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Chlamydomonas as a model for biofuels and bio-products production

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SUMMARY

Developing renewable energy sources is critical to maintaining the economic growth of the planet while protecting the environment. First generation biofuels focused on food crops like corn and sugarcane for ethanol production, and soybean and palm for biodiesel production. Second generation biofuels based on cellulosic ethanol produced from terrestrial plants, has received extensive funding and recently pilot facilities have been commissioned, but to date output of fuels from these sources has fallen well short of what is needed. Recent research and pilot demonstrations have highlighted the potential of algae as one of the most promising sources of sustainable liquid transportation fuels. Algae have also been established as unique biofactories for industrial, therapeutic, and nutraceutical co-products. *Chlamydomonas reinhardtii*'s long established role in the field of basic research in green algae has paved the way for understanding algal metabolism and developing genetic engineering protocols. These tools are now being utilized in *C. reinhardtii* and in other algal species for the development of strains to maximize biofuels and bio-products yields from the lab to the field.

Keywords

Chlamydomonas reinhardtii; recombinant proteins; biofuels; bio-products; molecular engineering

INTRODUCTION

Access to affordable and environmentally sustainable fuels and energy sources may be the greatest challenge of this century. With demand continuing to increase and new supplies costing ever more to extract, the availability of fossil fuels will inevitably shrink, resulting in rising energy prices worldwide. With the rising cost of energy also comes the rising cost of food, as food and fuel prices are closely linked. Recently, algae-based biofuels have been highlighted as one of the best current alternative source of renewable energy (Merchant *et al.*, 2011; Georgianna and Mayfield, 2012; Leite *et al.*, 2013; Oncel, 2013). Algae do not compete for arable land, have fast generation times, can grow in salt and waste water, and have the potential to produce more oil per acre than land plants (Dismukes *et al.*, 2008; Demirbas and Demirbas, 2011). While most focus has been on the production of biodiesel,

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algae can also be a source of other fuels, such as bioethanol, biohydrogen, and biogas (Jones and Mayfield, 2011; Oncel, 2013).

While algal biofuels hold significant promise to meet future energy demands, improvements are needed at all levels of production in order to realize this potential. Algal biofuels are not economically viable on their own at current production levels (Brownbridge *et al.*, 2013; Nagarajan *et al.*, 2013). One near-term solution is to couple biofuel production with high-value co-products to increase the commercial value of the entire algal biomass. Current high-value bio-products produced in algae include industrial and therapeutic proteins as well as nutraceuticals and other high-value small molecules (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Rasala and Mayfield, 2014).

As the most characterized algal species with the largest set of genetic tools and techniques, *Chlamydomonas reinhardtii* is an excellent model organism to understand and improve biofuels and bio-products production in algae. *Chlamydomonas reinhardtii* has led the field in the development of molecular tools for strain selection and engineering for green alga. By far, more recombinant proteins have been expressed in *C. reinhardtii* than all other algal species combined (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Rasala and Mayfield, 2014). Studies in *C. reinhardtii* have also helped elucidate the molecular mechanisms behind algal lipid and hydrogen metabolism (Merchant *et al.*, 2011; Torzillo and Seibert, 2013). *C. reinhardtii* is also among the first of the engineered algal species to be studied in commercial settings, which allows academic researchers to begin to understand the challenges of bringing transgenic algae to commercial-scale production (Scoma *et al.*, 2012; Gimpel *et al.*, 2014; Schoepp *et al.*, 2014).

BIOENGINEERING ALGAE

Chloroplast engineering

Over the last 70 years, *C. reinhardtii* has become the flagship alga for laboratory studies and genetic manipulation. The eukaryotic green alga has three modifiable genomes and is capable of producing a wide variety of protein products (Rosales-Mendoza *et al.*, 2012; Barrera and Mayfield, 2013; Rasala *et al.*, 2013b). The efficient manufacture of these products at commercial viability will require a myriad of genetic tools to enhance protein accumulation and bioactivity (Figure 1). To date, the chloroplast genome has been the primary target for engineering protein production, predominantly because it readily performs homologous recombination and is easily transformed (Boynton *et al.*, 1988). Typically, recombinant protein expressed from the chloroplast accounts for 1–10% of total protein (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Rasala and Mayfield, 2014).

One current limitation of chloroplast engineering in *C. reinhardtii* is the use of native promoters to achieve high levels of protein accumulation. The best exogenous gene expression achieved utilizes the endogenous *psbA* promoter (Manuell *et al.*, 2007). However, the effective use of the *psbA* promoter requires that the endogenous *psbA* regulatory region be removed due to a phenomenon known as auto-attenuation (Minai *et al.*, 2006; Rasala and Mayfield, 2011). This renders the organism non-photosynthetic, eliminating one of the unique attributes of algae. One way to alleviate this issue is to reintroduce the *psbA* gene

under the control of a different endogenous promoter (Gimpel *et al.*, 2014). This method results in photosynthetic *C. reinhardtii* that can produce recombinant proteins, albeit at reduced levels compared with the non-photosynthetic versions. Exogenous transcriptional regulation machinery have been used successfully in *C. reinhardtii* (Kato *et al.*, 2007), however exogenous promoters remain an underutilized resource for chloroplast engineering. Synthetic regulatory elements, such as UTRs, that modify gene expression have also been used in the *C. reinhardtii* chloroplast (Specht and Mayfield, 2012), although the successful use of fully synthetic promoters has yet to be described. A full understanding of synthetic promoter synthesis would allow for fine-tuning of gene expression, which is particularly useful for metabolic engineering.

In addition to engineering strains to produce maximal amounts of recombinant protein, chloroplast engineering is necessary for developing strains for robust growth, particularly in outdoor settings. Light intensity and availability is a major concern for maximizing growth rates and cell density. Excessive solar irradiation leads to photoinhibition, while low light flux due to weather or cell culture density can lead to reduced photosynthetic activity and slower growth. Chloroplast engineering for improved function under varying light conditions would allow maximum production of algae throughout the year. Early data have shown that the light antenna of *C. reinhardtii* can be modified to alter chlorophyll content and subsequently adjust its absorbance spectrum, altering photosynthetic productivity (Kirst *et al.*, 2012).

An alternative method of chloroplast engineering to alter photosynthetic activity has focused on the protein subunits of the photosystem itself. Photosystem II is a highly conserved protein complex, which performs the initial rate limiting step of photosynthesis. It is known that PSII is in part responsible for enhanced fitness in various light conditions (Mulo *et al.*, 2012). Recently, the D1 protein of *C. reinhardtii* was replaced with the homologous D1 proteins from *Synechococcus sp. 7942* (Vinyard *et al.*, 2013, 2014). These studies showed that complementation of the native D1 protein with natural variants from other species could increase photosynthetic efficiencies under varying light conditions, and also that this increased efficiency led to improved overall growth. In addition, due to *C. reinhardtii*'s unique low fluorescence background, these studies were the first to elucidate the mechanisms behind the optimized photosynthetic activity. Thus, not only were improved strains generated, but *C. reinhardtii* proved to be an ideal model for understanding PSII function.

Nuclear engineering

Although the chloroplast can effectively accumulate high levels of recombinant protein, nuclear transformation technology is required to fully engineer algae. Nuclear expression allows for organelle targeting or secretion of proteins, and enables more complex post-translational modifications of proteins such as glycosylation. In particular, proper protein localization is required for metabolic engineering, which relies on compartmentalization for some steps in syntheses. The nuclear genome of *C. reinhardtii* has been much more difficult to develop for recombinant protein production than the chloroplast. Low protein accumulation levels due in part to transgene silencing and insertion positional effects have

limited the use of the nuclear genome from a protein manufacturing standpoint (Cerutti *et al.*, 1997; De Wilde *et al.*, 2000; Wu-Scharf *et al.*, 2000). However, recently it was shown that fusing recombinant proteins to the Sh Ble antibiotic resistance gene, which requires high protein accumulation, could substantially increase recombinant protein accumulation (Rasala *et al.*, 2012). In addition, *C. reinhardtii* lacks strong constitutive promoters comparable to the viral promoters used in plants. While hybrid promoters have had some success in increasing transgene expression in *C. reinhardtii* (Schroda *et al.*, 2000), truly synthetic promoters may hold great promise for tight regulation of robust recombinant protein expression (Venter, 2007).

Homologous recombination

Homologous recombination (HR) allows for targeted gene knock-outs as well as targeted insertion for reducing positional effects for recombinant gene expression. This mechanism has enabled sophisticated engineering in the *C. reinhardtii* chloroplast; however at present, HR does not occur at sufficient levels in the nuclear genome to be of practical use. Knockdowns have been created by artificial microRNAs, which is also a valuable tool for genetic engineering (Molnar *et al.*, 2009; Zhao *et al.*, 2009; Moellering and Benning, 2010; Schmollinger *et al.*, 2010). Alternatively, a knockout strain library is currently being developed by insertional mutagenesis. Because of the scale of this project, a high-throughput screen known as ChlaM-meSeq has been established to identify genes disrupted by insertional mutagenesis. In a pilot screen, a pool of insertional mutants was shown to cover 39% of known protein coding genes (Zhang *et al.*, 2014). Although each of these methods have been valuable for progress of the field, a rapid method for developing novel knock-outs and targeted gene introduction is essential for future development of *C. reinhardtii.*

To date, methods of identifying HR in *C. reinhardtii* have not been reliable, nor have there been ways to significantly increase the low basal rate of HR in Chlamydomonas (Sodeinde and Kindle, 1993; Gumpel *et al.*, 1994; Nelson and Lefebvre, 1995). However, there have been many advances in targeted gene delivery across other platforms in the past decade. Transcription-activator-like effectors (TALEs) allow one to design a unique DNA-binding target sequence and the addition of a nuclease can achieve targeted restriction digestion and subsequent homology directed repair (Li *et al.*, 2011). Successful gene targeting by a TALE has been shown by a subsequent increase in endogenous ARS expression (Gao *et al.*, 2014). However, to this point, TALENs have not successfully been utilized for targeted gene disruption in *C. reinhardtii*. The CRISPR/Cas9 system is an alternative method for inducing targeted gene delivery. Here, a guide RNA will direct restriction by the Cas9 nuclease to a specific sequence of DNA. The CRISPR/Cas9 system has been successful in mammals, fish, fungus, and plants (tobacco, Arabidopsis, sorghum and rice; Jiang *et al.*, 2013). Cas9 has recently been transiently expressed in *C. reinhardtii*, although stable cell lines have not been established (Jiang *et al.*, 2014a). Successful transient expression shows that Cas9 is capable of modifying the *C. reinhardtii* genome.

The *C. reinhardtii* nuclear genome generally performs repairs of double-stranded breaks almost exclusively by non-homologous end joining (NHEJ) and errors are often introduced during the process. Unfortunately, because *Chlamydomonas* relies so heavily on NHEJ as a

repair mechanism, even if TALENs or the CRISPR/Cas9 systems are developed in *C. reinhardtii*, it is unlikely that a large percentage of gene delivery will occur by targeted homology repair. NHEJ machinery is comprised of three main subunits: Ku70, Ku80, and DNA ligase IV. In yeast, Ku70 and Ku80 form a heterodimer that binds to double stranded breaks and recruits DNA ligase IV to mediate NHEJ (Kanaar *et al.*, 1998). The *C. reinhardtii* genome contains homologues to each of these genes. Based on a study in *Pichia pastoris*, one strategy may be to stifle expression of these repair proteins in *Chlamydomonas* in order to increase the level of HR activity (Näätsaari *et al.*, 2012).

High-throughput screening

High-throughput screening allows detection of rare phenotypes including those following gene insertion or mutagenesis. One of the fastest quantitative methods of screening mutants is fluorescence-activated cell sorting (FACS). This technique has been utilized for detection of high lipid accumulating mutants by a process utilizing Nile Red, a lipid dye, to analyze lipid levels in a set of mutants generated using EMS (Xie *et al.*, 2014). This method successfully isolated *C. reinhardtii* strains that accumulated 23–58% higher fatty acid content by dry mass when compared to their parent strain. Alternatively, Terashima *et al.* (2014) developed a high lipid-accumulating mutant pool by insertional mutagenesis, a strategy termed *Chlamydomonas* High Lipid Sorting (CHiLiS). This method generates a detectable insertion that can be used to rapidly identify the disrupted gene(s). Characterization of the strains isolated by this method showed a significant increase in triacylglycerol accumulation. These papers demonstrate that rapid screening can identify mutants with altered metabolism for the production of biofuels, which can easily be translated beyond *C. reinhardtii.* In addition to fluorescent stains, fluorescent protein reporters in *C. reinhardtii* have been shown to effectively sort using FACs (Rasala *et al.*, 2013a). By tagging recombinant proteins with fluorescent protein tags, production strains can be rapidly screened and genes that affect recombinant protein accumulation can be identified. Fluorescent reporters fused to antibodies have also been successfully used to sort for wild isolates with specific cell wall components (Jiang *et al.*, 2014b). Development of techniques using these reporters will be necessary to rapidly isolate and engineer production strains.

Ultimately, high-throughput screening technologies will be an essential tool for generating strains for outdoor production. High-throughput screening allows one to select exclusively based on desirable traits, rather than validating transformation with linkage to an antibiotic marker. Horizontal gene transfer of antibiotic resistance cassettes to environmental microbes poses a theoretical threat to human health and safety. Therefore, high-throughput screening mechanisms that avoid generation of transgenic algae with drug resistant markers, and that can be scaled up to outdoor ponds for economically feasible biofuels production, are an area where more research is required.

ALGAL BIOFUELS

Biodiesel

Chlamydomonas reinhardtii has been a key model in understanding complex algae lipid metabolism. Current efforts have focused on the use of triacylglycerols (TAGs) as a first generation biodiesel. TAGs from algae are also of interest as alternatives to plant-based edible oils (Klok *et al.*, 2014). Traditionally, *C. reinhardtii* has not been seen as an oleaginous species (James *et al.*, 2011). However, studies have shown that natural levels of lipids can vary widely in *C. reinhardtii* strains (Siaut *et al.*, 2011). In addition, wild-type and mutant *C. reinhardtii* can accumulate significant amounts of TAGs in response to nitrogen or salt stress (Wang *et al.*, 2009; Li *et al.*, 2010; Work *et al.*, 2010; Goodson *et al.*, 2011; James *et al.*, 2011; Siaut *et al.*, 2011). In fact, *C. reinhardtii* studies have helped elucidate the mechanisms behind lipid accumulation in response to nitrogen deprivation (Boyle *et al.*, 2012; Msanne *et al.*, 2012; Goodenough *et al.*, 2014). *Chlamydomonas reinhardtii* has also been an excellent model to identify algal genes involved in TAG metabolism (Merchant *et al.*, 2011; Klok *et al.*, 2014).

Targeted overexpression of putative TAG metabolic genes in *C. reinhardtii* has been met with mixed success. Overexpression of type 2 diacylglycerol acyltransferases (DGATs) DGAT2–1 and DGAT2–5 led to increased lipid content, while DGAT2-a,b,c overexpression had no effect (Deng *et al.*, 2012; La Russa *et al.*, 2012). Overexpression of acyl-ACP (acyl carrier protein) esterase (AAE) led to an altered lipid profile but not an increase in lipid content (Blatti *et al.*, 2011). It is clear that even in *C. reinhardtii*, a better understanding of lipid metabolism is required in order to fully utilize algae's potential as a source of TAGs for biodiesel or edible oil production.

Biohydrogen

As an alternative to liquid fuels, *C. reinhardtii* has also been extensively studied as a model for the photoproduction of biohydrogen. Renewed interest in *C. reinhardtii* biohydrogen production began when significant and sustained H_2 production was demonstrated in sulfur starved strains (Melis *et al.*, 2000). The theoretical maximum light conversion efficiency for H_2 production is 13.4%, which is higher than the 11.2% limit for biodiesel (Torzillo and Seibert, 2013). In addition, purities of up to 98% can be achieved without the need of extraction (Torzillo *et al.*, 2009). However, several factors significantly limit the actual production of H_2 in algae. The most significant limitation is the sensitivity of hydrogenase to O_2, which inhibits hydrogenase activity by affecting transcription and protein maturation (Ghirardi *et al.*, 2007). Additional constraints include competition for electrons from alternative pathways, inefficient light conversion of large light-harvesting antennae, and inherent limitations of anaerobic growth (Torzillo and Seibert, 2013).

Studies in *C. reinhardtii* to address these limitations have included the generation of improved strains through either mutagenesis or targeted genetic engineering (Melis *et al.*, 2007; Esquível *et al.*, 2011; Torzillo and Seibert, 2013; Baltz *et al.*, 2014; Xu *et al.*, 2014). Studies have also shown significantly improved H_2 production through the optimization of growth conditions including periodic sulfur starvation (Melis *et al.*, 2000) and optimized

light, media and photobioreactor conditions (Torzillo and Seibert, 2013; Oncel *et al.*, 2014). However, despite these optimizations, the best light conversion efficiency reached has been approximately 3%. Even if these optimal conditions could be perfectly scaled to outdoor growth, the cost of algal H_2 would be well over US$8 per gallon gasoline equivalent (gge) (James *et al.*, 2009). However, continued research holds promise to optimize algae strains and growth conditions to make biohydrogen a competitive fuel in the future.

HIGH-VALUE BIO-PRODUCTS

Production of recombinant proteins in *C. reinhardtii* has been a fruitful area of research in recent years. Several recent articles have thoroughly reviewed the plethora of protein products that have been successfully expressed within *C. reinhardtii* (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Rasala and Mayfield, 2014). To date, 34 different protein co-products have been produced in algae, 29 of which were produced in *C. reinhardtii.*

The vast majority of targeted proteins have been therapeutic proteins. With a current market value of US$140 billion, therapeutic proteins provide an attractive co-product target to increase the economic value of algal biomass (Walsh, 2014). Therapeutic proteins produced include the UV-protectant metallothionein (Zhang *et al.*, 2006), antibody mimics 14FN3 and SAA-10FN3 (Rasala *et al.*, 2010), and anti-cancer proteins TNF-Related Apoptosis Inducing Ligand (TRAIL; Yang *et al.*, 2006), and allophycocyanin (Su *et al.*, 2005). The glycoprotein hormone erythropoietin has also been produced from the *C. reinhardtii* nucleus, demonstrating the ability to generate recombinant proteins with glycosylation, the most common post-translational modification found on protein therapeutics (Eichler-Stahlberg *et al.*, 2009; Walsh, 2014).

The largest class of therapeutic proteins that have been produced in *C. reinhardtii* are subunit vaccines (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Rasala and Mayfield, 2014). Vaccines are a particularly attractive target because algae are generally regarded as safe (GRAS) by the FDA. Therefore, if orally available vaccines can be produced, these vaccines may be stored and administered as lyophilized algal pellets. Edible algal vaccines would reduce the cost of vaccines by orders of magnitude and vastly increase their availability since algal vaccine pellets do not need to purified, do not require needle injection, and can be stored for up to 20 months at RT (Dreesen *et al.*, 2010; Gregory *et al.*, 2013).

Chlamydomonas reinhardtii has also been shown to be able to produce a novel type of antibody therapeutic. Monoclonal antibodies (mAbs) are the largest growing segment of protein therapeutics with almost 27% of the new approvals and six of the top 10 selling biotherapeutics products in 2013 (Walsh, 2014). In particular, antibody-drug conjugates (ADCs) are gaining in popularity as more effective cancer treatment. The most significant drawback of ADCs is the requirement for chemical linkage of the toxic molecule, which leads to increased production costs and potential off-target toxicity of free toxins. In contrast, the unique environment of the *C. reinhardtii* chloroplast is able to produce immunotoxins in which toxic proteins are genetically linked to the targeting antibody (Tran

et al., 2013a,b). This reduces the cost of production since the immunotoxin is produced as a single protein and this production method can also help eliminate off-target toxicity.

Protein co-products expressed in *C. reinhardtii* also include a range of nutraceuticals for use as feed additives and/or human nutritional supplements. Feed additives include phytase, xylanase, and flounder growth hormone (Kim *et al.*, 2002; Yoon *et al.*, 2011; Georgianna *et al.*, 2013). Mammary-associated serum amyloid A (MAA) is one of the best expressed bio-products in *C. reinhardtii*, accumulating to over 5% of total soluble protein (Manuell *et al.*, 2007). MAA can be used as a prophylaxis against enteric bacterial infections in humans as well as replacement for antibiotics in animal feed (Larson *et al.*, 2003; Mack *et al.*, 2003). Recombinant proteins have also been engineered to increase high-value nutritional compounds in *C. reinhardtii* such as organic selenium (Hou *et al.*, 2013), carotenoids (Baldo *et al.*, 2011; Couso *et al.*, 2011), and triacylglycerols (La Russa *et al.*, 2012). Carotenoids and triacylglycerols have the added benefit of being used in biofuels formulations (Merchant *et al.*, 2011; Peralta-Yahya *et al.*, 2011).

OUTDOOR CULTIVATION SYSTEMS

Large-scale microalgae cultivation can occur in either open or closed systems (Ugwu *et al.*, 2008; Singh *et al.*, 2011). Open systems range from small artificial ponds to large open bodies of water; however the most productive open systems currently implemented are shallow raceway ponds (Singh *et al.*, 2011). Although these open systems require large areas of land, they can be constructed in arid environments and avoid competition with preexisting agriculture (Georgianna and Mayfield, 2012). Raceways can be constructed relatively cheaply and require little maintenance (Singh *et al.*, 2011; Schoepp *et al.*, 2014). Open systems are inherently susceptible to contamination and thus are predominantly used to culture only robust photoautotrophic species (Ugwu *et al.*, 2008; Rasala and Mayfield, 2014). Heterotrophic growth in open systems is not a viable option due to the heightened susceptibility of contamination. Greenhouses can be used to cover smaller open systems in order to deter contamination and potentially increase productivity. Additionally, greenhouses allow for the growth of genetically engineered strains in contained ‘open’ systems, but these structures will add to the initial cost of a facility.

Closed systems are highly variable in design but overall provide a more productive environment while protecting the culture from contamination (Ugwu *et al.*, 2008; Singh *et al.*, 2011). More strains of algae can be cultivated in these closed systems, including mixotrophic and hydrogen-producing species of microalgae (Scoma *et al.*, 2012; Gimpel *et al.*, 2014). The productivity and sterility benefits of a closed system are countered by their heightened costs as they may cost up to 10 times that of open systems due to the additional infrastructure and operational costs (Del Campo *et al.*, 2007).

Recently, pilot facilities containing a variety of these systems have been built to test strains in a setting more comparable to conditions encountered at commercial facilities (Scoma *et al.*, 2012; Gimpel *et al.*, 2014; Schoepp *et al.*, 2014). Experiments with *C. reinhardtii* have been conducted in these outdoor systems to test the viability of the strain as a producer of biomass, biohydrogen and even high-value bio-products. These studies are invaluable for

understanding and addressing the challenges of bringing laboratory strains into commercial production.

OUTDOOR CULTIVATION OF *C. REINHARDTII*

Researchers at the University of California, San Diego have developed one of the first systems to measure research-scale production of algal strains in closed bags and open ponds. Biomass production (g L^{-1} day^{-1}) was reported for several algae and cyanobacteria species (Table 1; Schoepp *et al.*, 2014). *Chlamydomonas reinhardtii* (CC-1690) out produced eight other species in a 100-L closed system, but production was reduced by 80% when grown in an open 800-L pond. Lipid content was not measured in this study; however, reported average lipid content in *C. reinhardtii* is comparable to other species (Table 1; Griffiths and Harrison, 2009). This suggests that *C. reinhardtii* is competitive with other algal species in closed systems. While closed systems may be economical for high-value products, the cost and size limitations of closed systems may hinder sufficient production of lipid-accumulating strains for biofuel production (Figure 1).

Chlamydomonas reinhardtii was also tested in an outdoor system for its ability to produce biohydrogen gas. Scoma *et al.* cultivated *C. reinhardtii* heterotrophically in a 50-L tubular closed system. By starving the culture of sulfur, photosystem II was down-regulated to the point where oxygen consumption was greater than oxygen evolution, creating the necessary anaerobic conditions for biohydrogen production. However, the combination of uncontrolled solar intensities and nutrient deprivation can lead to photo-damage and the production of reactive oxygen species in microalgae, thus hindering production. To address this photoinhibition, Scoma *et al.* acclimated their cultures to natural light for 1 week before experimentation to induce physiological changes such as reduced chlorophyll, higher levels of xanthophylls, and increased photosynthetic and respiratory rates. When cells were allowed to acclimate to solar light, a more robust photoprotection system was observed, but biohydrogen production was still only equal to 20% of the laboratory results.

Recombinant protein production in *C. reinhardtii* has been demonstrated in a pilot commercial setting. Bovine Milk Amyloid A (MAA), a protein with anti-microbial properties, has previously been produced to high levels in *C. reinhardtii* in the laboratory (Manuell *et al.*, 2007). High accumulation of MAA in this strain required removal of the *psbA* gene, thus making the strain non-photosynthetic and unfit for outdoor growth. Recently, *psbA* was rescued using the alternative regulatory regions to eliminate issues of auto-attenuation (Gimpel *et al.*, 2014). The best rescued strain was then grown in 100-L hanging bags and accumulated MAA at approximately 3 mg L^{-1} of culture for a maximum yield of 11.8 g MAA kg^{-1} dry weight biomass. Even with lower photosynthetic efficiency and lower total soluble protein production versus the knockout laboratory strain, the production of MAA in *C. reinhardtii* has the potential to be highly profitable since it can be administered as a solid algal pellet at a cost 60 times lower than traditional purified bovine colostrum MAA.

CONCLUSION

Chlamydomonas reinhardtii with its extensive research history and genetic tool infrastructure has been an excellent system to begin to understand algal metabolism and strain development. *Chlamydomonas reinhardtii*'s ease of transformation, large toolset, and natural variety has led to strains with significantly increased biofuels and bio-product yields. The biggest successes with *C. reinhardtii* have been the production of high-value products at laboratory scale in closed systems (Almaraz-Delgado *et al.*, 2013; Barrera and Mayfield, 2013; Gimpel *et al.*, 2014; Rasala and Mayfield, 2014). However, *C. reinhardtii* remains limited by its naturally low oil content, sensitivity to high solar irradiation and its weak growth in outdoor ponds, thus making it a relatively poor choice for large-scale biofuels production (James *et al.*, 2011; Scoma *et al.*, 2012; Schoepp *et al.*, 2014). While genetic engineering may help to overcome some of *C. reinhardtii*'s limitations, it will be essential to also expand the knowledge and tools developed in *C. reinhardtii* to other algal species.

Algae are the most diverse organisms on the planet, able to grow in almost any environment, from marine systems to hot springs to desert soil crusts and even sewer drains (Norton *et al.*, 1996; Dufresne *et al.*, 2008; Parker *et al.*, 2008; Tirichine and Bowler, 2011; Blunt *et al.*, 2012). Through these adaptations, strains have naturally developed favorable traits for specific manufacturing applications. Many wild-type species of algae will be economically viable as production platforms for bio-products with minimal strain optimization. For instance, cold-tolerant microorganisms have to maintain higher levels of polyunsaturated fatty acids to maintain membrane fluidity at colder temperatures. These extremophiles make obvious choices for sources of omega-3 fatty acids. To date, several natural species have already demonstrated robust growth in commercial-scale systems, mostly for nutraceuticals (Oncel, 2013). In addition, over the past 20 years, we have expanded the use of genetic tools to a wide variety of algal species, albeit with varying success. These tools will be needed to engineer an arsenal of pest-resistant strains with optimal growth rates and production capabilities as well as to utilize algae as a protein biomanufacturing platform. The combination of improved strain selection and engineering in more diverse species is our best chance of developing algal strains for large-scale renewable energy and sustainable recombinant protein production in the future.

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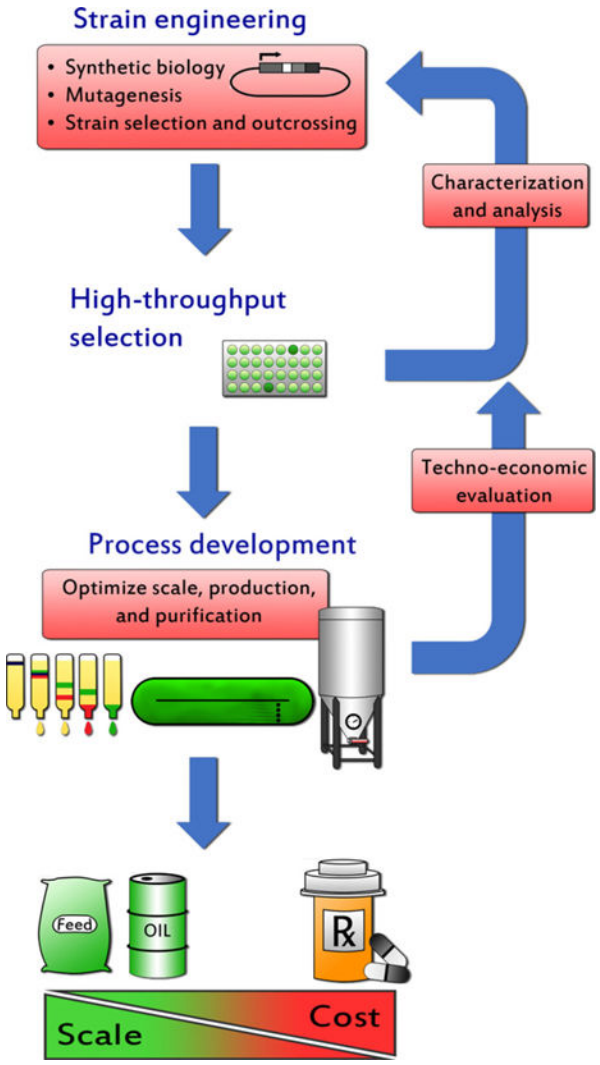

Figure 1.

Steps to engineering a production strain of green algae. Genetic manipulation using advanced tools will result in a desired phenotype. The isolated strain will be tested for scale-up and reanalyzed for characteristics such as growth rate, population density, recombinant protein accumulation, and lipid profile. If these traits do not overcome economic constraints of production, additional genetic manipulation in the laboratory is required. Once an ideal strain is identified, fuels and co-products can be extracted from scaled-up cultures.

Table 1

Biomass production of various algal species in an outdoor setting compared to average reported lipid content

Species	Closed system production ($g\ L^{-1}\ day^{-1}$)	Open system production ($g\ L^{-1}\ day^{-1}$)	Lipid content (nutrient-replete, % dry weight)
Scenedesmus dimorphus	0.095	0.090	26
Chlorella vulgaris	0.047	0.035	25
Chlamydomonas reinhardtii	0.078	0.015	21
Arthrospira platensis	0.040	0.018	13
Anabaena sp.	0.055	–	5
Porphyridium purpureum	0.074	0.036	11
Nannochloropsis salina	0.043	0.028	27
Dunaliella tertiolecta	0.039	0.031	15
Phaeodactylum tricornutum	0.021	–	21

Biomass production data from Schoepp *et al.* (2014) and lipid content from Griffiths and Harrison (2009).