower CO₂/O₂ specificity) may be attractive to increase productivity.

To harness the power of potentially superior RuBisCO variants, attempts were made to express natural or engineered RuBisCO proteins in RuBisCO deletion mutants of bacteria [38,39] and plants [40,41], as well as in the microalgal species *Chlamydomonas reinhardtii* [42]. However, so far these studies led to only limited improvements in growth, probably because of the modest number of variants screened and challenges in heterologous expression for many multimeric (type I) RuBisCO variants that often require activases or chaperone proteins. Pyrenoids – the microalgal subcompartments in which RuBisCO is often located to facilitate higher local CO₂ concentrations – are another complication facing the expression of heterologous RuBisCO in microalgae. It was recently determined that the pyrenoid compartment assembles by specific binding of pyrenoid-associated proteins to RuBisCO [43]. This assembly is likely disrupted when replacing a native RuBisCO by a heterologous candidate. However, the binding regions...
of the RuBisCO variant or of the pyrenoid-associated proteins could be engineered to support pyrenoid formation with a heterologous RuBisCO.

A potentially promising type I RuBisCO variant was discovered in the red alga *Grifithsia monilis* that has an unusually high CO2/O2 specificity factor of 167, from two- to fourfold higher than the values typically reported for C3 plants, green algae, and cyanobacteria [34,44–46]. However, the requirement for post-translational modifications of the *G. monilis* RuBisCO proteins and for the assembly of multiple subunits of this RuBisCO enzyme may explain its poor heterologous expression in tobacco plants [46]. Recent developments indicate that coexpression of chaperones that facilitate the assembly of specific RuBisCO enzymes could enable the heterologous expression of these proteins [47]. Therefore, developing techniques to assemble the red alga RuBisCO in microalgae could be an interesting approach towards enhancing CO2 fixation.

By contrast, the simpler homodimeric type II RuBisCO enzymes may be a better choice for heterologous expression. Metagenomic studies have revealed numerous RuBisCO-like proteins from non-domesticated prokaryotic and eukaryotic organisms [48,49]. Recently, 33 000 putative RuBisCO proteins were identified by this approach, from which 143 type II and type II/III RuBisCOs were selected and expressed in *Escherichia coli* and screened by *in vitro* assays to obtain catalytically superior variants [50]. The most active variant was a type II variant obtained from the bacterium *Gallionella* sp., which exhibited an eightfold improved turnover number compared to typical values reported for type I RuBisCO from green algae and plants [45]. However, the low affinity for CO2 of this bacterial RuBisCO (*Km* of 275 μM, compared to 39 μM in Chlamydomonas and 14 μM in plants) reduces the carboxylation efficiency compared to plants under normal atmospheric conditions [45,50]. Nevertheless, this protein could be a suitable candidate for culturing microalgae in reactors with CO2 supplementation.

In addition to RuBisCO, it is important to identify other rate-limiting steps in the CBB cycle to achieve optimal CO2 fixation. The CBB cycle reactions catalyzed by sedoheptulose bisphosphatase (SBP) and fructose bisphosphate aldolase (FBA), in addition to RuBisCO, are reported to be rate-limiting steps in plants and cyanobacteria [51,52]. Overexpression of other CBB enzymes was reported to enhance photosynthesis and growth rate in the cyanobacteria *Synechocystis*, *Synechococcus*, and *Anabaena* spp. [32,51,53,54]. Respectively overexpression of the cyanobacterial SBP and FBA in the microalgae *Chlorella vulgaris* and *Euglena gracilis* improved their biomass production by 1.2- and twofold, respectively [55,56]. By contrast, overexpression of native fructose 1,6-bisphosphatase in *C. reinhardtii* adversely affected growth rate and biomass production [57]. Obtaining improved variants of these enzymes via directed evolution and metagenomic studies should also be considered, alongside improvements to RuBisCO, as a means to increase the CO2 fixation via CBB cycle.

**Decreasing Carbon Loss via Photorespiration**

As described in Box 3, the oxygenation activity of RuBisCO, in addition to competing with the carbon fixation reaction, generates the toxic compound 2-phosphoglycolate (2-PG), especially under atmospheric CO2 concentrations. Hence, engineering more efficient alternative photorespiration pathways for recycling 2-PG could be an strong synthetic biology approach to tackle the losses of photorespiration in microalgae (Figure 2). Such approaches were already successfully demonstrated in plants, but not yet in microalgae. For example, the more energy-efficient glyceraldehyde photorespiration pathway, that is present in some bacteria [58,59], was introduced into *Arabidopsis thaliana* (arabidopsis) chloroplasts. The introduction of five genes encoding...
Box 3. The Calvin–Benson–Bassham Cycle and Other Natural CO₂ Fixation Pathways

CO₂ fixation in microalgae is primarily driven by RuBisCO as part of the CBB CO₂ fixation cycle [140]. There are four types of RuBisCO proteins, among which type I is the most abundantly found and harbors eight large and eight small subunits (L₈S₈). Type II RuBisCO found in some bacteria and dinoflagellates is structurally simpler because it comprises a dimer of the large subunit (L₂) and thus could be a suitable candidate for heterologous expression. The type III RuBisCO generally found in archaea comprises a dimer of the large subunit, as seen in type II, or multiple dimers attached to form a multi-subunit complex, (L₈)₂ [141]. RuBisCO enzymes are known to have relatively low catalytic rates (Kₘ = <10 s⁻¹) relative to other CO₂ fixation enzymes [142]. Furthermore, RuBisCO displays a ‘wasteful’ side-activity with oxygen in the presence of relatively high O₂ concentrations versus CO₂ (e.g., in ambient air that contains 20% O₂ but only 0.04% CO₂). This oxygenase reaction leads to the formation of 2-phosphoglycolate, which must be detoxified or recycled into the Calvin cycle via photorespiration pathways. In both plants and microalgae, 2-phosphoglycolate is recycled into the Calvin cycle via the C₂ cycle (see Figure 2 in main text) [143]. However, this C₂ cycle requires ATP input and releases some of the fixed CO₂ as well as fixed NH₃. Losses related to photorespiration have been reported to reduce photosynthetic efficiency in plants by up to 50% [144]. A study on microalgal cultivation in a photobioreactor, even in the presence of elevated CO₂, reported that photorespiration could lead to a 66% loss of fixed carbon, particularly because dissolved oxygen concentrations in the bioreactor can become very high as a result of photosynthesis [145].

In addition to the CBB cycle that harbors RuBisCO, six alternative natural CO₂ fixation pathways do not utilize RuBisCO for carboxylation: (i) the reductive tricarboxylic acid cycle, (ii) the Wood–Ljungdahl pathway, (iii) the 3-hydroxypropionate bicycle, (iv) the 3-hydroxypropionate-4-hydroxybutyrate cycle, (v) the dicarboxylate-4-hydroxybutyrate cycle, and (vi) the recently discovered reductive glycine pathway [69,70,72,74–76,146–148]. Among these six pathways, the CBB cycle is one of the least efficient CO₂ fixation route in terms of ATP consumption, because seven molecules of ATP are consumed to generate one molecule of pyruvate, in addition to the limitations of the carboxylating RuBisCO enzymes (see Table 1 in main text) [149]. Some other natural pathways consume much less ATP, and the carboxylating enzymes in these pathways are much more efficient than RuBisCO; however, several of these pathways only function under anaerobic conditions and/or require elevated CO₂ concentrations.

The alternatives discussed above for natural photorespiration pathways still release CO₂. Following a more ground-breaking approach, different synthetic pathways were proposed based on engineered enzymes that could lead to CO₂-neutral photorespiration. Such promising pathways, primarily based on the reduction of glycolate to glycolaldehyde via glycolyl-CoA with engineered enzymes (glycolyl-CoA synthetase and glycolyl-CoA reductase or glycolyl-CoA carboxylase), were so far only demonstrated in vitro [63,152]. However, these promising designs for synthetic photorespiration await in vivo testing in microalgae and other photosynthetic organisms.

Like many other photosynthetic organisms, microalgae have naturally evolved carbon-concentrating mechanisms (CCMs) to reduce the effects on photorespiration. For microalgae, this CCM involves multiple steps such as bicarbonate uptake, carbonic anhydrase-mediated interconversion of bicarbonate and CO₂, and localization of RuBisCO in pyrenoid compartments within the microalgal chloroplast [64,65]. Because CCMs involve multiple steps and pathways, the engineering strategies for improving this system are in the early stages. Overexpressing a bicarbonate transporter from C. reinhardtii improved the growth rate and biomass accumulation in N. salina by increasing the intracellular inorganic carbon concentration. However, the overexpression of the same gene in C. reinhardtii did not yield significant improvements [66,67].
Figure 2. Alternative Photorespiratory Pathways. The pathway in purple indicates the predicted natural photorespiratory pathway in a microalgal cell (and in plants) that transports glycolate outside the chloroplast and converts two glycolate molecules into glycerate via several metabolic steps, and glycerate is finally transported back into the chloroplast where it is fed as an intermediate into the Calvin cycle. During this process CO₂ and ammonia are released, resulting in a net loss of fixed carbon and energy. Two synthetic alternative photorespiratory pathways that have been demonstrated to improve biomass in C₃ plants are indicated in yellow and red. The pathway in yellow implements three enzymes from the Escherichia coli glycolate pathway, namely glycolate dehydrogenase (GDH), glyoxylate carboxylase (GCL), and tartronic-semialdehyde reductase (TSR). The malate cycle in red implements two enzymes – GDH from C. reinhardtii and malate synthase (MS) from Cucurbita maxima. In the synthetic pathways, CO₂ is released back into the chloroplast and can be again taken up by RuBisCO for the Calvin cycle, thereby potentially limiting energy and carbon losses and increasing the growth rate. Abbreviations: CBB, Calvin-Benson-Bassham; 3-PG, 3-phosphoglycerate, RuBisCo, ribulose-1,5-bisphosphate carboxylase-oxygenase.

Synthetic Carbon Fixation

Engineering an alternative and more efficient CO₂ fixation pathway is a promising and challenging, radical strategy to overcome the shortcomings of the RuBisCO enzyme, the CBB cycle, and photorespiration in microalgae (Table 1 and Box 4).

Some of the alternative carbon fixation pathways, including the highly ATP-efficient Wood–Ljungdahl pathway, include oxygen-sensitive enzymes, making them poorly compatible with oxygenic photosynthesis in microalgae. However, there are also aerotolerant natural carbon fixation pathways, of which the reductive glycine pathway (via serine) is probably the most attractive and ATP-efficient. In this pathway, CO₂ is first reduced to formate, which in subsequent steps is activated and further carboxylated to glycine, that can next be converted to serine and pyruvate, only consuming 2 ATP/pyruvate. The two carboxylation reactions in this pathway are both thermodynamically reversible, meaning that this pathway can only function under elevated CO₂ concentrations, which is often the case in photobioreactor setups. It has been recently demonstrated that this linear pathway can be engineered into heterologous hosts, at least for the
assimilation of formate. The pathway has been successfully implemented in the bacteria *E. coli* and *Cupriavidus necator* by using a combination of modular engineering and adaptive laboratory evolution [79,80]. The core part of the reductive glycine pathway, the production of glycine from formate, was also demonstrated to be functional in the yeast *S. cerevisiae* [81]. Although this pathway has not yet been introduced in photosynthetic organisms for the fixation of CO₂, the ubiquitous nature of all the enzymes involved and its simple linear nature make it a promising pathway to be also introduced into microalgae.

Moreover, the 3-hydroxypropionate bi-cycle and the 3-hydroxypropionate-4-hydroxybutyrate pathways can also operate under aerobic conditions. Both pathways fix CO₂ with propionyl-CoA carboxylase and acetyl-CoA carboxylase, which use the more soluble HCO₃⁻ instead of CO₂ as carbon source and may have advantageous kinetics over RuBisCO [68]. However, both systems require the generation of relatively complex and circular pathways involving many enzymatic steps, thus complicating their engineering, and especially in organisms with more limited genetic toolboxes such as microalgae. Although some modules of both pathways were

| CO₂ fixation pathway | Natural/synthetic (aerobic/anaerobic) | Total number of reactions | ATP required per pyruvate produced | Carboxylating enzyme | Refs |
|----------------------|---------------------------------------|---------------------------|------------------------------------|----------------------|-----|
| Calvin cycle         | Natural (aerobic)                     | 10                        | 7                                  | RuBisCO              | [68,69] |
| 3-Hydroxypropionate cycle | Natural (aerobic)         | 16                        | 7                                  | Acetyl-CoA carboxylase and propionyl-CoA carboxylase | [70] |
| Wood-Ljungdahl pathway | Natural (anaerobic)    | 8                         | <1                                 | Formate dehydrogenase and CO methylating acetyl-CoA synthase | [71] |
| Reductive TCA cycle  | Natural (anaerobic)                     | 9                         | 1–2                                | 2-Oxoglutarate synthase and isocitrate dehydrogenase | [68,72,73] |
| Dicarboxylate/4-hydroxybutyrate cycle | Natural                  | 14                        | 5                                  | Pyruvate synthase and PEP carboxylase | [68,74] |
| 3-Hydroxypropionate/4-hydroxybutyrate cycle | Natural (aerobic) | 16                        | 5                                  | Acetyl-CoA carboxylase and propionyl-CoA carboxylase | [68,75] |
| Reductive glycine pathway (via serine) | Natural (aerobic) | 7                         | 2                                  | Formate dehydrogenase and glycine synthase | [76] |
| Reductive glycine pathway (via glycine reductase) | Natural (anaerobic) | 8                         | 1–2                                | Formate dehydrogenase and glycine synthase | [76] |
| C4 glyoxylate cycle (MOG pathway) | Synthetic (aerobic) | 14                        | 10                                 | PEP carboxylase      | [68] |
|                      |                                       | 13                        | 6                                  | Pyruvate carboxylase  |     |
| CETCH 5.4            | Synthetic (aerobic)                     | 13                        | ~12¹                               | Crotonyl-CoA carboxylase/reductase | [77] |
| CETCH 6.0/7.0        | Synthetic (aerobic)                     | 14                        | ~6–9²                             | Crotonyl-CoA carboxylase/reductase and propionyl-CoA carboxylase | [78] |

Abbreviations: CETCH, crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA; MOG, malonyl-CoA/oxaloacetate/glyoxylate; PEP, phosphoenol pyruvate; TCA, tricarboxylic acid.

¹For the production of 1 pyruvate via the CETCH 5.4 and glyoxylate carboligase pathway, 2 ATP are required directly and 4 NADPH are additionally consumed for the production of pyruvate, which are not required in any of the other pathways [only 5 NAD(P)H or other types of reducing equivalents are necessary to reduce 3 CO₂ to 1 pyruvate]. These extra electrons are lost in the two oxidation reactions in the CETCH 5.4 pathway. Assuming a P/O ratio of 2.5, these 4 NADPH equivalents add an additional indirect cost of 10 ATP equivalents. 

²For the production of 1 pyruvate via the CETCH 6.0/7.0 and glyoxylate carboligase pathways, 4 ATP are required directly and 2 NADPH are additionally consumed for the production of pyruvate. The extra required electrons are lost in an oxidation reaction in the CETCH 6.0 pathway (methylsuccinyl-CoA oxidase). Assuming a P/O ratio of 2.5, these extra 2 NADPH equivalents add an additional cost of 5 ATP equivalents. However, in CETCH 7.0 the methylsuccinyl-CoA oxidase is replaced by methylsuccinyl-CoA dehydrogenase that can donate the electrons to ubiquinone, hence some additional ATP may be regenerated in an electron transport chain. We assume in this case that only one ATP is *lost* by the partial oxidation of NADPH, hence only 2 extra ATP are consumed indirectly.
In addition to the natural CO\(_2\) fixation pathways, synthetic pathways can be designed based on the large collection of characterized natural enzymes and potential engineered enzymes to develop an efficient CO\(_2\) fixation route (Box 4). Some of these pathways have been successfully characterized in vitro, but their full implementation in vivo, even in bacterial hosts, remains to be demonstrated. Altogether, even though the implementation of full synthetic CO\(_2\) fixation pathways in microalgae may seem to be a far-fetched goal, impressive recent progress in engineering functional CO\(_2\) pathways in several hosts is reassuring. Recently, genetic engineering approaches combined with adaptive laboratory evolution led to the establishment of a fully functional CBB cycle in the heterotrophic model bacterium E. coli [84], as well as in the yeast Pichia pastoris [85] that has a less advanced genetic toolbox. In both cases the introduction of the CBB cycle only required the addition of a limited number (3–8) of heterologous genes. However, this work, as well as the previously mentioned engineering of the reductive glycine pathway for formate assimilation, demonstrates the feasibility of the engineering of complete autotrophic pathways. Nevertheless, a major challenge in achieving this goal, especially for microalgae, is the major metabolic engineering effort that will be necessary to express and localize the high number of, mostly foreign, enzymes involved. However, recent developments in the genome-editing toolbox of microalgae are very promising. The characterization of inducible
promoters, the 2A signal peptide-based system for multi-cistronic expression of heterologous proteins, gene stacking techniques based on bidirectional promoters, the identification of resistance to multiple antibiotics, Cre recombinase-based marker recycling, and, last but not least, CRISPR-associated nucleases for precise genome editing (Box 1), are some recent developments that could be exploited for expressing a large number of heterologous proteins in microalgal strains [96–92]. We believe that the growing knowledge about efficient and transplantable CO₂ fixation pathways, together with state-of-the-art techniques for heterologous protein expression and for adaptive evolution, should be combined to develop microalgal strains with improved CO₂ fixation ability.

**Mixotrophic Routes for Carbon and Energy Source**

**Cofeeding of Organic Substrates**

In addition to cultivating microalgae under photoautotrophic conditions, some microalgae can also be grown heterotrophically owing to their capacity to utilize exogenous organic carbon sources. The concomitant use of light/CO₂ and organic carbon sources is termed mixotrophy. Mixotrophic cultivation of microalgae can lead to improved growth rates in comparison to phototrophic growth owing to the presence of additional carbon and energy sources [93]. However, mixotrophy does not consistently improve lipid or biomass yield across different microalgal species [94]. For example, photoautotrophic cultivation of marine *Chlorella* sp., *Nannochloropsis* sp., and *Chlorella vulgaris* UTEX 259 yielded higher lipid content compared to heterotrophic and mixotrophic cultivation [95,96]. These studies indicate that improvement of biomass or product yield in microalgae by mixotrophy will be species- and product-dependent.

Mixotrophic cultivation could also be a potential strategy to eliminate the energy-intensive process of aeration in photobioreactors that is required for CO₂ supply and oxygen release from the system [97]. The CO₂ required for photosynthesis can be generated by heterotrophic metabolism whereas the O₂ requirement for heterotrophic growth can be met by oxygenic photosynthesis, thereby creating an internal gas circulation to maximize the yield from substrate and avoid adverse oxygen accumulation [98]. An innovative mixotrophic cultivation strategy was recently reported to double photobioreactor productivity by completely eliminating the gas–liquid transfer of CO₂ and O₂ [97]. In this case, the CO₂ generated by heterotrophic metabolism of acetic acid was employed to fuel the CO₂ requirement of photosynthesis. In addition, phototrophic oxygen production maintained the dissolved oxygen concentration (DOC) for the heterotrophic metabolism of acetic acid. The heterotrophic biomass yield observed in this study was 0.50 (C-mol biomass per C-mol substrate), which is close to the theoretical maximum of 0.7 for aerobic heterotrophic organisms. The overall mixotrophic biomass productivity was almost twofold higher than for purely autotrophic growth in the same reactor setup [97].

In practice, the applicability of mixotrophy is limited to microalgal strains that can take up and degrade particular organic carbon sources. This limitation could be resolved by the expression of carbon transporters and missing enzymes for organic carbon metabolism in the host species. Expression of human glucose transporter GLUT1 in *Phaeodactylum tricornutum* improved the cell concentration by fivefold under mixotrophic conditions [99]. In addition, *C. reinhardtii* expressing the glucose transporter Hup1 from *Chlorella kessleri*, when grown on media supplemented with 100 mM glucose in absence of light, doubled the cell density in 12 h and then remained stationary throughout the experiment, whereas the wild-type strain did not grow in dark [100]. This strategy could be employed for the trophic conversion and mixotrophic cultivation of commercially relevant microalgae. From a
sustainability perspective, however, the advantage of direct conversion of \( \text{CO}_2 \) and sunlight as sustainable resources may be compromised by the reliance on organic (plant-based) carbon sources.

**Direct or Shuttle-Based Cofeeding of Electrons**

As an alternative or complement to light energy and organic substrates, electricity generated by renewable energy sources has been proposed as a sustainable biotechnological feedstock, and this could also be extended to microalgae [101]. Solar/photovoltaic (PV) cells can mediate more efficient conversions than biological water-splitting photosystems. Specifically, state-of-the-art PV systems can attain a solar-to-electricity efficiency of 20%, and can be combined with electrolysis to generate hydrogen at an overall solar to chemical energy conversion of 11%. By comparison, the maximum conversion efficiency of solar energy to biomass for microalgae is 3% when grown in outdoor bioreactors, and 1% for crop plants [14].

Feeding with (renewable) electricity for bioproduction has already been extensively explored in bacteria, and is mostly referred to as microbial electrosynthesis (MES) [102,103]. The uptake of external electrons from electrodes is achieved by microbes such as *Shewanella oneidensis* that are naturally capable of extracellular electron transfer (EET) [104]. EET is facilitated via various types of mechanisms that transfer the external electron into the ETC of the host species [105].

Alternatively, mediator molecules such as hydrogen or formate could facilitate the transfer of electrons from cathodes to non-electroactive microbes [106,107]. A proof of principle for mediator-based MES was demonstrated in the hydrogen-oxidizing bacterium *C. necator* by using hydrogen as the mediator of electron transfer. This study demonstrated biomass production with an overall solar energy to biomass conversion efficiency of 9.7%, compared to 3% and 6% for microalgae grown at pilot scale outdoors and under indoor laboratory conditions, respectively [108,109].

In microalgae, MES could be a potential strategy to enhance carbon influx and reducing power generation. Direct electron uptake, or uptake of an electron mediator molecule such as hydrogen or formate, could improve NAD(P)H generation in microalgae. In the case of formate, the \( \text{CO}_2 \) to H

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**Figure 3. Conceptual Figure of Microbial Electrosynthesis (MES) in Microalgae.** The blue lines indicate the pathways involved in MES based NADH regeneration. During the process, the water split at the anodic chamber releases electrons, and these can be directly fed into direct electron uptake systems, or can generate for example \( \text{H}_2 \) or formate that can act as mediator molecules. Abbreviations: CBB cycle, Calvin–Benson–Bassham cycle; RuBisCo, ribulose-1,5-bisphosphate carboxylase-oxygenase.
released by formate oxidation in microalgae to generate NAD(P)H could also be fixed via the CBB cycle (Figure 3). Similar to the use of organic substrates, this system could alleviate the problems associated with light limitation or could be an additional energy source for cultivating the microalgae at night. Although MES has solely focused on selected bacterial strains, the potential of this promising technology could be expanded to unicellular microalgae and cyanobacteria to improve the productivities of biomass and/or green chemicals.

Electricity-driven cultivation may already have been achieved in microalgae during pulsed electric field treatment – however, this was primarily to prevent bacterial contamination in algal cultures or to stimulate product release. For the microalgal and cyanobacterial species *C. vulgaris* and *Arthrospira plantensis*, respectively, this technique was reported to enhance cell growth, but it was not effective for *C. reinhardtii* [110,111]. Various assumptions have been made regarding the underlying mechanism that results in improved growth, but this remains elusive [112]. *C. vulgaris* and *A. plantensis* might be naturally able to transfer electrons via mechanisms such as EET, and these may be lacking in *C. reinhardtii*. Further studies could open up interesting possibilities by identifying microalgal species for which MES could be applied to improve the production of biomass or green chemicals. In addition to the direct supply of electrons, enzymatic conversion of dihydrogen could provide an interesting electron source. However, eukaryotic hydrogenase enzymes prefer the reduction reaction in which dihydrogen is produced, whereas prokaryotic hydrogenases preferentially oxidize the dihydrogen [113–115]. Implementing cyanobacterial hydrogenases that can directly uptake dihydrogen and utilize it as an electron source in microalgae could provide an additional source of electrons.

**Concluding Remarks**

The commercial production of microalgal products is hindered by the high cost of their phototrophic cultivation. Improving the influx of carbon as well as the generation of reducing power, while minimizing losses in their conversion after entering the host metabolism, should be the prime focus in tackling this issue. Host-specific studies on the CBB cycle and the identification of rate-limiting steps should stimulate the design of engineering strategies to improve CO₂ fixation capacity. Recent advances in metagenomics-based discoveries, in CRISPR-based genome editing, and in adaptive evolution strategies could be exploited to develop natural or synthetic CO₂ fixation pathways that will lead to microalgal hosts with improved productivity (see Outstanding Questions). Given the rapid development of the genome-editing toolbox for several microalgal strains, the challenging task of expressing the large number of enzymes involved in these pathways could be realized. Improving CO₂ fixation will require additional reducing power, and this can be produced either via alternative sources such as organic carbon and electricity or by improving the light reactions of photosynthesis. Expanding the spectrum of light available for photosynthesis together with the swift flow of electrons via the ETC will be instrumental for attaining an optimal generation of reducing power.

Genetic components for improving the productivity of slowly growing model producer microalgae could be potentially obtained from fast-growing microalgae. By contrast, developing reliable genome-editing tools for these fast-growing strains could help in engineering them to exploit their improved photosynthesis for the production of interesting commodities [116]. *Picochlorum celerii* is an interesting fast-growing strain for this approach. This strain is reported to have a 10-fold faster doubling time (<2 h) compared to most of model microalgal species [117]. In addition, its three- to ninefold improved photosynthetic rates, the ability of the strain to withstand the variation in light intensities, and its resistance to photoinhibition indicate that

**Outstanding Questions**

Among the various synthetic biology strategies, which strategy or combination of strategies is the most promising for improving microalgal productivity?

Transplanting the core photosystem subunits resulted in varying photosynthetic efficiencies in microalgae. Could replacing the entire photosystem rather than only the core subunit be feasible?

Does implementing alternative photosynthetic pathways affect cell viability? The native photosynthetic pathway is also reported to be crucial for specific functions such as photoprotection and maintenance of redox balance.

RuBisCO enzymes from prokaryotes are reported to be catalytically superior but have reduced specificity for CO₂. The opposite is observed in RuBisCO from higher plants. Can protein engineering be employed to develop a chimeric RuBisCO with an improved catalytic rate (as seen in primitive variants), and high CO₂ specificity (as observed in higher plants)?

What further developments in microalgal genome engineering will be necessary to implement entire synthetic carbon fixation pathways?

Mixotrophy studies are often focused on media supplemented with specific carbon sources. Could mixotrophy implemented with wastewater effluents make this strategy more sustainable?

Do microalgae such as *C. vulgaris* have extracellular electron transfer mechanisms? If not, what causes their improved growth during pulsed electric field treatment?

The photosynthetic and carbon fixation machineries of fast-growing microalgal variants could have higher catalytic rates and efficiencies. How laborious will it be to express these faster variants in model microalgal strains, and does this approach have potential for improving carbon fixation and reducing power?

Instead of implementing entire photosynthetic machineries from a fast-
it harbors interesting light reaction machineries, and potentially also interesting CBB enzyme variants [117]. Characterization of the light-harvesting complex (LHC) and ETC of this strain could pave the way for heterologous expression of these systems in other model microalgal organisms to improve the PE.

Taken together, the development of microalgal organisms as a major platform for green chemical production is challenging but feasible. Application of a single strategy (such as improving the CBB cycle or ETC, or reducing photorespiration), as has been done in the past, is probably insufficient to address current inefficiencies. To this end, the diverse strategies discussed in this review will need to be combined together in a model microalgal strain (Figure 4). Given the spectacular progress that has recently been made in the fields of synthetic biology and genetic engineering, crucial steps can now be taken towards engineering microalgae as a sustainable platform for the production of biomass and green chemicals.

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Declaration of Interests
The authors declare no conflicts of interest.

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