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SPECIALTY SECTION

This article was submitted to Bioenergy
and Biofuels,
a section of the journal
Frontiers in Energy Research

RECEIVED 27 June 2022

ACCEPTED 23 August 2022

PUBLISHED 13 September 2022

CITATION

Subramanian S and Sayre RT (2022), The
right stuff; realizing the potential for
enhanced biomass production
in microalgae.

Front. Energy Res. 10:979747.
doi: 10.3389/fenrg.2022.979747

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The right stuff; realizing the potential for enhanced biomass production in microalgae

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There is growing evidence that eukaryotic microalgae can become a more sustainable and profitable alternative than terrestrial crops to produce feed, fuels, and valuable coproducts. The major factor driving progress in algal biomass production is the potential of microalgae to produce substantially greater biomass per unit land area than terrestrial crops. To be financially feasible, however, current algal biomass yields must be increased. Given the fact that algal biomass production is in its infancy there exist multiple opportunities to improve biomass yields. For example, recent bioprospecting efforts have led to the identification of new microalgal strains having biomass yields that compete economically with plant biomass. Substantial increases in biomass yields have also been achieved using advanced genetic engineering approaches. Targeted improvements in photosynthetic efficiency have led to three-fold increases in algal biomass yields. One genetic tool that has seen limited application for algal biomass enhancement is advanced breeding genetics. The greater availability of algal genomes and recent advancements in breeding algae will further accelerate yield improvements. Genetic engineering strategies to increase biomass production will also be assisted by transcriptomic and metabolomic studies that help identify metabolic constraints that limit biomass production. In this review we assess some of the recent advances in algal strain selection, directed evolution, genetic engineering and molecular-assisted breeding that offer the potential for increased algal biomass production.

KEYWORDS

microalgae, biomass, biofuels, photosynthesis, genetics, carbon capture

Highlights

- Based on thermodynamic considerations five-fold increases in algal biomass production are theoretically feasible

Abbreviations: Bu, bushels; CBBC, Calvin-Benson-Bassham Cycle; Chl, chlorophyll; gdw, gram dry weight.



GRAPHICAL ABSTRACT

- Enhanced and stable algal biomass production strains have recently been identified that achieve biomass yields approaching those necessary for economical bioproduct production
- Molecular assisted breeding of algae has been shown to enhance biomass yields more than three fold
- Engineering the photosynthetic apparatus of algae has lead to three-fold increases in biomass production
- Future strategies for enhancing biomass production in algae are considered

Introduction

Among the major constraints limiting the commercialization of eukaryotic microalgae for biomass production are the high costs of cultivation, harvesting and processing. Relative to crop plants algal biomass production requires substantial capital and operating investments including lined ponds, pond aeration and mixing systems, and energy intensive algal harvesting and processing systems (Unkefer et al., 2017; Benedetti et al., 2018; Sun et al., 2018; Rajvanshi and Sayre, 2020). The high operating costs for algal biomass can largely be attributed to the fact that algal biomass concentration is only 0.1%–0.5% of the cultivation media substantial volumes of water must be managed to grow and harvest algae requiring specialized infrastructure and high energy costs that could exceed the energy content of the algal biomass. Offsetting these extensive cost factors, however, is the high growth rate of algae. High algal growth rates are associated with their potential to grow throughout the year, the absence of heterotrophic tissues not engaged in biomass production, and the presence of high efficiency photosynthetic systems including

active CO₂ concentrating systems that reduce photorespiratory carbon losses (Sayre, 2010; Wang et al., 2011; Subramanian et al., 2013). As a result, algae have the potential to produce as much as 5-fold more biomass per unit land area than terrestrial crop plants (Weyer et al., 2010; Subramanian et al., 2013). Consequently, there has been incredible interest to develop microalgae for low-cost production of feeds, fuels, high value bioproducts and to sequester atmospheric carbon dioxide to mitigate climate change (Olivares et al., 2016; Lammers et al., 2017; Unkefer et al., 2017; Marrone et al., 2018; Sayre, 2010). However, there remain multiple constraints that limit the commercialization of eukaryotic microalgae. Life cycle and techno-economic analyses of algal cultivation systems have indicated that only open-pond production systems can economically produce algal biomass that is cost competitive with crops (Chisti, 2013; Davis et al., 2014; Barry et al., 2016). But to be economically feasible, outdoor open-pond cultivation systems must produce stable biomass yields approaching 40 gdw/m²/day, corresponding to an overall solar to biomass conversion efficiency of ~3% (Bolton and Hall, 1991; Kruse et al., 2005a; Kruse et al., 2005b; Zhu et al., 2008; Lammers et al., 2017). Given that the maximum theoretical efficiency for solar energy conversion into the chemical energy of algal biomass by photosynthesis is approximately 11% there is the potential to achieve 2- to 3-fold increases in biomass yields (Bolton and Hall, 1991; Kruse et al., 2005a; Kruse et al., 2005b; Zhu et al., 2008; Weyer et al., 2010). Thus, substantial improvements in biomass accumulation are theoretically possible.

When considering strategies to increase algae biomass yields it is informative to consider genetic and agronomic practices that have been successfully used to increase biomass production in terrestrial crops. Over the last 75 years great strides have been

achieved in the development of higher yielding and more robust crops. These achievements have largely been the result of advanced agronomic practices to better manage resource inputs and the application of modern agricultural breeding techniques to improve crop genetics. Crop breeding strategies were initially based on the application of genetic linkage analyses between traits of interest and more tractable or scorable traits. In the last 40 years molecular assisted breeding and genomic selection techniques have further accelerated the development of improved crops. The application of modern breeding systems including, the use of inbred lines, hybrid sterility mechanisms, and transgenic modifications, has led to over a 7-fold increase in maize yields between 1940 and 2015 (168 bu/acre). Most of these improvements in crop yield have been achieved through the development of herbicide resistance genes, and the introduction of pest, pathogen and stress tolerance traits. Surprisingly, few projects focusing enhanced carbon capture for biomass yield improvements have reached the stage of commercial deployment.

In contrast to terrestrial crops, the greatest enhancements in microalgal biomass yields have been realized through the identification of more robust algal species and through directed selection and transgenic approaches rather than through traditional breeding approaches (Kumar et al., 2013; Kumar et al., 2016; Crozet et al., 2018; Fayyaz et al., 2020; Dementyeva et al., 2021; Mosey et al., 2021). In the following sections we address why that is the case and review recent advances that have led to further improvements in eukaryotic microalgal biomass production.

Strain selection for enhanced biomass yield

The emerging consensus is that the most economically feasible means to produce algal biomass is to cultivate algae in continuously mixed, open pond culture systems (Chisti, 2013; Davis et al., 2014; Barry et al., 2016; Lammers et al., 2017; Marrone et al., 2018). These pond systems typically range in depth from 15 to 30 cm. While greater algal biomass yields per unit area are possible in closed photobioreactor (PBR) systems these systems have several inherent disadvantages when operated at large scale including, greater capital and operating expenses than open pond systems, challenges associated with the formation of biofilms that reduce light penetration and the management of pathogens or herbivores (Chisti, 2013; Davis et al., 2014; Barry et al., 2016; Lammers et al., 2017; Marrone et al., 2018; Rajvanshi and Sayre, 2020). Overall PBR-based algal biomass production systems are estimated to be 2- to 2.5-fold more expensive to operate than open pond production systems (Chisti, 2013; Davis et al., 2014; Barry et al., 2016). Due to their open nature algal species that are cultivated in ponds must have fast growth rates to out compete weedy invasive species and be

resistant to biotic and abiotic stressors including large temperature and light intensity shifts, limiting daylength periods at high latitudes, precipitation, competing weedy algal species, pathogens, and herbivores (Barry et al., 2016; Neofotis et al., 2016; Olivares et al., 2016; Marrone et al., 2018; Rajvanshi and Sayre, 2020). As a result of these challenges substantial efforts have gone into screening or bioprospecting for algal strains that not only have high growth rates but that can also tolerate abiotic or biological stressors that lead to pond crashes. Given that there are over 164,000 known species of algae bioprospecting for high biomass producing algal strains particularly those best suited for the local environments where they are cultivated continues to provide opportunities for enhancing algal biomass yields (Marrone et al., 2018; Guiry and Guiry, 2021).

Since the mid-1970s, the United States Department of Energy has supported multiple algae bioprospecting efforts to identify high biomass yielding and stable strains suitable for commercial biomass production (Weissman and Goebel, 1987; Barry et al., 2016; Olivares et al., 2016). To date, algal strains having the highest and most stable biomass production rates are dominated by species from two phyla, the Chlorophyta and Ochrophyta. These include species from the genera, *Chlorella*, *Picochlorum*, *Scenedesmus*, *Cyclotella*, *Tetraselmus* and *Nanochloropsis* (Table 1). Many of these species have demonstrated long-term biomass production (>100 days) yields approaching 40 gdw/m²/day 22, (Huesemann et al., 2018; Dahlin et al., 2019; Gonzalez-Esquer et al., 2019; Aketo et al., 2020; Mucko et al., 2020; Cano et al., 2021; Krishnan et al., 2021). Averaged over a yearlong growing season algae having a growth rate of 40 gdw/m²/day could yield as much as 145 metric tons dw/ha/yr having an average energy density of 21 kJ/gdw. In comparison, corn yields in the United States Midwest average 11.5 metric tons dw/ha/yr having an average energy density of 16.7 kJ/gdw. Thus, the annual areal energy yield for algal biomass grown at a rate of 40 gdw/m²/day is 16-fold greater than for corn. Given that the corn growing season is only 3 months, however, the relative efficiency of algal biomass production is about 4-fold greater than for corn on an annualized basis.

Assessing relative biomass yields between strains and treatments can be challenging due to the lack of standardized practices for measuring biomass yields or net productivity. Algal biomass yields may be quantified based on optical density, cell numbers per unit volume, dry weight per unit volume and dry weight yield per unit land area. Furthermore, since algae are grown under a variety of environmental conditions it can be challenging to assess relative yields between different studies. Given that solar energy inputs are based on the photon flux density per unit area it is useful to assess biomass yields based on areal productivity per unit time (gdw/m²/day) so that energy conversion efficiencies can be directly assessed.

Another important consideration in choosing algal strains for biomass production is the biochemical composition (lipid, carbohydrate and protein makeup) of the algae. Many algae

TABLE 1 Relative biomass yields from high biomass producing strains of algae.

Species	Biomass yield (gdw/m ² /day)	Outdoor growth location
<i>Chlorella sorokiniana</i> (Huesemann et al., 2018)	36	Mesa, AZ, United States
<i>Picochlorum celery</i> (Krishnan et al., 2021)	31–40	Mesa, AZ, United States
<i>Picochlorum renovo</i> (Dahlin et al., 2019)	34	Mesa, AZ, United States
<i>Tetraselmis</i> sp. (Laws et al., 1986)	37	Kauai, HI, United States
<i>Scenedesmus</i> UTEX393 (Davis and Laurens, 2010)	27	Mesa, AZ, United States
<i>Nanochloropsis</i> (Boussiba et al., 1987)	25	Israel
<i>Cyclotella</i> sp. (Weissman and Goebel, 1987)	30	California, United States
<i>Chaetoceros muelleri</i> (Weissman and Goebel, 1987)	32	California, United States

species can also facultatively adjust their relative lipid and carbohydrate levels depending on culture conditions (Boussiba et al., 1987; Negi et al., 2016; Sarwer et al., 2022). In a metanalysis of the biomass composition of the Chlorophyta and Ochrophyta it was determined that the Chlorophytes had on average 33% protein, 16% lipid, 14% carbohydrate and 12% ash content per unit dry weight. In contrast, the Ochrophytes had 33% protein, 21% lipid, 14% carbohydrate, and 19% ash content by dry weight. Therefore, the ratio of carbohydrate to lipid (0.9) was greater for the Chlorophytes than for the Ochrophytes (0.5) (Finkel et al., 2016). Thus, the Ochrophytes were on average more energy dense than the Chlorophytes.

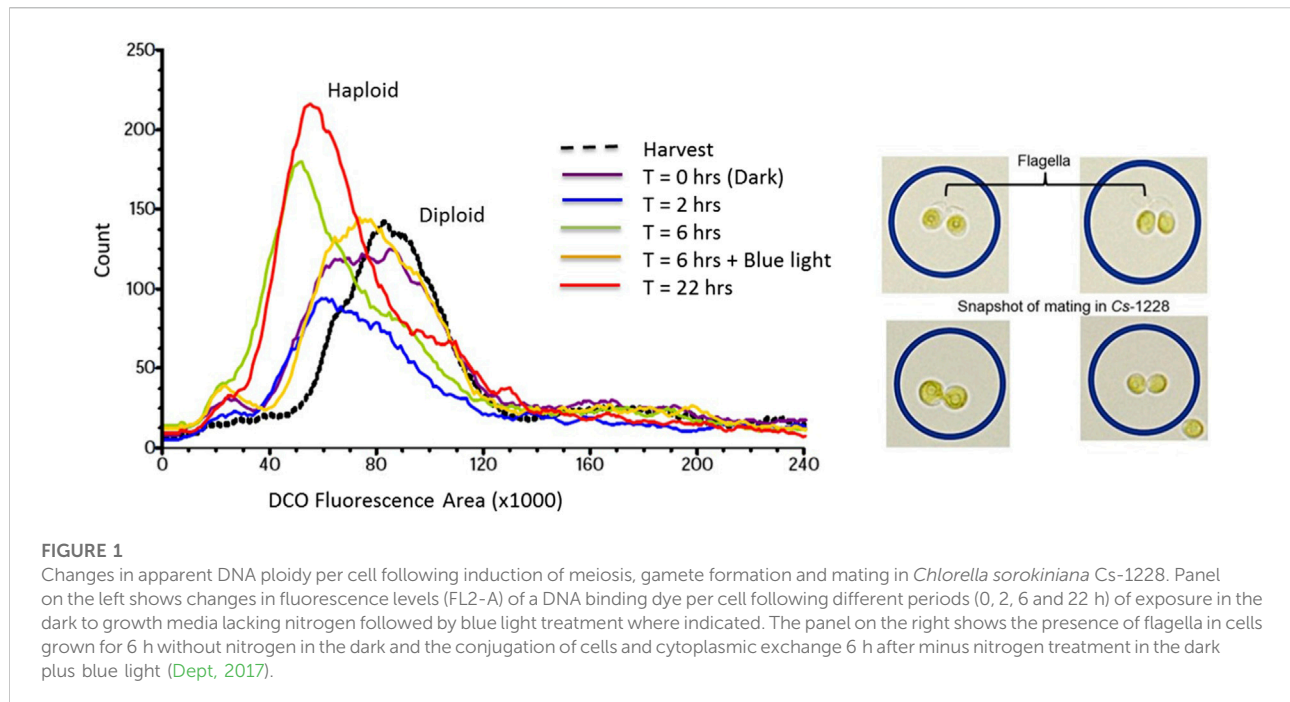
Recently, one genus of algae, *Picochlorum*, has emerged as one of the highest and most stable biomass producers (Dahlin et al., 2019; Gonzalez-Esquer et al., 2019; Mucko et al., 2020; Cano et al., 2021; Krishnan et al., 2021). *Picochlorum* are marine algae having a wide global distribution that tolerate high salinity and temperatures (40 C) well allowing for cultivation in a broad range of environments (Zhu et al., 2008; Dahlin et al., 2019; Krishnan et al., 2021). Furthermore, recent studies have indicated that some *Picochlorum* species have greater photosynthetic efficiency than many algal species (Cano et al., 2021). This enhanced efficiency is associated with ability to self-adjust their light harvesting antenna size to optimize light use efficiency in dynamically changing light environments (Perrine et al., 2012; Negi et al., 2020; Cano et al., 2021). In addition, *P. celery* has been shown to be able to both adjust its light harvesting antenna size as well as accumulate high levels of photoprotective carotenoids involved in non-photochemical quenching of excess radiation (Cano et al., 2021). Recently, molecular toolboxes for the genetic manipulation of *P. celery* have been developed including genetic transformation (transformation systems and the development of selectable marker genes) and genome editing tools (Cas9) allowing for additional engineering opportunities to improve biomass yields.

Thermodynamic modeling of the energy efficiency of converting solar photons into chemical energy of biomass in algae indicates that the type of major energy product stored has an impact on the overall solar to chemical energy conversion

efficiency. Storage of reduced carbon as starch has been shown to be more energy efficient than lipid storage (Subramanian et al., 2013; Finkel et al., 2016; Sun et al., 2018; Aketo et al., 2020; Changko et al., 2020; Negi et al., 2020). The greater energy storage efficiency of starch versus lipids is largely due to the loss of previously reduced carbon during lipid synthesis that occurs with the decarboxylation of pyruvate to produce acetyl CoA for lipid synthesis. This loss of previously fixed carbon does not occur during starch accumulation. It follows to reason that screening techniques for the selection of high biomass production strains efforts should focus on high starch content strains rather than high lipid strains.

Recently, a green algal species *Pseudoneochloris* sp. Strain NKY372003 was shown to accumulate very high starch levels relative to other algal strains (68% of total biomass) (Aketo et al., 2020). In addition, *Pseudoneochloris* has the ability to increase its cell volume by over 100-fold. In comparative growth studies, the volumetric biomass yield (8.1 gdw/L) of *Pseudoneochloris* was shown to be 47% greater than for *Tetraselmis* (5.5 gdw/L) one of the higher known areal biomass producers (37 gdw/m²/day) (Table 1). These results suggest that alterations in the allocation of fixed carbon between starch and lipid storage could substantially impact algal biomass yields. Given the fact that starch has a substantially higher mass density (1.54 g/cm³) than lipids (0.91 g/cm³), simple sedimentation or isopycnic centrifugation selection systems could be used to efficiently bioprospect for high starch/high biomass strains. Due to the greater interest in algal lipids for biofuel production, however, less research effort has focused on bioprospecting for high starch containing strains than for high lipid strains.

As previously mentioned, development of management systems for the control of pathogens in open pond production systems is only in its infancy (Richmond et al., 1990; Davis and Laurens, 2010; Olivares et al., 2016; Lammers et al., 2017). Pathogen/herbivore infections can cause the very rapid in (as little as 2 days) and complete collapse of algae pond production systems. A variety of strategies have been used to control pathogens in algal cultivation systems. Chemical pathogen management practices for pathogen and herbivores have



included the use of ammonia as a nitrogen source, phosphite as a source of phosphorous in strains engineered to oxidize phosphite, salts, and antibiotics (Lammers et al., 2017; Changko et al., 2020). Physical management practices include taking advantage of the differential sheer sensitivity of algae and versus pathogens to mechanical forces such as pumping or sonication (Lammers et al., 2017; Marrone et al., 2018). Competing weedy algae and pathogens can also be controlled using pulsed rather than continuous applications of macro-nutrients. The greatest success in managing pond crashes, however, has been achieved through the selection of algal strains that are naturally resistant to pathogens (Krishnan et al., 2021; Pang et al., 2022). In large part the molecular basis for disease resistance is not known. Interestingly, some species of *Picochlorum* have been shown to grow heterotrophically on bacteria presumably by phagocytosis opening the possibility for breeding or engineering this trait into algal production strains to control bacterial pathogens (Pang et al., 2022). Overall, pathogen control in algae remains one of the major opportunities for increasing in algal biomass yield.

Breeding algae for increased biomass production

Improvements in terrestrial crop production yields over the last 80 years can largely be attributed to improved agronomic practices and the application of advanced genetic breeding approaches for crop improvement. More recently, molecular assisted breeding and genomic selection tools have been

applied to crop breeding and have accelerated the development of advanced crops with desirable properties. Molecular assisted breeding has been made possible through the development of low-cost and efficient genome sequencing tools. In contrast to crop plants, however, there has been little application of molecular-assisted breeding tools for the genetic improvement of algae except for the model species *Chlamydomonas*. *Chlamydomonas* is particularly amenable to breeding for enhanced yields since its dominant (mating type) generation is haploid, the dominant growth generation for *Chlamydomonas* is haploid. Ecotypes of different haploid mating types have been identified and used for breeding purposes. Recently, novel recombinant *Chlamydomonas* strains having three-fold increases in biomass yield relative to their parental strains have been generated through genetic crosses and strong selection regimes (Lucker et al., 2022). The increases in yield in the progeny were correlated with widespread alterations in gene frequencies between the two parental strains.

Unlike *Chlamydomonas*, however, the dominant generation for many algae is diploid. Thus, the challenge for mating many algae strains is how to induce gametogenesis and conjugation. Induction of meiosis, gamete formation and subsequent mating in diploid algae is currently an inexact science. The most common approach to induce meiosis in algae is nutrient stress in the dark (Huang and Beck, 2003; Přibyl P Light is a, 2013). In addition, exposure of gametes to a short pulse of blue light activates the phototropin receptor and can induce mating in some algal strains (Pfeifer et al., 2010). One approach to assess the effectiveness of various environmental treatments on

TABLE 2 Transgenic modifications of green algae for enhanced biomass production.

Host	Target gene	Phenotype	Cultivation system	Biomass increase relative to wild type
<i>Chlamydomonas</i> (Negi et al., 2020)	Light-regulated chlorophyll a oxygenase expression mediated by NAB1 translational repressor	Small light harvesting antenna when grown in high light and large light harvesting antenna when grown in low light	Simulated diurnal pond environment in PBR	100–180%
<i>Chlamydomonas</i> (Perrine et al., 2012)	Reduced chlorophyll a oxygenase expression	30% reduction in antenna size, Chl <i>a/b</i> ratio = 5 for transgenic vs. 2.2 for wild type	Simulated diurnal pond environment in PBR	40%
<i>Chlamydomonas</i> (Shokravi et al., 2021)	Overexpression of activated NAB1 gene repressor of LHCII expression	17% reduction in antenna size. Chl <i>a/b</i> = 2.2 for transgenic vs. 2.0 for wild type	PBR supplemented with 3% CO ₂ and a continuous light intensity of 700 μmol photons/m ² /sec	30%
<i>Chlamydomonas</i> (Beckmann et al., 2009)	Reduced global expression of LHC family members	30% reduction in Chl content/cell, Chl <i>a/b</i> ratio = 4	Photoheterotrophic growth in presence of acetate under continuous illumination at 1,000 μmol photons/m ² /sec	Not determined, but 45% faster growth rate
<i>Chlamydomonas</i> (Mussgnug et al., 2007)	Phototropin knock out mutants	Global alterations in photosynthesis, cell cycle control and carbon allocation	Simulated diurnal pond environment in PBR	100%
<i>Phaeodactylum</i> (Fu et al., 2017)	Expression of eGFP in the cytoplasm	Increased light use and reduced NPQ	Simulated outdoor growth in PBR supplemented with 1% CO ₂	50%
<i>Chlorella</i> (Yang et al., 2017)	Overexpression of cyanobacterial CBB Cycle enzyme fructose 1,6-bisphosphate aldolase	1.3-fold higher aldolase activity	Low light (40 μmol photons/m ² /sec) intensity shaker flasks	6%

gametogenesis and mating is tracking changes in DNA ploidy levels per cell in real time. This can be accomplished by quantifying relative changes in cellular DNA content using DNA binding fluorescent dyes coupled with fluorescent activated cell counting (Dept, 2017). As shown in Figure 1, *Chlorella sorokiniana* Cs-1228 cells starved of nitrogen in the dark for 6 h had half the DNA content of cells grown in nitrogen replete media indicative of the production of gametes under nitrogen stress (Dept, 2017). Gamete induction was observed in as little as 2 h after transfer of cells to media without nitrogen in the dark (Figure 1). At 22 h after nitrogen deprivation in the dark there was a small shift to higher DNA content/cell suggesting some level of mating and production of diploid cells. But the additional treatment of cells with a brief pulse of blue light (1–2 μmol photons/m²/s for 5 min) resulted in a near complete shift to diploid cells indicative of mating. The conversion of diploid to haploid cells competent for mating during dark nutrient stress treatments was further supported by the appearance of flagella required for mating at cells 6 h after treatment in the dark without nitrogen (Figure 1). Mating was also demonstrated by cytoplasmic exchange in blue-light treated cells (Figure 1) (Dept, 2017).

The development of procedures to induce gametogenesis and mating in other polyploid algae opens the door for the use of advanced genome wide association studies, molecular breeding tools and selection for enhanced performance characteristics and biomass yield (Přibyl P Light is a, 2013; Luckner et al., 2022). Overall, it is expected that the potential to

increase biomass yields is great using breeding approaches given the vast genetic diversity that exists in natural algal ecotypes and the possibility to engineer algae (Baker et al., 2007; Ng et al., 2017; Crozet et al., 2018; Naduthodi et al., 2020; Ng et al., 2020; Shokravi et al., 2021).

Directed and adaptive evolution of algae for increased biomass yield

Biomass production can also be optimized for particular environments using directed or adaptive evolution approaches. When coupled with the generation of mutant populations for selection, additional improvements in yield resulting from new genotypes may also be achieved. Significantly, additional understanding of the metabolic factors that regulate growth can be achieved through comparative genomic, transcriptomic, proteomic and metabolomic studies of improved strains selected through directed evolution. For example, *Haematococcus pluvialis* strains selected for improved growth under high (15%) CO₂ conditions were shown to have enhanced photosynthesis and glycolysis associated with the increased expression of genes involved in photosynthetic electron transport including PetH (ferredoxin–NADPC reductase) and ATPF0A (F-type H⁺-transporting ATPase subunit), and genes involved in respiration including, the PetJ (cytochrome c6) gene involved in NADPH generation. Additionally, genes of the C3 and

C4 photosynthetic pathways in plants were overexpressed in improved strains including Pdk, pyruvate, orthophosphate dikinase and FBA, fructose-bisphosphate aldolase. These genes facilitated carbon uptake under high CO₂ growth conditions (Li et al., 2017). Among the greatest improvements in biomass yield was achieved using directed evolution approaches under high CO₂ growth conditions. As much as a 3-fold increase in algal biomass production (3.7 gdw/L) was achieved after 31 rounds of selection under high (10–20%) CO₂ (Li et al., 2015). This increase in biomass yield was associated with the accumulation of greater amounts of starch per cell. It is important to note however, that the molecular adjustments resulting in increased biomass yields may not be the same for slow growing versus fast growing parental strains. If the biomass yield of the parental strain is low to begin with, modest metabolic changes resulting in large relative increases in biomass yield may not be applicable to very fast-growing strains. The metabolic bottlenecks limiting growth for each strain must be evaluated.

Engineering algae for increased biomass yield

Metabolic engineering strategies to increase biomass yields can be separated into at least four different categories including increasing carbon source strength, increasing carbon sink strength, alterations in the expression of master regulatory genes that control global carbon partitioning and flux rates, and strategies that control the cell cycle and cell division rates. First, we describe genetic manipulations that increase carbon source strength or rates of photosynthesis (Table 2).

Based on relative thermodynamic efficiency, the earliest steps in photosynthesis involved in light capture and utilization, i.e., the conversion of chlorophyll excited states into charge separated states driving photosynthetic electron transfer processes, are collectively the least efficient processes in biomass production (Subramanian et al., 2013; Okada et al., 2020; Vecchi et al., 2020). This is largely attributed to the fact that photosynthetic electron transfer rates saturate at 20–25% of full sunlight intensity in all algae and plants (Perrine et al., 2012). This is due to the fact that at full sunlight intensities the rate of photon capture by the light harvesting complexes is 5- to 10-fold faster than the rate-limiting steps in photosynthetic electron transport, i.e., the oxidation of plastoquinol by the cytochrome b6/f complex (Baker et al., 2007; Perrine et al., 2012). In addition, as the proton gradient increases across the thylakoid membrane following illumination rates of plastoquinol oxidation are further diminished (Baker et al., 2007; Kiirats et al., 2009).

When addressing electron transport or metabolic steps having rate-limiting kinetics one strategy to alleviate bottlenecks is to overexpress the enzyme(s) or enzyme complex that are rate limiting. For example, to compensate

for the slow rate of RuBP carboxylase/oxygenase (RuBisCO) catalysis plants and algae substantially overexpress the enzyme RuBisCO). In algae the cytochrome b6/f complex, catalyzes the rate-limiting step in electron transport. The cytochrome b6/f complex is present in equal stoichiometry with the photosystem I and II reaction center complexes (Kiirats et al., 2009). Thus, it is conceivable that elevating the relative number of cytochrome b6/f complexes coupled with accelerated dissipation of the proton gradient by the ATPase complex, could compensate for the slow rate of plastoquinol oxidation. To date, however, there have been no reports of successfully overexpressing the cytochrome b6/f complex to address this bottleneck in electron transport. This inability to substantially alter the stoichiometry of cytochrome b6/f complexes may in part be due to the unique requirements for assembling stable cytochrome b6/f complexes.

An alternative strategy for addressing inefficiencies in coupling chlorophyll excited state transitions to electron transport rates has been to reduce the optical cross-section or size of the light harvesting antenna to a level where the rate of photon capture corresponds with the rate of electron transfer. Reductions in light harvesting antenna size have been achieved by multiple means in mutant and engineered *Chlamydomonas*. One of the more successful strategies for reducing light harvesting antenna sizes has been reduction in chlorophyll *b* (Chl *b*) accumulation. Chl *b* is present only in the peripheral, nuclear-encoded, light harvesting pigment/protein complexes associated with reaction center complexes (photosystem II and I) and is not present in the chloroplast-encoded reaction center proximal antenna proteins or the core reaction center complex proteins. The core reaction center proteins only contain only Chl *a* (Melis, 2009; Perrine et al., 2012). Reduction in Chl *b* levels destabilizes the light harvesting protein subunits resulting in the turnover of Chl *b*-binding proteins effectively reducing the peripheral light harvesting antenna size (Melis, 2009; Perrine et al., 2012; Negi et al., 2020). The earliest studies on reduction of Chl *b* levels in algae were those carried out by Melis and colleagues (Melis, 2009). Their studies on antennae size modulation focused on the selection for mutants that did not accumulate Chl *b*. When grown under photoheterotrophic conditions the Chl *b*-less mutants showed some improvements in yield. However, when grown strictly photosynthetically chlorophyll *a* oxidase mutants, unable to produce any Chl *b*, had substantially reduced growth rates compared to their wild-type parents (Perrine et al., 2012).

But all things in moderation. By fine tuning Chl *b* levels by partially but not completely suppressing chlorophyll *a* oxygenase (CAO) activity it was determined that moderate reductions in light harvesting antenna size resulted in the greatest increases in photosynthetic efficiency. The highest biomass producing strains were shown to have lost one LHCII trimer complex relative to wild-type algae, equivalent to a Chl *a/b* ratio of 5 (Perrine et al., 2012). Transgenic algae having Chl *a/b* ratios lower than 5 (larger antenna sizes) including wild-type algae (Chl *a/b* = 2.0–2.5) or

algae having Chl *a/b* ratios greater than 5 (smaller antenna) had reduced photosynthetic rates and reduced abilities to tolerate high light stress due to a reduced capacity for non-photochemical quenching of excess captured energy. Significantly, biomass yields for algae having Chl *a/b* ratios of 5 was 40% greater than those of wild-type algae when grown in photobioreactors simulating outdoor pond cultivation conditions (Perrine et al., 2012). Similar observations were made for plants engineered to have reduced light harvesting antenna sizes. Optimal biomass production in *Camelina* was achieved in plants having a Chl *a/b* ratio of 5 (Friedland et al., 2019).

Reductions in light harvesting antenna complexes have also been achieved by overexpression of the NAB1 protein which regulates the expression of multiple light harvesting complex protein (Beckmann et al., 2009). The NAB1 protein is a translational repressor that binds to the 5' end of transcripts encoding members of the light harvesting complex. Overexpression of the activated (reduced disulfides) NAB1 protein resulted in a 17% reduction in antenna size in transgenic *Chlamydomonas* corresponding to a Chl *a/b* ratio of 2.2. Biomass yields were 30% greater in NAB1 transgenics relative to the parental wild-type strain when grown in photobioreactors supplemented with elevated CO₂. Additional approaches have been used to engineer light harvesting antenna size. Mussgnug et al (Mussgnug et al., 2007) described a more generalized approach to reduce light harvesting antenna size by expressing an RNAi construct that targeted a common DNA sequence present in the 5' untranslated region of multiple LHCI family members. They observed a 30% reduction in Chl content/cell leading to a Chl *a/b* ratio of 4. Under mixotrophic growth conditions the transgenics had a 45% faster growth rate than wild type, however, no biomass studies were reported.

To date, the greatest biomass increases resulting from genetic engineering approaches have been achieved through the light regulation of light harvesting antenna size (Negi et al., 2020). Dynamic regulation of light harvesting antenna size was achieved by co-expression of a light-regulated NAB1 translational repressor along with coexpression of a modified CAO gene having a 5' NAB1 binding light response element expressed in a CAO knock strain. In contrast, under high light conditions light harvesting antenna sizes were reduced due to increased NAB1 expression. In contrast, under low-light growth conditions antenna sizes were increased due reduced NAB1 expression in transgenic strains. It was also observed that sensitivity to photoinhibition was reduced relative to wild type cells in the transgenic strains relative to wild type.

Another novel approach to alter light harvesting efficiency has been the expression of light absorbing proteins in the stroma of chloroplasts. The expression of the green fluorescent protein in the chloroplasts of the diatom *Phaeodactylum tricornerutum* resulted in a 50% increase in biomass yield (0.23 gdw/L/day) under simulated pond growth conditions supplemented with 1% CO₂ (Fu et al., 2017). This increase in biomass yield was only observed when the

transgenics were grown under light intensities (400 μmoles photons/m²/sec) that were greater than those that light-saturate photosynthetic electron transport. Interestingly, non-photochemical quenching in GFP expressing strains was reduced under high-light growth conditions relative to wild type. These results suggest that GFP was shading the light harvesting apparatus resulting in reduced energy dissipation through NPQ allowing for enhanced photosynthetic electron transport at high light levels.

One of the more elegant strategies to enhance algal biomass production has been the genetic manipulation of master growth regulatory genes that globally regulate multiple genes involved in biomass accumulation. One such algal regulatory gene is the blue light photoreceptor, phototropin (Im et al., 2006; Pfeifer et al., 2010; Petroustos et al., 2016). In *Chlamydomonas* phototropin regulates the expression of multiple gene systems including genes involved in the sexual life cycle, eye spot size, and genes involved chlorophyll, carotenoid, and chlorophyll binding protein synthesis (Im et al., 2006; Petroustos et al., 2016). Negi et al. (Negi et al., 2017) demonstrated that phototropin knock out mutants had higher Chl *a/b* ratios than wild type cells ranging from 2.9 when grown under low light to 3.4 when grown under high light conditions associated with a reduction in light harvesting antenna size. In addition, cell division rates and biomass accumulation rates were substantially increased in phototropin knock out mutants. RNAseq experiments indicated that the expression of multiple genes involved photosynthesis, carbon metabolism, and cell division rates were significantly altered in phototropin mutants. Phototropin mutants had a 2- to 5-fold increase in the expression levels of the Rieske Fe-S protein (cytochrome b6f complex), the Calvin-Benson-Bassham Cycle (CBB) enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase, sedoheptulose 1,7 bisphosphatase glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, and genes involved in starch synthesis including ADP glucose pyrophosphorylase, starch synthase, and genes involved in respiration and fatty acid biosynthesis. In addition, the SNRK1 gene that shares homology with the major growth regulatory genes KIN10 and KIN11 in plants had ten-fold higher expression levels in phototropin mutants (Baena-González et al., 2007; Negi et al., 2017). Genes involved in cell cycle control including NIMA (never in mitosis), NEK2, NEK6 (NIMA related kinases), RCC1 (regulator of chromosome condensation, cyclin and cyclin-dependent kinases: Cyclin-dependent kinases, and MAT3 a homolog of retinoblastoma protein (MAT3/RB) were also upregulated 2-to 15-fold in phototropin mutants relative to wild type. Overall, there was a two-fold increase in biomass yield in phototropin mutants relative to wild-type cells when grown under environmental conditions that mimicked outdoor light and temperature conditions (Negi et al., 2017). However, there is some evidence that phototropin mutants may be more susceptible to photoinhibition than wild type (Li et al., 2017)

These results indicate that the coordinated regulation of source and sink strength genes as well as cell cycle control genes can enhance biomass accumulation in algae.

In contrast to efforts to improve biomass yields through alterations in light use efficiency, there have been limited efforts to improve algal biomass yields through manipulation of CBBC enzymes or by increasing carbon sink strength. There have, however, been substantial efforts to increase lipid accumulation in microalgae through genetic engineering and genome editing approaches often at a loss in biomass accumulation (Gonzalez-Esquer et al., 2019; Fayyaz et al., 2020; Ng et al., 2020; Shokravi et al., 2021). One approach to increase biomass accumulation through manipulation of the CBBC activity was achieved through enhancement of fructose 1,6-bisphosphate aldolase activity, an enzyme playing a major role in carbon flux control in the CBBC (Yang et al., 2017). Transgenic *Chlorella* cells overexpressing fructose 1,6-bisphosphate aldolase were shown to have 20% higher rates of photosynthesis but only a 6% increase in biomass yields relative to wild type. The small increase biomass yields may be due to the low light conditions under which the algae were cultivated. Additionally, it has been demonstrated that genetic complementation of *Chlamydomonas* mutants impaired in starch synthesis (ADP glucopyrophosphorylase mutants) resulted in reduced generation of potentially toxic reactive oxygen species indicating the critical role starch synthesis plays as an electron sink (Saroussi et al., 2019). Finally, starch hyperaccumulation mutants impaired in glycolysis were shown to have 72% higher cell numbers at stationary phase than wild-type cells (Koo et al., 2017). Collectively, these results demonstrate the critical role carbon metabolism and starch accumulation plays in overall algal biomass accumulation.

Finally, biomass yields are also determined by cell cycle kinetics and changes in cell volume (Li et al., 2016). The regulation of cell cycle activity and cell size in algae, however, is less well understood than central metabolism but offers significant opportunities for manipulation of biomass production (Li et al., 2016).

Conclusion

Microalgae are gaining increased attention as possible alternative food sources, platforms to produce high value co-products, biofuels, and for long-term carbon sequestration in geologic formations. Recent life cycle analyses of algal production systems have determined that algal biomass production rates are one of the major bottlenecks impeding the commercialization of algal biomass, bioproducts and biofuel products. Thus, the need for enhanced algal biomass production to make algae commercially competitive with other biomass production platforms.

Theoretical and empirical evidence indicate that algae that accumulate starch as their major reduced energy reserve have faster growth rates and greater biomass accumulation potential than algae that predominantly store lipids as energy reserves. Since carbon skeletons for lipid production are in large part derived from starch it is apparent that the focus on identifying algal strains that are high lipid accumulators may not be the most effective bioprospecting strategy for identifying algal strains that have higher biomass yield. A potentially better strategy for identifying new commercial strains of algal for coproduct production may be to identify the highest starch accumulating species.

Substantial progress has, however, been made in identifying algal strains that have stable, long-term biomass production in outdoor environments. Genome sequencing and the development of molecular toolboxes including genetic transformation, enhanced and inducible gene expression cassettes, and genome editing tools will substantially expand the potential of algae as sustainable platforms for the production of high value biomolecules and for carbon sequestration. Progress has been made in improving light use efficiency in photosynthesis resulting in nearly a 200% increase in biomass yield in simulated outdoor growth studies. Surprisingly, there has been less research focus on increasing carbon flux through the CBBC or increasing carbon sink strength to improve biomass yields.

Perhaps, the greatest unrealized potential for improvements in algal biomass yields is through the application of molecular assisted breeding of algae. With the increased availability of annotated algal genomes, isolation of ecotypes from diverse environments, a growing understanding of methods to induce gametogenesis and mating and the availability of high throughput screening and selection systems for identifying fast growing progeny of sexual crosses the future for algae having enhanced biomass yields is bright.

Author contributions

RS drafted the review article. SS contributed the sections on *Chlorella sorokiniana* mating and Figure 1.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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