ADVANCED TECHNOLOGIES AND PERSPECTIVES ON SUSTAINABLE MICROALGAE PRODUCTION

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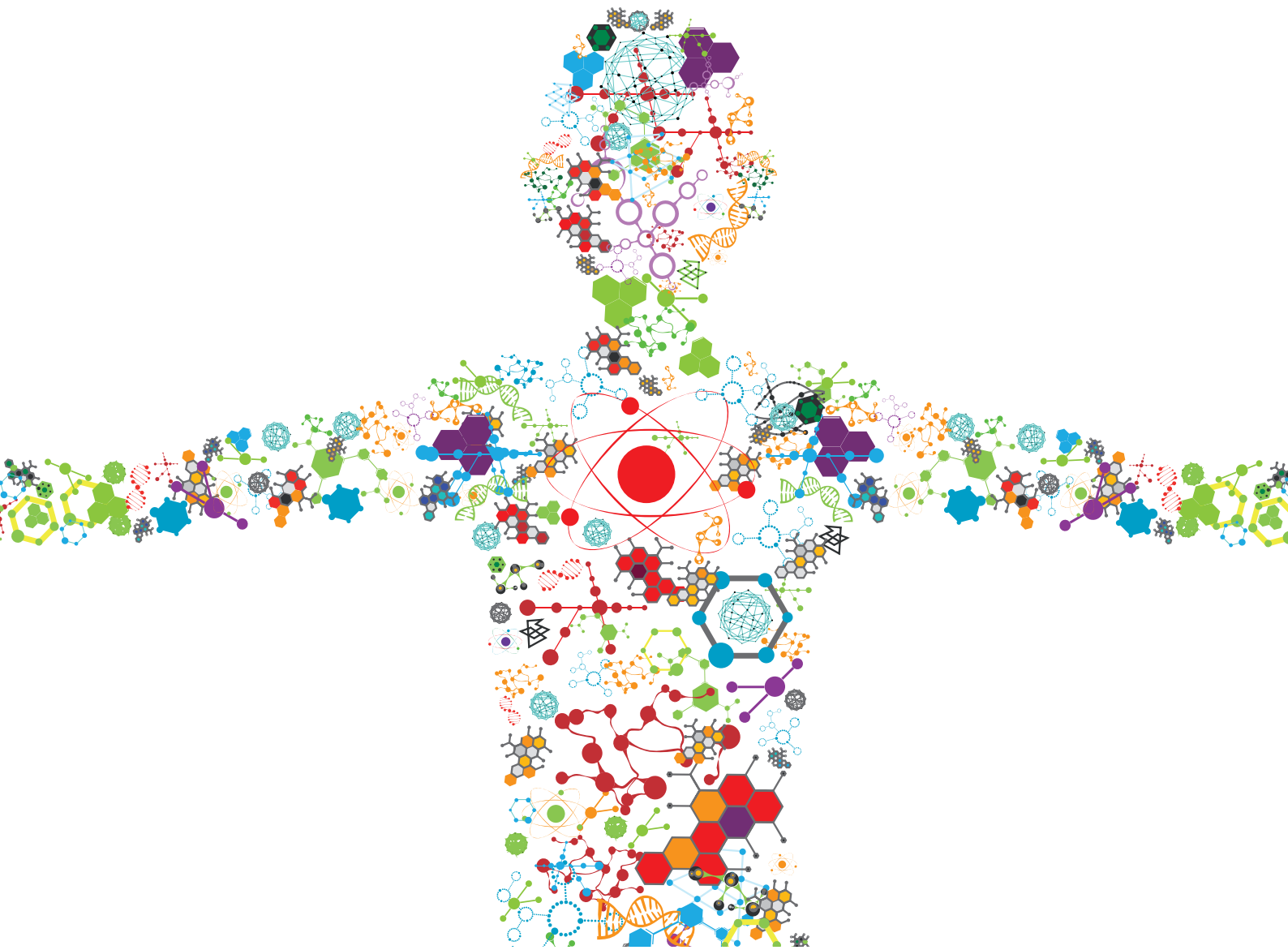

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ADVANCED TECHNOLOGIES AND PERSPECTIVES ON SUSTAINABLE MICROALGAE PRODUCTION

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Dr. Fan holds patents related to microalgae production. The other Topic Editors declare no competing interests with regards to the Research Topic theme

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EDITORIAL
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Editorial: Advanced Technologies and Perspectives on Sustainable Microalgae Production

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Keywords: algae, sustainable food production, safe environment, carbon sequestration, omega-3 fatty acid food security

Editorial on the Research Topic

Advanced Technologies and Perspectives on Sustainable Microalgae Production

Algae are photoautotrophs like plants, produce their food by fixing atmospheric carbon dioxide in the presence of sunlight. They produce half of the total atmospheric oxygen, hence supporting all lifeforms on the earth. They can be unicellular or multicellular, microalgae or macroalgae and occur naturally in moist areas, freshwater, and marine environments. Being the primary producers in marine ecosystems, they form the base of most aquatic food webs. They multiply rapidly, have short generation time, and hence can spread across large areas in less time. They can withstand harsh environments without compromising survival due to their highly adaptive nature and can accumulate a wide range of products like fatty acids (oils), carbohydrates, chromophores (carotenoids, chlorophyll, phycobiliproteins), antioxidants, vitamins, enzymes, polymers, peptides, toxins, and sterols. These high-value products find diverse applications in biofuel production, food/feed supplements, nutraceuticals, cosmetics, natural colouring agents, and therapeutic and pharmaceutical fields. Apart from these, algae became extensively used in bioremediation, wastewater treatment, biochar and biofertilizers.

Microalgae has gained high interest in the global bioenergy sector as a third-generation biofuel feedstock with, potential to meet global transportation fuel demand. The algal biomass serves as sources of biofuels such as biodiesel, bioethanol, biohydrogen, biomethane, and gasoline. Algae-based fuels are carbon-neutral because they tend to not disturb carbon emission-fixation balance and could significantly reduce the rising threats of global warming, unlike coal-based fossil fuels. Microalgal strains with higher lipid and/or fermentable sugar content are exploited for biodiesel and bioethanol production. These strains are isolated, cultivated under controlled conditions of optimized pH, temperature, nutrients, and light in photobioreactors and open raceway ponds over large areas, followed by harvesting and downstream processing to obtain the required product. The natural oil content in algae comes in the range of 20–50 percent of its dry weight and the oil yield far exceeds that from terrestrial crops. However, algal cultivation requires higher maintenance and harvesting costs, and advanced technologies for conversion to biofuels. Achieving indefinite availability of algal biofuels in higher volumes for fulfilling future demands is a big challenge in terms of capital investment and technological support. This makes algal-based biofuel production not commercially viable and economically feasible when compared to other conventional biofuels.

Various cost-effective strategies for improving commercial viability of algae-based biofuels have been proposed, one of them being co-production of value-added products from algae. The algal biomass is a rich natural source of antioxidants, polyunsaturated fatty acids (PUFAs), vitamins, and minerals and has the potential to serve as nutrient-enriched supplements to food diets, in therapeutic, cosmetic, and

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pharmaceutical applications. Their supplementation in small quantities would be suffice for meeting adequate nutritional demands of the larger population. Microalgal PUFAs like omega-3-fatty acids [Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA)] and omega-6-fatty acids [Arachidonic acid and gamma-linolenic acid (GLA)] offer many health-promoting benefits and treatment of diseases related to the cardiac and vascular system, nervous system, arthritis, obesity, mental health, and various autoimmune disorders. The supplementation of these nutrient-rich algal sources into dietary intakes of poultry, aquaculture, animal feeds and human diets enhance food quality and overall health of the consuming population. This ensures greater food security to maintain the nutritional status of population, foreseeing a disease-free healthy future. Considering the reckless usage of fossil fuels and shrinking of cultivable land, the Alga, primary producer of aquatic ecosystem may gain a significant place in ensuring the food security, environment conservation by sequestering CO_2 and liberation of oxygen in near future.

AUTHOR CONTRIBUTIONS

MA-Concept, foresight, drafting and communication.

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ORIGINAL RESEARCH
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Isolation and Characterization of Novel Chlorella Vulgaris Mutants With Low Chlorophyll and Improved Protein Contents for Food Applications

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Microalgae are widely used as food supplements due to their high protein content, essential fatty acids and amino acids as well as carotenoids. The addition of microalgal biomass to food products (e.g., baked confectioneries) is a common strategy to attract novel consumers. However, organoleptic factors such as color, taste and smell can be decisive for the acceptability of foods supplemented with microalgae. The aim of this work was to develop chlorophyll-deficient mutants of *Chlorella vulgaris* by chemically induced random mutagenesis to obtain biomass with different pigmentations for nutritional applications. Using this strategy, two *C. vulgaris* mutants with yellow (MT01) and white (MT02) color were successfully isolated, scaled up and characterized. The changes in color of MT01 and MT02 mutant strains were due to an 80 and 99% decrease in their chlorophyll contents, respectively, as compared to the original wild type (WT) strain. Under heterotrophic growth, MT01 showed a growth performance similar to that of the WT, reaching a concentration of 5.84 and 6.06 g L^{-1}, respectively, whereas MT02 displayed slightly lower growth (4.59 g L^{-1}). When grown under a light intensity of 100 μmol m^{-2} s^{-1}, the pigment content in MT01 increased without compromising growth, while MT02 was not able to grow under this light intensity, a strong indication that it became light-sensitive. The yellow color of MT01 in the dark was mainly due to the presence of the xanthophyll lutein. On the other hand, phytoene was the only carotenoid detected in MT02, which is known to be colorless. Concomitantly, MT02 contained the highest protein content, reaching 48.7% of DW, a 60% increase as compared to the WT. MT01 exhibited a 30% increase when compared to that of the WT, reaching a protein content of 39.5% of DW. Taken together, the results strongly suggest that the partial abrogation

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of pigment biosynthesis is a factor that might promote higher protein contents in this species. Moreover, because of their higher protein and lower chlorophyll contents, the MT01 and MT02 strains are likely candidates to be feedstocks for the development of novel, innovative food supplements and foods.

Keywords: heterotrophic cultivation, microalgae, nutritional applications, pigments, protein, random mutagenesis, scale-up

INTRODUCTION

The consumer demand for health-promoting and nutritional-rich foods has been increasing over the last few years. Microalgae are a sustainable biological resource with a well-balanced biochemical profile, rich in protein and bioactive compounds such as carotenoids and essential fatty acids that provide potential benefits for human health (Lucas et al., 2018). Nevertheless, from the thousands of microalgal strains currently described and identified, only a narrow number of strains are currently approved for human consumption. In the EU, *Arthrospira platensis* ("spirulina") and *Chlorella vulgaris* are approved for human consumption due to a long history of safe use, being well-established in the market, while odontella aurita and tetraselmis chui were recently approved as novel foods by the european food safety authority (EU, 2017/2470).

Microalgal biomass is widely commercialized worldwide in the nutraceutical sector as food supplements (e.g., tablets and capsules), while in the food market they are normally incorporated as a natural food colorant or as a healthy supplement, able to enhance the nutritional value of conventional food products (e.g., bars, pasta and cookies; Sahni et al., 2019). Nevertheless, the incorporation of microalgae in food products faces challenges mainly due to their organoleptic characteristics, including a strong color, taste and odor (Lafarga, 2019). The sensory attributes of foods are directly linked to the consumer acceptance whereby the color is the first parameter observed by the consumer and can be decisive for whether or not to include the food in their diet (Delwiche, 2012). Therefore, microalgal-based food products that are usually green in color comes with very low sensorial acceptance by the consumer. Moreover, chlorophyll, the pigment responsible for the green color of microalgae and higher plants, usually imparts a grassy taste to tea (van Lelyveld and Smith, 1989). Therefore, these less favorable organoleptic characteristics of microalgal biomass need to be modified in order to improve its acceptance in food products.

Alternative strategies to improve the organoleptic qualities of food containing microalgal biomass have included the extraction of the target compounds with the concomitant removal of chlorophyll or the addition of ingredients such as chocolate to improve the final flavor and color (Lucas et al., 2018). Another option could be isolation of novel microalgal strains with improved organoleptic characteristics. Random mutagenesis is an interesting cell modification tool for food applications, as it is not considered a method that generates genetic modified organisms (GMOs), because it does not introduce any foreign genetic material into the target cell (Zimny et al., 2019, directive 2001/18/ec). By exposure of the target cells to physical (e.g., UV light) or chemical mutagenic agents (e.g., ethyl methane sulfonate), strains with improved characteristics are generated. Upon mutagenesis, it is important to apply a selection procedure to screen for the desired mutants, e.g., abiotic stress factors such as light intensity. Furthermore, when the genes of the carotenoid biosynthetic pathway are targeted, specific inhibitors can be used such as compactin, diphenylamine, nicotine or norflurazon (Cordero et al., 2011; Chen et al., 2017; Huang et al., 2018).

Accordingly, the aim of this work was to develop chlorophyll-deficient mutants of *C. vulgaris* by chemically induced random mutagenesis in order to obtain biomass with different pigmentations for nutritional applications. The heterotrophic growth performance under light and dark conditions of wild type (WT) and established mutants was evaluated as well as their proximate biochemical composition and pigment profile. One of the mutants was scaled up to evaluate the growth performance in 5-L and 200-L fermenters and determine their feasibility as future feedstocks for the food industry.

MATERIALS AND METHODS

Wild Type Inoculum and Growth

Chlorella vulgaris was obtained from Allmicroalgae Natural Products S.A. culture collection. The cryopreserved cultures stored in liquid nitrogen were transported to the Center of Marine Sciences (University of Algarve) on dry ice. The inoculum was transferred to a 50 mL centrifuge tube containing 20 mL of culture medium, comprising 0.1% glucose, 0.25% yeast extract and 0.5% peptone. The culture was later divided into several 250-mL Erlenmeyer flasks with a working volume of 50 mL containing the same medium and incubated in an orbital shaker at 28 ± 2°C under constant shaking (100 rpm).

Random Mutagenesis and Selection of Chlorophyll-Deficient Mutants

Cells of *C. vulgaris* growing exponentially (3.2×10^6 cells mL^{-1}) were concentrated 10-fold by centrifugation (3,000 *g*, 3 min) and treated with 150, 200, 250, 300, 350, and 400 mM ethyl methane sulfonate (EMS, Merck, USA) for 1 h under mild agitation in the dark (FAO/IAEA, 2018). By addition of sodium thiosulfate to a final concentration of 5%, the reaction of EMS was stopped, and cells were pelleted by centrifugation at 3,000 *g* for 3 min. Cells were washed thrice with sterile distilled water and incubated for 24 h in the dark to prevent light-dependent DNA repair. For the determination of the cell survival rate, cells were plated onto Plate Count Agar (PCA; VWR, Portugal) in serial dilutions and incubated at 30°C for 72 h in the dark. The mutant

selection was carried out by visual observation of the plates in dim light. A colony with yellow color was picked, sub-cultured several times on PCA plates and subsequently transferred into liquid media. This yellow mutant was grown to exponential phase and subjected to a second round of random mutagenesis using 300 mM EMS. This time, mutant selection was performed on PCA plates with the carotenoid biosynthesis inhibitor norflurazon, which blocks phytoene desaturase (Breitenbach et al., 2001; Koschmieder et al., 2017). To choose the lowest concentration that inhibits cell growth of the mutant, cells were previously spread onto 2, 4, 8, and 10 μM of norflurazon plates. Only at 10 μM the authors obtained plates without any colonies, whereas lower concentrations led to a lawn of cells. Therefore, upon mutagenesis, cultures were plated onto PCA plates containing 10 μM of norflurazon and incubated at 30°C in the dark for 1 week. Herbicide-resistant white colonies were sub-cultured several times, first on plates containing norflurazon and afterwards on plates without herbicide to confirm the phenotypic stability of the mutants.

Experimental Trials in Erlenmeyer Flasks

Experimental trials were conducted to evaluate the heterotrophic growth performance and biochemical composition of WT and established mutants under dark and light conditions. The trial was conducted in 250-mL Erlenmeyer flasks, with a final working volume of 50 mL, using a heterotrophic basal medium (HM; Barros et al., 2019) supplemented with glucose (20 g L^{-1}). Cultures were then placed in two orbital shakers at 30°C and 200 rpm. A spotlight was kept on top of one orbital shaker using a photon flux density of 100 μmol m^{-2} s^{-1} (light condition), while the other orbital shaker was covered with aluminum foil (dark condition). All experimental trials were carried out in triplicate.

Growth Comparison of Wild Type vs. Mutant in 5-L and 200-L Fermenters

The seed for heterotrophic growth was obtained sequentially in 50- and 250-mL cultures in, respectively, 250-ml and 1000-mL Erlenmeyer flasks, in order to reach a volume of 5 L in a bench-top fermenter (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany), which was later used to inoculate a 200-L fermenter, developed and assembled in-house. Temperature in both fermenters was maintained at 30°C and pH at 6.5 by addition of ammonia solution (24% m m^{-1}). As in the Erlenmeyer flask tests, HM medium was used (Barros et al., 2019), but glucose was added in fed batch so that a non-limiting concentration of 1 to 20 g L^{-1} was kept. Samples were collected aseptically for supernatant analysis or biomass concentration analysis. Throughout the growing period the air inlet flowrate was adjusted to maintain ∼1 vvm. Accordingly, the agitation rate ranged from 100 to 1,200 rpm, so that the dissolved oxygen in the medium was not a limiting factor for culture growth.

Sampling and Growth Assessment

Sampling of each culture was done twice a day in order to analyze growth parameters, namely optical density (OD) at 600 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), pH and optical microscopy (Zeiss Axio Scope A1, Oberkochen, Germany).

Dry weight (DW) determination was only possible for the samples of cultures grown in fermenters. Culture samples were filtered using pre-weighed 0.7 μm GF/C 698 filters (VWR, Pennsylvania, USA) and dried at 120°C until constant mass was obtained using a DBS 60–30 electronic moisture analyzer (KERN & SOHN GmbH, Balingen, Germany). All dry weight samples were washed with demineralized water to remove growth medium salts. Whenever the previous procedure could not be carried out, a DW vs. optical density correlation developed in-house for this strain was used. Biomass productivity was obtained by equation 1 and growth rate by equation 2.

$$P\left(g\,L^{-1}\,d^{-1}\right) = \frac{DW_f - DW_i}{t_f - t_i} \quad (1)$$

$$\mu\left(d^{-1}\right) = \frac{ln\left(DW_f/DW_i\right)}{t_f - t_i} \quad (2)$$

Proximate Composition

The protein content was determined by CHN elemental analysis, according to the procedure provided by the manufacturer using a Vario el III (Vario EL, Elemental Analyzer system, GmbH, Hanau, Germany). The final protein content was calculated by multiplying the percentage of nitrogen by 6.25.

The lipid content was determined using the Bligh and Dyer (1959) method described in Pereira et al. (2011) with minor modifications. Briefly, freeze dried samples were extracted with methanol through bead-milling with glass beads, using a Retsch MM 400 mixer mill at 30 Hz for 3 min to ensure effective cell disruption. The tubes were centrifuged (10,000 *g*) and the supernatants were collected to new vials. The pellets suffered a second extraction and both methanol supernatants were pooled. Chloroform and water were added to the methanol (2:1:2) and the tubes were vortexed for 5 min. Afterwards, the samples were centrifuged to obtain a biphasic system and the lipid extract was separated. A known volume of the extracts was transferred to pre-weighed tubes, evaporated and weighted in order to determine the lipids gravimetrically.

The ash content was determined by burning the freeze-dried biomass in a furnace (J. P. Selecta, Sel horn R9-L, Barcelona, Spain) at 550°C for 6 h. The carbohydrate content was determined by difference of the remaining macronutrients.

Chlorophyll Content

A culture volume corresponding to 10 mg of biomass was taken from each sample and centrifuged for 15 min, at 2,547 *g* (HERMLE Labortechnik GmbH, Wehingen, Germany). Chlorophyll extraction was performed in acetone by successive zirconia bead milling. The supernatant was collected by centrifugation and re-extraction of the biomass was performed until colorless. The absorbance of the supernatant was measured in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) at 630, 647, 664 and 691 nm. The chlorophyll *a* content was then estimated according to the

following equation by Ritchie (2008):

$$Chl_a = -0.3319\,Abs_{630} - 1.7485\,Abs_{647} + 11.9442\,Abs_{664} - 1.4306\,Abs_{691} \quad (3)$$

Carotenoid Profile

The extraction of carotenoids was carried out on ice and under dim light to avoid oxidation. Approximately 5 mg of freeze-dried biomass was weighed in a glass vial, ∼0.6 g of glass beads (425–600 μm) and 1 mL of ice-cold methanol containing 0.03% butylhydroxytoluene (BHT) were added. Cells were disrupted using a Retsch MM 400 mixer mill at 30 Hz for 3 min. To collect the supernatant, the samples were centrifuged for 3 min at 21,000 *g*. The remaining biomass was extracted repeatedly with 1 mL of methanol/BHT by vortexing for 10 s, followed by centrifugation until both the pellet and the supernatant became colorless. The extracts were combined, and methanol was evaporated under a gentle nitrogen flow. Prior to HPLC analysis, the extracts were resuspended in 1 mL of methanol and filtrated through 0.22 μm PTFE filter to remove suspended particles.

Carotenoid analysis was performed by HPLC as described previously (Schüler et al., 2020). Briefly, a Dionex 580 HPLC System (DIONEX Corporation, USA) consisting of a PDA 100 Photodiode-array detector, STH 585 column oven set to 20°C and a LiChroCART RP-18 (5 μm, 250 × 4 mm, LiChrospher) column was used. Carotenoid separation was achieved using a mobile phase composed of solvent A acetonitrile:water (9:1; v v^{-1}) and solvent B ethyl acetate with the following gradient: 0–16 min, 0–60% B; 16–30 min, 60% B; 30–32 min 100% B and 32–35 min 100% A. All carotenoids were detected at 450 nm and 280 nm and analyzed with Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, USA). The quantification was carried out using calibration curves of neoxanthin, violaxanthin, lutein, zeaxanthin and β-carotene standards (Sigma-Aldrich, Portugal). Phytoene was identified by its specific absorbance profile at 280 nm and only quantified as equivalent to lutein. Injection volume of both extracts and standards was 100 μL.

Statistical Analysis

Statistical analyses were performed with R software (version 3.6.1). Statistical significance was tested using analysis of variance (one-way ANOVA) and Tukey HSD *post-hoc* at a 0.05 probability level.

RESULTS AND DISCUSSION

Development of Mutants

In the first stage of this work, chlorophyll-deficient mutants of *C. vulgaris* were obtained by random mutagenesis using the alkylating agent ethyl methane sulfonate (EMS). Different concentrations of EMS were tested on the WT to find the concentration, which resulted into a survival rate between 5 and 10% (**Figure 1**). The selection of the correct survival rate is critical to increase the likelihood that the survivors contain at least one mutation, but also to avoid the selection of cells containing multiple mutations, which are often detrimental to growth. The selection and further scale up of the mutants were carried out in the absence of light and with glucose as carbon source to suppress the need for energy supply via photosynthesis, and thus promoting the growth of chlorophyll-deficient mutants. After treatment of the cells with a concentration of 300 mM of EMS, a yellow colony indicating the absence of chlorophyll emerged onto the plate. The repeated sub-cultivation on solid media of this mutant, MT01, confirmed the stability of the yellow color throughout 10 generations. Most probably, a mutation in the photosynthetic machinery is the reason for the reduction of chlorophyll in this mutant (Tiwari et al., 2019).

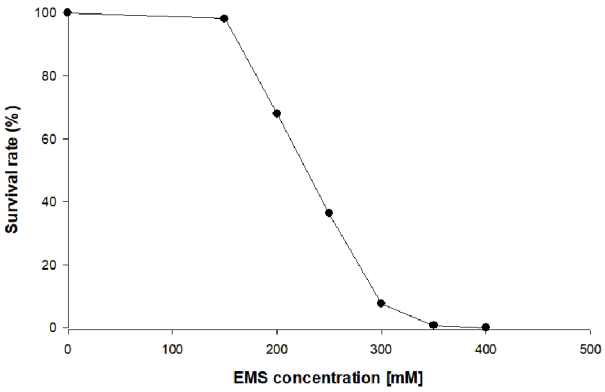

FIGURE 1 | Survival rate of heterotrophic *Chlorella vulgaris* upon exposure to different ethyl methane sulfonate (EMS) concentrations on plate count agar (PCA) plates.

Thereafter, a second random mutagenesis was conducted on MT01, with subsequent selection of mutants by their resistance to the carotenogenic pathway inhibitor norflurazon. A wide range of concentrations of norflurazon was tested to find out that 10 μM was the minimal lethal concentration to MT01. This selection procedure gave rise to white colonies with resistance to the bleaching herbicide norflurazon. After sub-cultivation, only one mutant maintained the white color when the herbicide was removed from the media over 10 generations. This mutant, MT02, most probably contains an irreversible mutation in the phytoene desaturase gene leading to the inhibition of the following steps within the carotenoid and/or plastoquinone biosynthetic pathways (McCarthy et al., 2004; Qin et al., 2007). Other studies on *Chlorella zofingiensis* and *Chlorella sorokiniana* used a similar approach to obtain mutants with accumulation of zeaxanthin or lutein, respectively (Chen et al., 2017; Huang et al., 2018). In those cases, the inhibitors diphenylamine or nicotine were used to select for mutations in genes coding for enzymes involved in carotenoid biosynthesis.

Wild Type vs. Mutants in Dark and Light Conditions

Growth Performance

C. vulgaris WT and mutants were grown in 250-mL Erlenmeyer flasks under light and dark conditions, to assess the effect of light

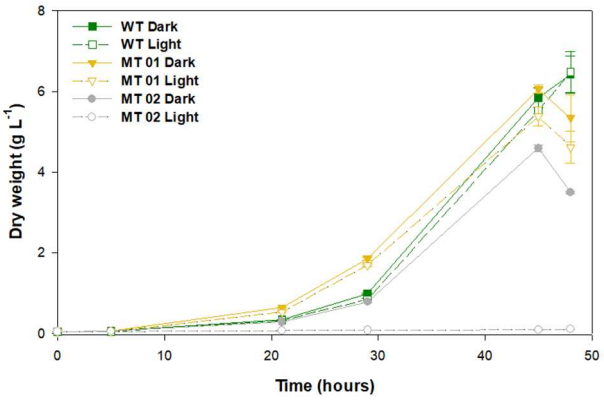

FIGURE 2 | Growth curves of wild type and mutants, under light and dark conditions grown in 250-mL Erlenmeyer flasks for 48 h.

on growth performance and biomass color (**Figure 2**). After a lag phase of about 20 h the cultures grew exponentially until the depletion of glucose, which led to cell death after 48 h.

The WT along with the yellow mutant MT01, both in the dark, reached the highest DW after 45 h of growth, 5.84 and 6.06 g L^{-1}, respectively. Under light conditions, the WT and MT01 achieved a similar DW ($p > 0.05$), 5.52 and 5.38 g L^{-1}, respectively, but significantly lower than that obtained under dark conditions (**Figure 2**). Several pale-green *C. vulgaris* mutants reported in literature also showed biomass productivity similar to that of the WT strain used, however, under autotrophic conditions (Shin et al., 2016; Dall'Osto et al., 2019). Furthermore, those mutants showed with increasing light intensity higher biomass productivities (up to a 68% increase) than that of the WT. Those studies further showed that the changes observed not only improved growth performance, but also the pigment profile, at the cost of higher sensitivity to light. Interestingly, all these phenotypes were associated to smaller antenna sizes in the photosynthetic machinery of the mutants (Shin et al., 2016; Dall'Osto et al., 2019).

The white mutant MT02 displayed a significantly lower biomass concentration in the dark compared to the WT and MT01 ($p < 0.05$), attaining a maximum DW of 4.59 g L^{-1} after 45 h of growth. Moreover, MT02 was not able to grow under light conditions, achieving only 0.08 g L^{-1} of DW at the end of the assay. Similarly, Kamiya (1985) also described light, particularly blue light, as inhibitory for growth, cell division and glucose uptake for colorless *Chlorella* mutants. Nonetheless, in the dark, MT02 displayed a promising growth performance, which was statistically indistinguishable from that of the WT ($p > 0.05$). In spite of enhancing pigment content, exposure to excess light might lead to a more or less noticeable growth inhibition, which in this case was observed not only in the white MT02 mutant growth, but also in the WT and yellow MT01 mutant cultures exposed continuously to light.

Pigment Profile

Macroscopically, WT cultures displayed a green color and acquired a more intense green color when grown under a spotlight (**Figure 3**). MT01 cultures presented an intense yellow color under dark conditions, which was reversed back to green when cultures were exposed to light conditions. On the other hand, MT02 cultures exhibited a white tonality and absence of any other color under dark conditions, while no biomass was produced under light conditions.

In order to characterize the color of WT and mutant strains under light and dark conditions, the chlorophyll and carotenoid content of the cultures were analyzed. It is evident that light significantly increased the chlorophyll content of WT and mutant cultures ($p < 0.05$) (**Figure 4**). Although, MT01 and WT exhibited equivalent growth performances ($p > 0.05$), MT01 contained significantly lower chlorophyll content than the WT ($p < 0.05$) under both light and dark conditions. WT cultures displayed the highest chlorophyll content, 9.16 mg g^{-1} under dark conditions, which was enhanced to 14.06 mg g^{-1} in the presence of light. MT01 cultures grown in the dark registered 1.69 mg g^{-1} of chlorophyll, while under light exposure 8.02 mg g^{-1} of chlorophyll was detected, which granted them the green coloration. In fact, no significant differences were observed between the chlorophyll content of WT grown in the dark and the light grown MT01 displaying a pale green color ($p>0.05$). This is in agreement with studies of *C. vulgaris*, where EMS-induced light green mutants with a 50% reduced chlorophyll content compared to the WT were selected (Shin et al., 2016; Dall'Osto et al., 2019). However, cultures in those studies were grown under autotrophic conditions as the objective was to enhance biomass productivities and photosynthetic efficiencies.

The MT02 mutant, however, displayed only residual chlorophyll contents grown in the dark (0.045 mg g^{-1}). Although not easily visible, after some days of light exposure, MT02 started to acquire a pale green tonality, which was evidenced by the detection of an increased chlorophyll content in the biomass as compared with the dark cultured biomass (0.25 mg g^{-1}; $p < 0.05$). This is in accordance with studies on EMS-induced white mutants of *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, which showed a pale green color due to a 40-fold decrease in chlorophyll content compared to the WT (Kamiya, 1985; McCarthy et al., 2004). However, with the mutants developed in this work, which are heterotrophically cultivated, it is possible to maintain a stable non-green color under dark conditions.

The carotenoid profile of *C. vulgaris* WT was mainly composed of lutein and β-carotene, while neoxanthin, violaxanthin and zeaxanthin were only detected in minor quantities (**Table 1**). The carotenoid profile of MT01 showed the same characteristics as compared with the WT, however, with lower contents of 0.93 ± 0.01 and 1.70 ± 0.13 mg g^{-1} DW in the dark, respectively. As lutein is the major carotenoid, this can explain the yellow color of MT01 under dark conditions (**Figure 3**). Huang et al. (2018) also obtained a yellow *Chlorella* mutant by random mutagenesis with similar growth performances of the wild type strain. That mutant strain displayed a dysfunction in carotenoid ketolase enzyme, which prompted zeaxanthin accumulation (up to 7.00 mg g^{-1}) enhanced by high-light irradiation, nitrogen depletion and glucose feeding. Those treatments also led to the accumulation of

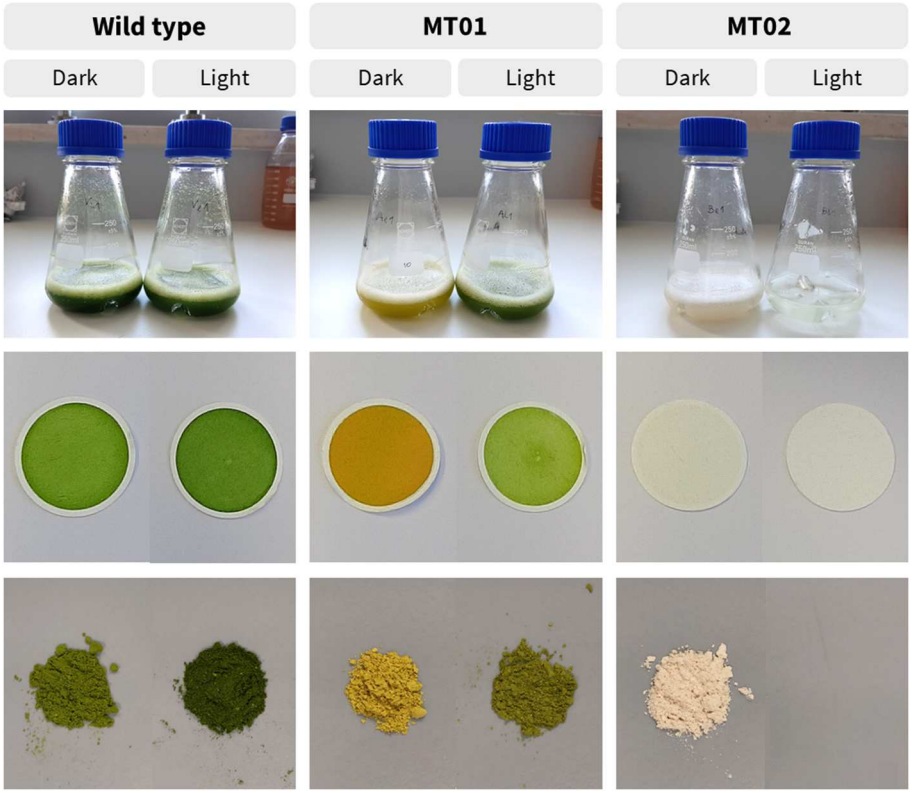

FIGURE 3 | Different coloration of wild type and mutant cultures, dry weight filters and freeze-dried biomass, grown under light and dark conditions in 250-mL Erlenmeyer flasks, after 42 h.

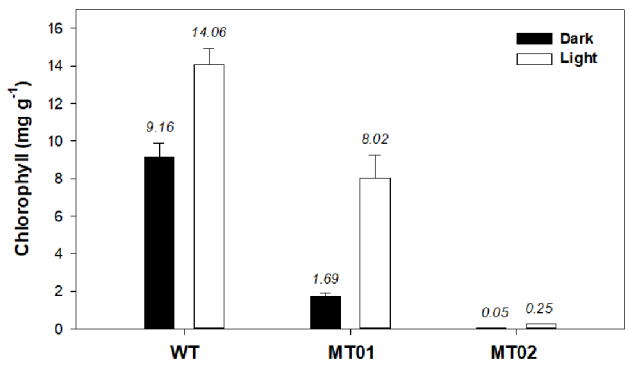

FIGURE 4 | Chlorophyll content (mg g^{-1}) of *Chlorella vulgaris* wild type and mutants grown in 250-mL Erlenmeyer flasks under dark and light conditions. Values are given as means ± standard deviation of three biological replicates ($n = 3$).

other carotenoids, such as β-carotene (7.18 mg g^{-1}) and lutein (13.81 mg g^{-1}), which together imparted their yellowish hues to the biomass. In addition, Dresbach and Kowallik (1974), which also established a chlorophyll-free *C. vulgaris* mutant pointed out that carotenoid biosynthesis might be enhanced by permanent irradiation with blue light. Moreover, several positive effects on human health such as the reduced risk for cardiovascular disease and age-related macular degeneration as well as cancer prevention have been attributed to lutein (Astorg, 1997; Ma et al., 2012; Han et al., 2015). Therefore, it could be interesting to study the accumulation of this pigment in MT01 by testing other stressing or stimulating factors, such as nitrogen depletion, glucose feeding and other light wavelengths or intensities.

Increased light intensity seems to promote the induction of carotenoids in both WT and MT01 by about 1.6-fold (**Table 1**). This is most probably related with the function of carotenoids, as they are important pigments involved not only in light harvesting, but also in the protection of the photosynthetic apparatus from excess light (Mulders et al., 2014). As expected, the content of violaxanthin decreased with the concomitant increase of the photoprotective xanthophyll zeaxanthin (**Table 1**). Remarkably, the content of β-carotene in MT01 cultivated under light conditions increased 10-fold compared with cells under dark conditions, confirming the importance of this carotenoid as photoprotective pigment in this microalga. Conversely, as its white color indicated already, all colored carotenoids were absent in the MT02 mutant; the only carotenoid detected was the colorless phytoene with 2-fold higher concentrations as compared with the WT under dark conditions (**Table 1**). Phytoene is a linear carotenoid without a conjugated system of double bonds, which has already been

TABLE 1 | Carotenoid content of *Chlorella vulgaris* WT and chlorophyll-deficient mutants MT01 and MT02 grown in 250 mL Erlenmeyer flasks under light and dark conditions.

Culture	Condition	Neoxanthin (mg g^{-1} DW)	Violaxanthin (mg g^{-1} DW)	Lutein (mg g^{-1} DW)	Zeaxanthin (mg g^{-1} DW)	β-carotene (mg g^{-1} DW)	Phytoene (mg g^{-1} DW)*
WT	Dark	0.085 ± 0.008^{b}	0.043 ± 0.007^{a}	1.280 ± 0.077^{b}	0.007 ± 0.001^{b}	0.284 ± 0.036^{b}	0.194 ± 0.010^{e}
	Light	0.181 ± 0.012^{a}	0.033 ± 0.007^{ab}	1.853 ± 0.060^{a}	0.010 ± 0.001^{a}	0.585 ± 0.047^{a}	0.252 ± 0.012^{d}
MT01	Dark	0.005 ± 0.001^{d}	0.033 ± 0.010^{ab}	0.858 ± 0.003^{c}	0.003 ± 0.001^{c}	0.034 ± 0.001^{c}	0.320 ± 0.004^{c}
	Light	0.038 ± 0.009^{c}	0.016 ± 0.006^{b}	1.167 ± 0.079^{b}	0.009 ± 0.001^{ab}	0.322 ± 0.026^{b}	0.363 ± 0.008^{b}
MT02	Dark	0	0	0	0	0	0.414 ± 0.010^{a}
	Light	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

*Different letters indicate significant differences ($p > 0.05$) between strains and treatments. Values are given as means ± standard deviation of three biological replicates ($n = 3$). n.a., not analyzed due to insufficient biomass sample. *Values calculated as lutein-equivalent contents.*

TABLE 2 | Proximate composition of macronutrients of *Chlorella vulgaris* WT and mutants presented as percentage of dry weight.

Culture	Condition	Proteins (% DW)	Lipids (% DW)	Carbohydrates (% DW)	Ashes (% DW)
WT	Dark	30.5 ± 0.8^{e}	15.4 ± 1.9^{a}	48.8 ± 2.9^{a}	5.4 ± 0.5^{d}
	Light	35.3 ± 0.4^{d}	15.8 ± 1.5^{a}	42.2 ± 1.8^{b}	6.6 ± 0.7^{c}
MT01	Dark	39.5 ± 0.9^{c}	18.4 ± 1.8^{a}	32.0 ± 1.1^{c}	10.1 ± 0.2^{b}
	Light	45.5 ± 0.8^{b}	14.3 ± 2.3^{a}	27.5 ± 3.3^{c}	12.7 ± 0.4^{a}
MT02	Dark	48.7 ± 1.3^{a}	14.9 ± 2.4^{a}	27.1 ± 2.1^{c}	9.3 ± 0.2^{b}
	Light	n.a.	n.a.	n.a.	n.a.

Different letters indicate significant differences ($p > 0.05$) between strains and treatments. Values are given as means ± standard deviation of three biological replicates ($n = 3$). n.a., not analyzed due to insufficient biomass sample.

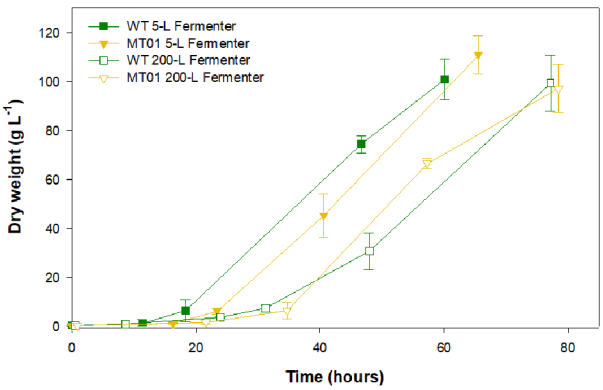

FIGURE 5 | Growth curves of wild type vs. MT01 mutant in 5-L and 200-L fermenters. Values are given as means ± standard deviation of three biological replicates ($n = 3$).

reported to be ineffective in photoprotection (León et al., 2005). This is most probably the reason why MT02 was not able to grow under light conditions. Phytoene, however, has gained interest in the cosmetic industries due to its absorption of UV radiation, anti-inflammatory and anti-oxidant effects (Meléndez-Martínez et al., 2018). Therefore, it would be interesting to study the accumulation of this carotenoid in the *C. vulgaris* MT02 strain under specific growth conditions to maximize its production.

Proximate Composition of Main Macronutrients

The comparison of the composition of main macronutrients revealed significant differences between WT, MT01 and MT02 in terms of protein, ash, and carbohydrate contents (**Table 2**). MT02 grown in the dark displayed the highest protein content, 48.7% of DW, followed by MT02 grown in the light and dark conditions, 45.5 and 39.5% of DW, respectively ($p < 0.05$). The WT displayed the lowest protein content under light and dark conditions, 35.3 and 30.5% of DW, respectively ($p < 0.05$). On the other hand, the highest carbohydrate content (48.8 and 42.2% of DW, in the dark and in the light, respectively) was achieved by the WT ($p < 0.05$), while MT01 and MT02 presented similar carbohydrate contents (27.1–32.0% of DW; $p > 0.05$). Interestingly, despite the great variations found in chlorophyll content between cultures and conditions, no significant differences in total lipid content were detected, which ranged from 14.3 to 18.4% of DW in all cultures and conditions ($p > 0.05$). The WT revealed the lowest ash content (5.4 and 6.6% of DW in the dark and in the light, respectively), followed by MT01 and MT02 grown in the dark (9.3–10.1% of DW), whereas MT02 grown under light conditions displayed the highest ash content (12.7% of DW; $p < 0.05$). The conditions (light vs. dark) affected protein, carbohydrate and ash significantly, resulting in higher content of both protein and ash, and lower content of carbohydrates, when cells were exposed to light ($p < 0.05$).

Both higher amounts of proteins and lower amounts of chlorophyll detected in both mutants may suggest a truncated chlorophyll antenna size of the photosystems as reported in other chlorophyll-deficient mutants (Polle et al., 2002; Shin et al., 2016; Dall'Osto et al., 2019). Those chlorophyll-deficient mutants have been characterized with similar or even higher protein levels, namely chlorophyll-binding proteins and thylakoid membrane proteins (Polle et al., 2002; Gu et al., 2017). Furthermore, a previous report revealed that higher light exposure induces the accumulation of proteins; thus, in this case, low light might have induced the synthesis of larger photosynthetic units, resulting in higher protein content in the light (Seyfabadi et al., 2011). While

TABLE 3 | Mean and maximum biomass productivities and growth rates of *Chlorella vulgaris* WT and mutant MT01 in 5- and 200 L fermenters.

Strain/fermenter	Mean productivity ($g\ L^{-1}\ d^{-1}$)	Batch maximum productivity* ($g\ L^{-1}\ d^{-1}$)	Mean specific growth rate (d^{-1})	Batch maximum specific growth rate* (d^{-1})
WT 5-L Ferm	42.44 ± 5.31^{a}	48.22	2.67 ± 0.32^{ab}	2.92
MT01 5-L Ferm	41.03 ± 1.56^{a}	42.11	2.98 ± 0.04^{a}	3.01
WT 200-L Ferm	30.98 ± 2.25^{b}	33.06	2.38 ± 0.08^{b}	2.47
MT01 200-L Ferm	30.07 ± 1.47^{b}	31.73	2.46 ± 0.26^{ab}	2.64

Same letters in superscript after the values denote significant statistical differences ($p > 0.05$) between values on the same column. Values are given as means ± standard deviation of three biological replicates ($n = 3$).

**Batch maximum productivity and batch maximum specific growth rate correspond to maximum mean productivity and mean specific growth rate obtained among the three replicates, respectively.*

the higher content of carbohydrate found in the dark conditions was probably due to the accumulation of polysaccharides such as starch. In addition, increased ash content in chlorophyll-free biomass has also been previously reported (Li et al., 2016), suggesting that the mineral metabolism might have also been affected in the mutants. Overall, WT cultures revealed proximate composition values within those previously reported for *C. vulgaris* grown in heterotrophic conditions (Kim et al., 2019; Canelli et al., 2020), while MT01 and MT02 displayed significantly higher protein contents. Therefore, the low ash associated with high protein contents of mutants, adds to these cultures improved nutritional profiles with commercial interest for their application as feedstocks for food products.

Scale-Up Case Study: MT01 Growth Validation in 5-L and 200-L Fermenters

In order to validate the previous results, the WT and MT01 growth performance was compared at a larger scale in 5-L and 200-L fermenters (**Figure 5**).

In the 5-L fermenters, growth was similar for both strains ($p > 0.05$) reaching a maximum DW of 100.94 and 110.85 g L^{-1} for the WT and MT01 cells, respectively, ∼60 h upon inoculation. Similarly, no significant differences ($p > 0.05$) were observed in the growth of MT01 and WT in the 200-L fermenters as shown by the key process indicators (KPI; **Table 3**). Final DW here obtained was of 99.39 and 97.13 g L^{-1} for WT and MT01 strains, respectively, after ∼75 h. These values are below those previously reported for the WT strain of 174.5 g L^{-1} (Barros et al., 2019). Nevertheless, the aforementioned dry weight was obtained after 7 days of growth, whereas in this run only 3 days are considered. A similar scale-up case study for a mutant of *Chlorella pyrenoidosa* was obtained by Song et al. (2018). In this case, the mutant obtained yielded 81.9 and 84.9 g L^{-1} of biomass in the 5-L and 2,000-L fermenters, respectively. As in this study, the authors point out to the homogeneity and growth patterns of their mutant upon scale-up as a strong indicator of the suitability of the mutant strain for industrial biomass production.

Concurrently, there were no statistical differences ($p > 0.05$) in the maximum nor in the average specific growth rate of WT and MT01 growth in the scales tested: 5 L and 200 L. This is an excellent indicator of the robustness of this mutant for industrial scale heterotrophic production. Maximum productivities were also similar for both strains throughout scale-up ($p > 0.05$). On the other hand, the average productivity was higher ($p < 0.05$) for both strains in the 5-L fermenter compared to the 200-L, given the shorter lag phase observed in these growth curves. In fact, the KPI for the WT and MT01 strains in the 200-L fermenter are well in accordance with the previously reported for the WT grown in the same 200-L fermenter – productivity of 27.54 ± 5.07 g L^{-1} d^{-1} and mean growth rate of 0.92 ± 0.11 d^{-1} (Barros et al., 2019). Furthermore, the biomass productivity and specific growth rate obtained for the MT01 strain were higher than those previously obtained for a *C. pyrenoidosa* mutant (19.68 g L^{-1} d^{-1} and 1.44 d^{-1}, respectively) using a reactor with a volume of 2,000 L (Song et al., 2018).

CONCLUSIONS

The established *Chlorella vulgaris* strains with yellow (MT01) and white (MT02) colors showed high biomass productivities comparable to the wild type. The color change in MT01 and MT02 cells were due to a 5- and 180-fold decrease in chlorophyll contents and the presence of lutein and phytoene, respectively, when the cells were grown heterotrophically in the dark. Both mutants displayed improved protein contents compared to that of the WT with a 60% increase under heterotrophic growth. MT01 was successfully scaled up to industrial 200-L fermenters, reaching a concentration of about 100 g DW L^{-1}. Because of this growth performance as well as improved organoleptic and nutritional characteristics, both new strains MT01 and MT02 show a high potential for applications in the food and nutraceutical industries for novel products based on microalgal biomass.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LS, EG, and MT conceived, designed, and performed the experiments, prepared the figures/tables, and wrote the manuscript. AM and PD performed the experiments on the generation of the mutants and drafted the manuscript. AM and MS performed the growth trials and fermentation experiments and drafted the manuscript. MC and IM performed the analysis of the proximate biomass composition, prepared figures/tables, and drafted the manuscript. BC performed the fermentation experiments, statistical analysis, and drafted the manuscript. HP and AB conceived, designed, and supervised the experiments and wrote the manuscript. JV and JS conceived and designed the experiments, reviewed the manuscript, and contributed to the funding. All authors contributed to the final approval of the article.

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Effective Harvesting of *Nannochloropsis* Microalgae Using Mushroom Chitosan: A Pilot-Scale Study

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For efficient downstream processing, harvesting remains as one of the challenges in producing *Nannochloropsis* biomass, a microalga with high-value omega-3 oils. Flocculation is an effective, low-energy, low-cost method to harvest microalgae. Chitosan has been shown to be an effective food-grade flocculant; however, commercial chitosan is sourced from crustaceans, which has disadvantages including concerns over heavy-metal contamination. Thus, this study tests the flocculation potential of mushroom chitosan. Our results indicate a 13% yield of chitosan from mushroom. The identity of the prepared chitosan was confirmed by Fourier-transform infrared (FTIR) spectroscopy. Furthermore, results show that mushroom chitosan can be an alternative flocculant with >95% flocculation efficiency when tested in 100-mL jar and 200-L vertical column photobioreactor. Applications in a 2000-L raceway pond demonstrated that thorough mixing of mushroom chitosan with the algal culture is required to achieve efficient flocculation. With proper mixing, mushroom chitosan can be used to produce food-grade *Nannochloropsis* biomass suitable for the production of vegan omega-3 oils as a fish oil alternative.

Keywords: *Nannochloropsis*, flocculation, mushroom, chitosan, harvesting, vegan, omega-3

INTRODUCTION

Microalgae are photosynthetic microorganisms that grow in various environments. In recent years, research on microalgae has shifted from their use as biofuel to the production of nutraceuticals such as omega-3 fatty acids, carotenoids, and protein. *Nannochloropsis* sp. is a marine microalga, which contains high amounts of omega-3 fatty acids in the form of eicosapentaenoic acid (EPA) that has been proposed as a suitable vegan alternative for fish oil (Chua and Schenk, 2017). Its protein content can reach 36% of the biomass (Schulze et al., 2016) and even 46% according to our own data. Because of these abundant high-value products, *Nannochloropsis* sp. has gained interest from investors for large-scale cultivation.

In large-scale microalgae cultivation systems, one of the main challenges is harvesting the cells (Mathimani and Mallick, 2018). Conventional methods of harvesting include filtration and centrifugation. However, because of the small cell size (2–5 μm) of *Nannochloropsis*, these conventional harvesting methods require expensive equipment, which are also energy-intensive. Hence, harvesting can cost up to 30% of the total capital investment (Milledge and Heaven, 2013). An easy, simple, and low-cost method of harvesting is by flocculation (Vandamme et al., 2013). The most common and cheapest flocculant used is alum (Vandamme et al., 2013). However, this method contaminates the final harvested biomass with high amounts of aluminum, which makes the product not suitable for human and animal consumption. Another well-studied flocculant is chitosan (Vandamme et al., 2013; Chua et al., 2019). Chitosan is a linear polysaccharide derived from the deacetylation of the abundant natural polymer chitin, which is mainly composed of *N*-acetyl-D-glucosamine monomer units (Dimzon and Knepper, 2015). Numerous research papers have already proven the effectivity of chitosan to flocculate microalgae cells (Şirin et al., 2012; Xu et al., 2013). We have previously shown the importance of the pH of the chitosan–microalgae mixture to have high flocculation efficiencies (Chua et al., 2019). All chitosan samples tested were sourced from crustacean shells. However, crustacean-sourced chitosan has several disadvantages including heavy metal contamination (Ghormade et al., 2017). Previous studies have shown that chitosan can also be extracted from mushrooms (Yen and Mau, 2006; Erdogan et al., 2017) or even mushroom wastes (Wu et al., 2004). Thus, in this study, we produced and tested the effectiveness of mushroom chitosan for flocculating *Nannochloropsis* cells. The results were further verified in large-scale cultures, i.e., a 200-L vertical column photobioreactor culture and a 2000-L raceway pond culture.

MATERIALS AND METHODS

Microalgae Culture

Nannochloropsis oceanica BR2 (Genbank accession JQ423160) was obtained from the microalgae culture collection of the University of Queensland Algae Biotechnology culture collection (Lim et al., 2012; Brown et al., 2019). The species was initially grown in a 250-mL flask using 20 g/L Ocean Nature Sea Salt (Aquasonic Pty. Ltd., NSW, Australia) enriched with f/2 medium (per L water): 75 mg $NaNO_3$, 5 mg $NaH_2PO_4{\cdot}H_2O$, 30 mg $Na_2SiO_3{\cdot}9H_2O$, 3.15 mg $FeCl_3{\cdot}6H_2O$, 4.36 mg $Na_2EDTA{\cdot}2H_2O$, 9.8 μg $CuSO_4{\cdot}5H_2O$, 6.3 μg $Na_2MoO_4{\cdot}2H_2O$, 22 μg $ZnSO_4{\cdot}7H_2O$, 10 μg $CoCl_2{\cdot}6H_2O$, 180 μg $MnCl_2{\cdot}4H_2O$, 200 mg thiamine HCl, 1 μg biotin, and 1 μg cyanocobalamin (Guillard, 1975; Ma et al., 2018; Chua et al., 2019). The culture was continuously illuminated with fluorescent light (70 μmol photons $m^{-2}s^{-1}$) and aerated with filtered (through 0.2-μm pore size membrane filter) air. The culture was then gradually scaled up to the larger volumes (2 and 20 L) until it was transferred to outdoor cultures with volumes of 200 and 2000 L. The 200-L culture was grown in a vertical column photobioreactor with a diameter of 36 cm and a height of 2 m. The culture was maintained at pH 8.2 for optimum growth by bubbling CO_2 (food-grade, 99.99% pure) at 1 vvm. Apart from maintaining the pH, CO_2 was also the sole source of carbon for the culture. On the other hand, the 2000-L culture was grown in a 10 m^2 raceway pond that had 1 m wide channels and a depth of 10 cm. The pond was mixed using an air-lift system and pH was controlled and maintained at 8 by automatic additions of CO_2.

Preparation of Chitosan From Mushroom

Chitosan was prepared from 50 g of Shiitake mushroom powder (Austral Herbs, NSW, Australia) following the method by Mohammed et al. (2013) with some modifications. Briefly, the mushroom powder was mixed with 5% NaOH solution in 1:8 ratio of powder to NaOH solution. The mixture was stirred at 120 r/min for 2 h at 60°C. Then, the sample was washed three times with distilled water. The crude chitin was deacetylated by refluxing in 50% (w/v) NaOH for 2 h at 100°C. The resulting liquor was then centrifuged, and the pellet was continuously washed until the pH was neutral. Finally, the pellet was lyophilized to obtain the crude chitosan. The entire procedure was carried out with 500 g mushroom powder for testing in the large-scale microalgal cultures.

Characterization of the Prepared Mushroom Chitosan

The crude mushroom chitosan was characterized using a Fourier-transform infrared (FTIR) spectrophotometer (Thermo Scientific Nicolet 700) fitted with an attenuated total reflectance accessory and a diamond crystal internal reflection element. The resulting spectrum was compared to the commercial chitosan (Sigma).

Elemental Analyses of Chitosan

Elemental analyses for heavy metals in chitosan samples were performed in duplicates as previously described (Aslam et al., 2019). Included in the analyses were two crustacean chitosan samples (Sample 1: Biomedical Chitosan, Australia; Sample 2: Qingdao Yunzhou Biochemistry Co., Ltd., China) and the mushroom-derived chitosan from the present study (Sample 3).

Testing the Prepared Mushroom Chitosan for *Nannochloropsis* Flocculation

A similar method was used to test for the flocculation efficiency of the prepared mushroom chitosan as described in Chua et al. (2019). The optimized parameters (chitosan concentration of 25 ppm, culture optical density of 2, adjustment of pH to 6 after chitosan addition, and increase of final pH to 10 after mixing the chitosan) were used for the test.

The prepared mushroom chitosan was compared to commercial chitosan and mushroom powder. All samples were suspended in 1% acetic acid. Samples were collected at mid-height at 5, 15, and 30 min and the absorbance of the

samples was measured at 440 nm to evaluate the flocculation efficiency. The flocculation efficiency was calculated using Eq. 1:

$$\text{Flocculation efficiency(in \%)} = \left(1 - \frac{OD_t}{OD_0}\right) \times 100 \quad (1)$$

where OD_0 and OD_t are the OD values of the cultures before and after the flocculation test, respectively. The culture absorbance was measured at 440 nm since this is the absorption maximum of chlorophyll *a* which is abundantly present in *Nannochloropsis*.

Further, a pilot scale testing of mushroom chitosan for flocculation was performed on *Nannochloropsis* cultivated in a 200 L vertical column photo-bioreactor and 2000 L open raceway pond. Both these cultures were maintained at pH 8 through CO_2 supplementation. The final pH of the cultures was set by adding KOH. The same method was used to calculate the flocculation efficiency.

Fatty Acid Quantification and Profiling

Fatty acid methyl esters from chitosan-harvested *N. oceanica* BR2 biomass were quantified by gas chromatography-mass spectrometry (GC-MS) as previously described (Ma et al., 2018). The analysis was done in triplicates.

Statistical Analysis

The lab scale flocculation test was performed in triplicates. Tukey's multiple comparisons test was used to test the significance among groups. Comparisons with p-values < 0.05 considered as statistically significant.

RESULTS

Preparation of Mushroom Chitosan

Mushroom chitosan was prepared from mushroom chitin via alkaline treatment. From the mushroom powder used, 17.22 g of crude extract was obtained after the first alkaline treatment. The first alkaline treatment was necessary to remove the protein contaminants (Wu et al., 2004; Mohammed et al., 2013; Erdogan et al., 2017). The amount of crude extract was further reduced after the second alkaline treatment to 6.16 g, which equates to a 12.32% yield. For the 500 g-mushroom powder, 157.02 g of crude chitin were obtained resulting in a 31.4% yield. After the second alkaline treatment, 68 g of crude chitosan were obtained for a final yield of 13.60%. **Figure 1A** shows the appearance of the mushroom chitosan after it has been lyophilized. The final yields obtained for both the small scale and large-scale extractions were comparable to those in literature (Yen and Mau, 2006; Di Mario et al., 2008).

Infrared spectroscopy (**Figure 1B**) results indicated that chitosan was successfully prepared from the extracted mushroom chitin. Peaks at 3400–3200 cm^{-1} correspond to the N–H and O–H stretching. The peaks around the 1660 cm^{-1} region correspond to the C = O stretching from the amide group while the peak at 1600 cm^{-1} is the amine peak (Dimzon and Knepper, 2015). Finally, the peaks at 1024, 1373, and 2870 cm^{-1} correspond to the C–O bending, C–H bending, and C–H stretching from the polymer backbone, respectively.

Flocculation Efficiency of the Prepared Crude Mushroom Chitosan

The crude mushroom chitosan was tested on *N. oceanica* BR2 using previously optimized conditions which were: culture OD of 2, 25 ppm chitosan, adjusting the pH to 6 after chitosan addition, and increasing the final pH to 10 after mixing the chitosan into the culture (Chua et al., 2019). Similar to the results in Chua et al. (2019), no flocculation was observed without increasing the final pH to 10. Results indicated that that the crude mushroom chitosan can induce flocculation similar to the commercial chitosan ($p > 0.05$) with flocculation efficiency values > 94% after 5 min (**Figure 2**). On the other hand, the mushroom powder only yielded an average flocculation efficiency value of 66% even after 30 min.

Testing of Mushroom Chitosan in a Pilot-Scale Harvesting

The effectiveness of mushroom chitosan to induce flocculation of *N. oceanica* BR2 was tested at pilot scale using a 200-L vertical column photo-bioreactor and a 2000-L open raceway pond. **Figures 3A,B** show the 200-L column photo-bioreactor before and after the flocculation procedure, respectively. In this case, a flocculation efficiency of 98.3% was achieved. As for the 2000-L raceway pond, it had an initial OD of 2.9. So, a 50-mL sample was obtained before testing. Flocculation was observed after adding chitosan into the 50-mL sample as shown in **Figure 3D** indicating that 25 ppm of mushroom chitosan is still effective. Mixing was performed by using an air-lift system, and the chitosan was poured in and mixed for 10 min. **Figures 3F,G** show some of the flocs that formed after the chitosan was added and these flocs were not present before chitosan addition as shown in **Figure 3E**. The entire procedure yielded 64% flocculation efficiency after 1 h of settling. After 24 h, samples were collected at different points of the pond, and the average OD was 0.787, yielding 73% flocculation efficiency. **Figure 3C** summarizes the flocculation results of the 200- and 2000-L culture.

To determine which type of chitosan (mushroom- or crustaceae-derived) is a safer option for human consumption, elemental analyses were performed using two samples of crustacean and one sample of mushroom chitosan from the present study. These showed that heavy metals varied greatly for the two crustacean-derived samples, with chromium and nickel levels as high as 47.44 and 27.21 mg/kg, respectively, while mushroom-derived chitosan did not contain any concerning heavy metal contamination (**Supplementary Table S1**).

Fatty Acid Profiling of Mushroom Chitosan-Harvested *N. oceanica* BR2 Biomass

The mushroom chitosan-harvested biomass has EPA levels up to 41.3(±0.3)% of the total fatty acid content. Other fatty acids detected were palmitic acid (C16:0, 15.0 ± 0.6%), palmitoleic acid (C16:1, 34.4 ± 0.8%), oleic acid (C18:1, 2.8 ± 0.3%),

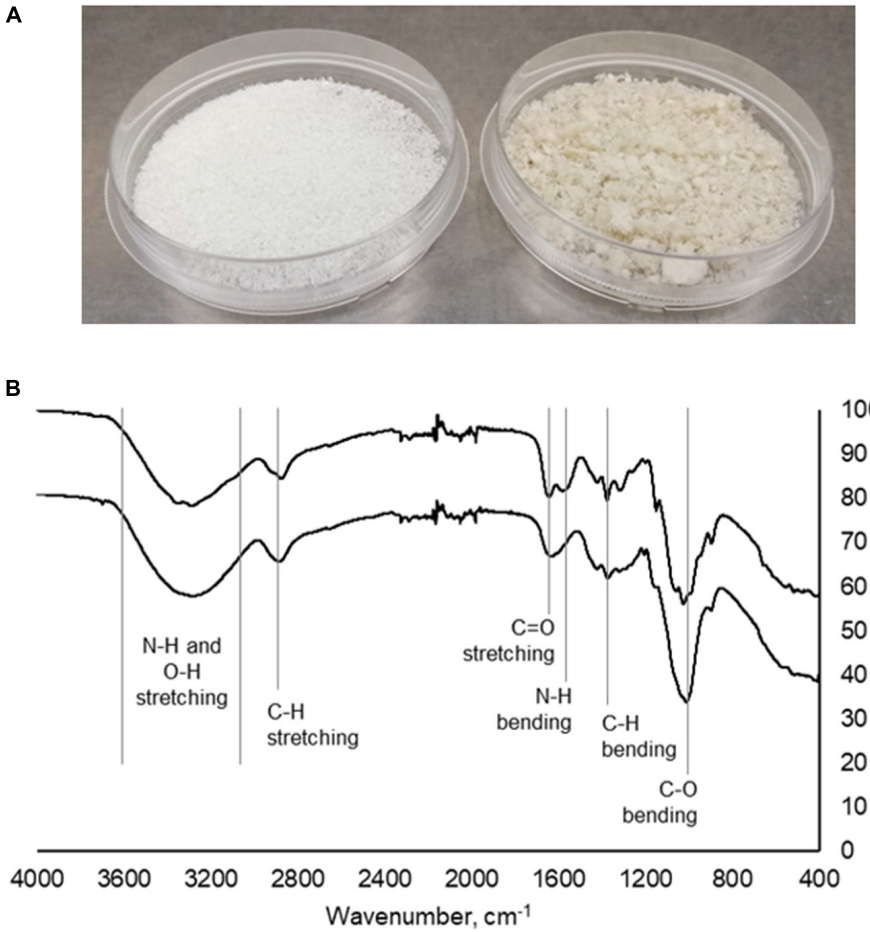

FIGURE 1 | (A) Commercial chitosan (left) and crude mushroom chitosan (right). **(B)** Infrared spectra of commercial chitosan (top) and mushroom chitosan (bottom).

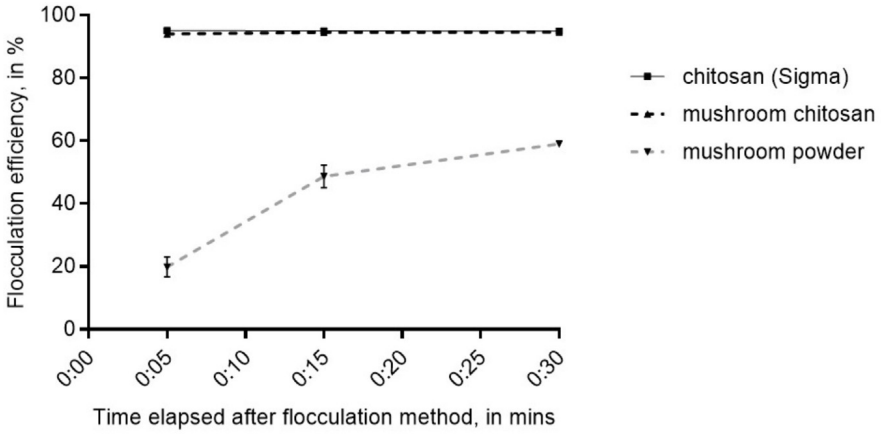

FIGURE 2 | Flocculation efficiency for mushroom chitosan compared to the commercial chitosan from crustacean. Shown are mean values ± SE of three replicates.

linoleic acid (C18:2, 1.8 ± 0.2%), and arachidonic acid (C20:4n-6, 4.7 ± 0.3%). **Supplementary Table S2** lists the fatty acid profile of the mushroom-harvested *N. oceanica* BR2 biomass together with profiles from other studies for comparison.

DISCUSSION

Chitin is popularly known to be extracted from the shells of crustaceans such as crabs and shrimps. Earlier estimates have

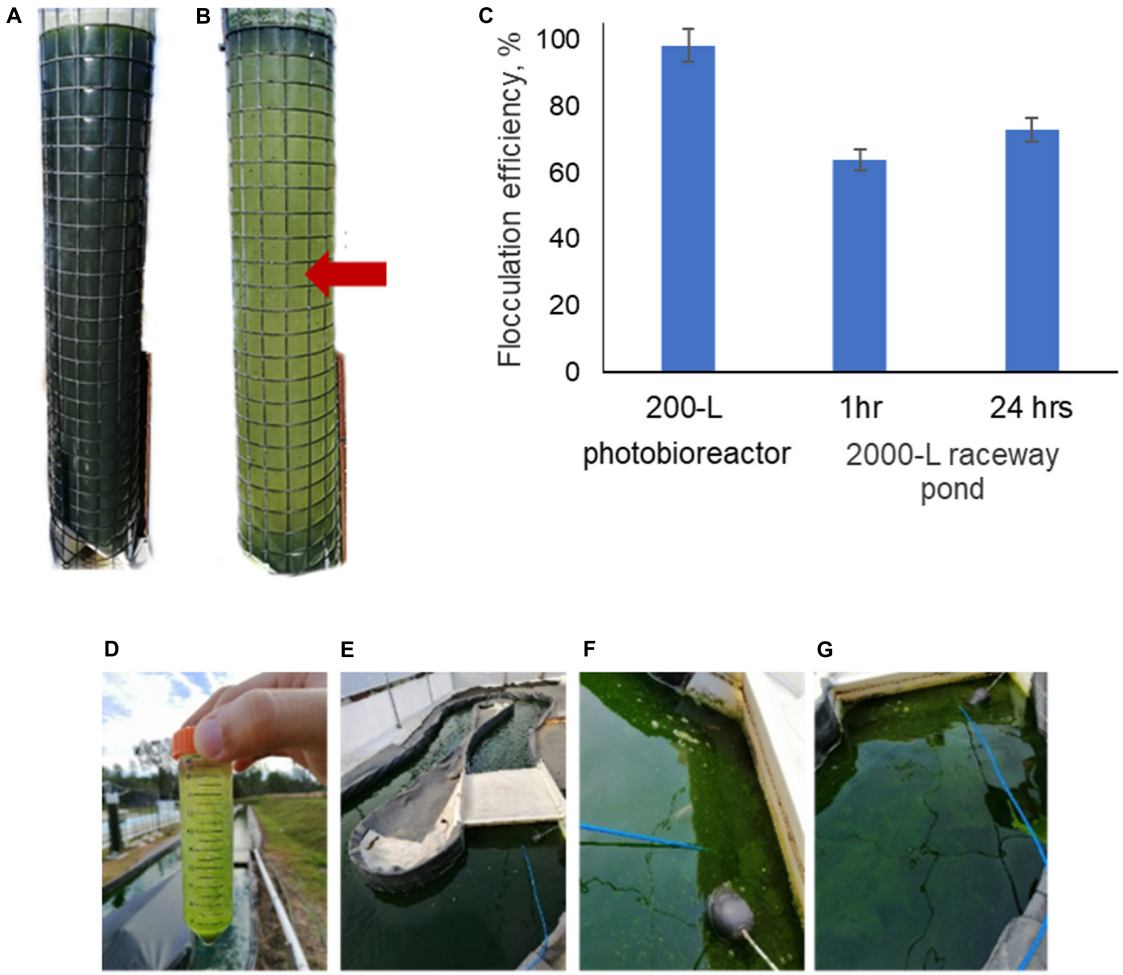

FIGURE 3 | Mushroom chitosan-mediated flocculation of *N. oceanica* in a 200-L tower culture: **(A)** before flocculation and **(B)** after flocculation. The resulting flocculation efficiencies are presented in **(C)**. The red arrow indicates the sampling point; and in a 2000-L raceway pond: **(D)** testing on a 50-mL sample, **(E)** before flocculation, **(F,G)** after flocculation.

shown that more than 80,000 tons of chitin is obtained from marine by-products (Ghormade et al., 2017). However, there are some disadvantages to marine-derived chitosan including seasonal variation and possible heavy metal contamination (Ghormade et al., 2017; Abo Elsoud and El Kady, 2019). This was also confirmed in the present study (**Supplementary Table S1**). In addition, fungal chitosan is suitable for vegans and is free from allergenic shrimp protein, which can be included in the final harvested biomass (Arcidiacono and Kaplan, 1992; Dhillon et al., 2013).

To theoretically estimate the comparative production expenses of chitosan from mushroom and crustaceans, the literature outlines that due to the inconsistent structure of chitin and chitosan from crustaceans, fungal (mushroom in this case) may represent a better alternative (Di Mario et al., 2008; Hassainia et al., 2018; Jones et al., 2020). Furthermore, fluctuations in seasonal supply of various animal sources and challenges in raw material standardization cause high variability in terms of deacetylation degree and molecular mass. These results may interfere in final flocculation efficiency of the chitosan. Unlike crustacean chitin, fungal chitin has more consistent physical and chemical properties, is not limited by seasonal and regional variation, and does not require the aggressive acid treatment that crustacean chitin needs for purification and demineralization to remove calcium carbonate and other minerals (Di Mario et al., 2008; Hassainia et al., 2018; Jones et al., 2020). In addition, from an environmental economics and sustainability point of view, crustacean chitosan production is likely to generate more waste than fungal chitosan. In the traditional process of chitin extraction from crustaceans, calcium and proteins are removed by HCl and NaOH, respectively. The remaining material is usually bleached with $KMnO_4$ or H_2O_2 and deacetylation is performed with hot concentrated alkaline or acidic solution. These harsh treatments can result in considerable amounts of wastes and deleterious trace contaminants (Bierhalz et al., 2016). Therefore, chitosan produced from mushroom waste is safer, more environmentally friendly, more reliable in its supply, and suitable for vegetarians.

A difference in color between the crude mushroom chitosan and the commercial chitosan was observed, which could be because the mushroom chitosan was not purified. The yellowish color was also obtained by Yen and Mau (2006). Further purification may be conducted by refluxing the crude powder in HCl or acetic acid (Wu et al., 2004; Darwesh et al., 2018). Decolorization may also be performed to improve the color (Yen and Mau, 2006; Mohammed et al., 2013). However, these processes will increase the cost of producing the chitosan flocculant. Nevertheless, even without the purification step, IR spectroscopy revealed the successful preparation of mushroom chitosan. The same characteristic peaks were observed in the IR spectrum of the mushroom chitosan when compared to the commercial chitosan.

Chitosan has been demonstrated as a good alternative bio-flocculant (Xu et al., 2013; Chua et al., 2019). Our flocculation test results confirm that mushroom chitosan was successfully prepared and has similar flocculation properties as the commercial chitosan. Mushroom powder did not flocculate the cells, which indicates that chitosan is the active ingredient and can only be obtained after deacetylation. Recently, Pugazhendhi et al. (2019) discussed the chemical mechanism of cationic polymers for microalgae flocculation. Because chitosan is cationic at acidic pH; thus, the lowering of the pH was necessary. In another study by Blockx et al. (2018), they demonstrated that high pH is necessary to flocculate microalgae in seawater medium. Thus, it was necessary to increase the pH after chitosan addition. This demonstrates that mushroom chitosan can indeed be used to harvest *Nannochloropsis* biomass that would then be suitable for vegetarians or even vegans. The fatty acid profile, which was not different from those in literature, further supports that the mushroom chitosan-harvested biomass can indeed serve as an alternative source of fish oil.

Previous studies (**Table 1**) have tested different flocculants to harvest *Nannochloropsis* spp. In the current study, mushroom chitosan has resulted in almost similar flocculation efficiency of >94% as compared to the previously reported chemical-based flocculants such as $Al_2(SO_4)_3$ and $FeCl_3$, but at much lower concentrations. Therefore, in this case, chitosan has advantage over chemical flocculants, which are not recommended for food-grade applications of harvested biomass. On the other hand, mung bean protein concentrate can be avoided since it can drive the food debate over its use for harvesting purpose. Tanfloc showed a comparable flocculation efficiency and at an even lower concentration compared to the mushroom chitosan concentration used in this study. However, it is not clear if Tanfloc can be used in food production as it is currently sold for water and wastewater treatment (TANAC, S.A.[1]). To our knowledge, this is the first study to report the use of mushroom chitosan for microalgae flocculation.

TABLE 1 | Comparison of different flocculants used for *Nannochloropsis* sp. harvesting.

Flocculant	Concentration	Flocculation efficiency	References
Aluminum sulfate	82.5 ppm	>95%	Chua et al., 2019
Ferric chloride	82.5 ppm	>95%	Chua et al., 2019
Tanfloc	10 ppm	98%	Roselet et al., 2016
AFlok-BP1	160 ppm	92%	Fuad et al., 2018
Mung bean protein concentrate	20 mL/L	>90%	Kandasamy and Shaleh, 2017
γ-Polyglutamic acid	22 ppm	96%	Zheng et al., 2012
Mushroom chitosan	25 ppm	>94%	This study

Higher flocculation efficiencies were observed in the 200-L vertical column photobioreactor compared to the air-lift raceway pond. The low flocculation efficiency was likely caused by the suboptimal mixing of the chitosan into the pond culture, as the mixing was significantly more efficient in the 50-mL and 200-L cultures (>95 and 98.3% yield, respectively). The raceway pond was slowly mixed using an air-lift system achieved with microbubbles, which may have also affected the performance since the bubbles disrupted the large flocs. Even after 24 h, the flocculation efficiency did not reach >90%. This result clearly indicated that the cells have not interacted properly with the chitosan. Bleeke et al. (2015) and Pugazhendhi et al. (2019) discussed the importance of mixing speed, intensity, and time. Mixing using a paddle-wheel system may provide a better flocculation performance as the mechanism less disruptive to the large flocs as they pass through. Koley et al. (2017) have demonstrated the effectivity of chitosan with flocculation efficiencies of ∼90% to flocculate *Scenedesmus obliquus* and *Chlorella vulgaris* cultured in raceway ponds. However, the cultures had to be pumped into 1000-L tanks, which was easier to mix with a large motor-driven stirrer. Further optimization on the mixing of the chitosan into the pond culture would be necessary along with economic feasibility studies to improve the attractiveness of chitosan for use in microalgae harvesting.

CONCLUSION

Mushroom chitosan was prepared by extracting and deacetylating chitin from mushroom powder and was verified using FTIR. Results showed that the prepared mushroom chitosan had similar flocculation efficiency as commercial crustacean-derived chitosan. Furthermore, chitosan can be sustainably prepared utilizing the wastes from mushroom industries and using it for harvesting promotes the chemical-free harvesting protocol for microalgae for food and/or feed applications. While chitosan was also found suitable for harvesting of large culture volumes, the requirement for efficient mixing should be considered. The availability of mushroom chitosan harvested *Nannochloropsis* offer an affordable and sustainable fish oil replacement product suitable for vegans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

[1]http://www.tanac.com.br/en/

AUTHOR CONTRIBUTIONS

EC conducted the experiments, analyzed the results, and wrote the original draft. AS assisted in the large-scale experiments. EE assisted in preparing the mushroom chitosan. ST-H prepared the resources for the large-scale experiments. PS supervised the entire project and acquired the necessary funding. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00771/full#supplementary-material

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Improving Fucoxanthin Production in Mixotrophic Culture of Marine Diatom *Phaeodactylum tricornutum* by LED Light Shift and Nitrogen Supplementation

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Fucoxanthin (Fx), a kind of primary carotenoids in brown seaweeds and diatoms, has attractive efficacy in human's healthcare including loss weight, the prevention of diabetes and Alzheimer's disease. Marine diatom *Phaeodactylum tricornutum* is now realized as a promising producer for commercial Fx production due to its higher content of Fx than brown seaweeds with easily artificial cultivation and Fx extraction. In the present study, to improve Fx production in *P. tricornutum,* the mixotrophic cultures were applied to optimize initial cell density, light intensity, light regime and nitrogen supplementation. The results showed that the higher initial cell density (1×10^7 cells mL^{-1}) and lower light intensity (20 μmol m^{-2} s^{-1}) were favorable for biomass production and Fx accumulation. The maximal Fx content [16.28 mg g^{-1} dry weight (DW)] could be achieved under blue light (BL), but the highest biomass concentration (5.53 g L^{-1}) could be attained under red: blue light (R: B, 6:1) in the batch culture. A novel two-phase culture approach was developed to increase the biomass concentration to the highest value (6.52 g L^{-1}) with the maximal productivity of Fx (8.22 mg L^{-1} d^{-1}) through light shift from R:B ratio (6:1) in phase 1 to R:B ratio (5:1) by enhancing BL and tryptone addition in phase 2. The content and intracellular amount of Fx were also increased 8% and 12% in phase 2 compared to phase 1. The expression levels analysis revealed that genes encoding phytoene synthase (PSY), zeaxanthin epoxidase (ZEP), and fucoxanthin-chlorophyll-protein b (FCPb) were upregulated significantly, with downregulation of the gene encoding violaxanthin de-epoxidase (VDE), leading to the improvement of Fx in phase 2. The present study demonstrated the two-phase culture strategy could promote Fx productivity through enhancing biomass production and increasing Fx content, indicating that strengthening BL coupled with adding tryptone were effective to facilitate Fx production by mixotrophic cultivation of marine diatom *P. tricornutum*.

Keywords: fucoxanthin, *Phaeodactylum tricornutum*, light regime, two-phase culture, qRT-PCR

INTRODUCTION

Fucoxanthin is a kind of primary carotenoids and draws increasing attention because of its functions of anti-oxidant, anti-obesity, and anti-cancer as well as effects against Alzheimer's disease (Vilchez et al., 2011; Fu et al., 2015; Xiang et al., 2017). The commercial source of Fx is mainly from brown seaweeds, which are difficult to meet the market demands due to the low productivity, low quality and high cost. Currently, marine diatom *Phaeodactylum tricornutum* is realized as a promising producer for commercial production of Fx since it grows fast and contains higher amount of Fx [1% to 6% of dry weight (DW)], which is over one hundred-fold of brown seaweeds (Rajauria et al., 2017; McClure et al., 2018). *P. tricornutum* is usually cultivated in deep tanks and open ponds for aquaculture, as well as in artificial photobioreactors recently for industrial purpose under autotrophic mode (Gao et al., 2017; Delbrut et al., 2018). Importantly, most strains of *P. tricornutum* could use glycerol and urea as organic carbon and nitrogen source, and biomass could reach to 3∼15 g L^{-1} under mixotrophic mode (Garcia et al., 2005; Huang et al., 2015; Nur et al., 2019). However, the mixotrophic *P. tricornutum* has not been applied in large-scale cultivation system because of the technological barriers mainly on the big risk of contamination when use organic nutrients in open system (Matsumoto et al., 2017). Compared to the autotrophic culture, the mixotrophic culture enhanced the cell growth rate and biomass production, but reduced the photosynthesis activity, leading to the decreased content of photosynthetic pigments (Liu et al., 2009). The content of carotenoids in *P. tricornutum* under mixotrophic conditions was usually 0.5∼0.7% of DW, in which Fx portion was even lower (Ceron-Garcia et al., 2013; Patel et al., 2019). It seems difficult to achieve high biomass and high Fx content simultaneously, resulting in the low productivity of Fx by mixotrophic *P. tricornutum*. Therefore, developing an applicable approach of mixotrophic *P. tricornutum* is vital to commercial production of Fx.

Light regime, including light intensity, light quality and light/dark cycle, is indispensable in the mixotrophic cultivation. In *P. tricornutum*, Fx binds with Chl $a + c$ and proteins to form FCP complex (Durnford et al., 1999), playing an important role in light harvesting and non-photochemical quenching (NPQ) (Havurinne and Tyystjarvi, 2017; Wang et al., 2019). Different from other microalgae, the specific structure of FCP in *P. tricornutum* allows it to capture blue-green light and supports its application in artificial cultivation system (Wang et al., 2019). At present, the impacts of light quality (presented as light spectrum) on *P. tricornutum* were usually investigated under autotrophic mode. For example, red light could promote cell growth and blue light (BL) could enhance Fx accumulation (Sirisuk et al., 2018; Wang S. et al., 2018). Also, blue LED light could save 50% and 75% energy input compared to red-blue LED light and white fluorescent light, respectively, which is beneficial for industrial use (Wang S. et al., 2018). Under green light, the intracellular amount of Fx was similar with white light (WL), while the ratio of FCP in thylakoid membrane proteins was significantly increased (Zhao, 2015). Accordingly, a two-phase culture with different light regimes has been used for promoting biomass and Fx production. For example, a hetero-photoautotrophic two-phase cultivation with white: blue light (1:1) induction was used in marine diatom *Nitzschia laevis* for Fx accumulation (Lu et al., 2018). Two-phase culture using different light regimes has also applied in inducing lipid accumulation in *P. tricornutum* (Sirisuk et al., 2018; Jung et al., 2019). It is noteworthy that light is not only energy source in the mixotrophic culture of microalgae, but also a role of inducing factor in accumulation of biomass and bioactive compounds in diatom. So far, it is still lack of reports for improving Fx production by mixotrophic *P. tricornutum* under different light regimes.

The whole genome sequencing of *P. tricornutum* was completed in 2008, which provides the biological basis for transcriptome analysis of gene expression and regulation (Bowler et al., 2008). Transcriptome and metabolome analysis indicated that the central-carbon metabolism, especially glycolysis, was enhanced by glycerol, the organic carbon source used in the mixotrophic mode, leading to the increase of cell growth rate and the final cell density (Villanova et al., 2017). The expression levels of genes involving Fx biosynthesis were investigated in recent years, but the reports are rare. For instance, the genetic engineering study demonstrated that the overexpression of *DXS* and *PSY* genes could result in the significantly increase of Fx content (Kadono et al., 2015; Eilers et al., 2016). Under autotrophic condition, the most of genes involving Fx biosynthesis, including *PSY*, *PDS*, *ZDS*, *LCYB*, and *ZEP* were upregulated by blue or green light (Coesel et al., 2008; Valle et al., 2014). It is still very limited to know the regulation of genes expression in Fx biosynthesis pathway under different light regimes and two-phase culture mode under mixotrophic conditions.

In the present work, the mixotrophic *P. tricornutum* was cultivated in shake flasks to optimize the growth conditions for improving Fx production. Initial cell density, LED light intensity and light quality were firstly investigated to obtain optimal parameters, subsequently a two-phase culture approach was developed for promoting Fx productivity by LED light shift and nitrogen supplementation. The expression levels of several key genes in Fx biosynthesis pathway were analyzed by quantitative real time polymerase chain reaction (qRT-PCR), aiming to reveal the metabolic regulation in two-phase culture process.

Abbreviations: Chl *a*, chlorophyll *a*; CRISO, carotenoid isomerase; Ddx, diadinoxanthin; DMAPP, dimethylallyl diphosphate; Dtx, diatoxanthin; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FCP, fucoxanthin-chlorophyll-protein; Fx, fucoxanthin; G3P, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl diphosphate; GPPS, geranyl pyrophosphate synthase; IDI, isopentenyl diphosphate: dimethylallyl diphosphate isomerase; IPP, isopentenyl pyrophosphate; LCYB, lycopene β-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-Carotene desaturase; ZEP, zeaxanthin epoxidase.

MATERIALS AND METHODS

Microalgal Strain and Seed Culture

Marine diatom *P. tricornutum* CCMP 1327 was kindly provided by Dr. Hanhua Hu in Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. The seed culture was

applied in 250-mL Erlenmeyer flasks containing 100-mL modified f/2 medium (Guillard, 1975) under mixotrophic condition. Temperature at 20°C was setup with continuous illumination of 10 μmol m^{-2} s^{-1} under white LED light and rotating speed of 150 r/min in shaking incubator. The modified f/2 medium contained (per liter): 20 g sea salt, 9.20 g glycerol (0.10 mol L^{-1}), 10 mg $NaH_2PO_4.H_2O$, 30 mg $Na_2SiO_3.9H_2O$, 3.15 mg $FeCl_3.6H_2O$, 4.36 mg $Na_2EDTA.2H_2O$, 9.80 μg $CuSO_4.5H_2O$, 6.30 μg $Na_2MoO_4.2H_2O$, 22 μg $ZnSO_4.7H_2O$, 10 μg $CoCl_2.6H_2O$, 180 μg $MnCl_2.4H_2O$. In addition, 1.17 g L^{-1} of tryptone and 0.30 g L^{-1} of urea (1:1, N mol/N mol) were added into the medium to final concentration of total nitrogen (TN) at 0.02 mol L^{-1}. The medium was autoclaved at 121°C for 20 min followed by urea addition using stock solution filtered through 0.45 μm membrane. The seed culture in late logarithmic phase was harvested by centrifugation and re-suspended in the medium above as the inoculum for subsequent experiments.

Optimization of Initial Cell Density and Light Intensity

A series of initial cell densities (1×10^6, 4×10^6, 7×10^6, 1×10^7 cells mL^{-1}) were investigated by adding the inoculum and culturing for 14 days. Then, various light intensities (10, 20, 30, 40, 50, 100, 150, 200 μmol m^{-2} s^{-1}) were evaluated at initial cell density of 1×10^7 cells mL^{-1} by cultivation for 12 days. The other culture conditions were same as the seed culture. Cell density, glycerol and nitrogen concentration were measured every 2 days during the cultivation. Biomass, content and volumetric concentration of Fx were measured at the end of cultivation.

Optimization of Light Regimes and Nitrogen Supplementation

White Light and Red: Blue Light

To explore the effects of different LED light qualities on biomass production and Fx accumulation, the full-spectrum WL and different red: blue lights (R:B, 0:1, 6:1, 1:1, 1:2, 1:0) were evaluated at initial cell density of 1×10^7 cells mL^{-1} under 20 μmol m^{-2} s^{-1} of light intensity. Cell density, biomass, glycerol and nitrogen concentration were measured every 2 days during the cultivation. The content and volumetric concentration of Fx were analyzed at the end of cultivation.

Light Shift and Nitrogen Supplementation in Two-Phase Culture

Two-phase culture approach was investigated to promote biomass and Fx production by light shift and nitrogen supplementation through two batch cultures.

In batch 1, R: B light (6:1) at 20 μmol m^{-2} s^{-1} was used in the mixotrophic cultivation for 6 days in phase 1, and then shifted to BL at 20 μmol m^{-2} s^{-1} with tryptone (T), urea (U) or the mixture (T:U = 1:1, N mol) addition in the medium to final concentration of TN (0.02 mol L^{-1}) in phase 2. The culture without nitrogen addition in phase 2 was set as the control.

In batch 2, the two-phase culture with light shift to BL and tryptone addition (BL + T) was set as the control. In the experimental groups, R: B light (6:1) at 20 μmol m^{-2} s^{-1} was used to culture for 6 days in phase 1, then BL was strengthened alone to form various R: B lights (5:1, 3:1, 1:1) at 25 μmol m^{-2} s^{-1}, or shifted to pure green light (GL) at 20 μmol m^{-2} s^{-1} with tryptone addition in phase 2. The cell growth and nutrient consumption were monitored every 2 days during the cultivation. Fx production were analyzed at the end of culture. The cells in the culture of R: B light (5:1) at 25 μmol m^{-2} s^{-1} on the 6th, 8th, 10th, 12th day were collected for Fx detection and total RNA isolation.

Analytical Methods

Cell Growth and Biomass Concentration

Cell density was determined by CytoFLEX flow cytometry (Beckman-Coulter, United States). Specific growth rate (μ, d^{-1}) of cells was calculated by the following formula:

$$\mu\ (d^{-1}) = (\ln N_t - \ln N_0)/(t - t_0) \quad (1)$$

where N_t and N_0 are the cell density (cells mL^{-1}) at time t(d) and time t_0 (d) (Chen et al., 2017).

2-mL cell suspension was collected in a pre-weighed tube and centrifuged at 3300 × g for 3 min. The pellet was washed twice and dried in a 60°C oven to a constant weight for biomass measurement. The biomass productivity ($P_{Biomass}$, mg L^{-1} d^{-1}) was calculated by following formula:

$$P_{Biomass}(mg\ L^{-1}\ d^{-1}) = (DW_t - DW_0)/(t - t_0) \times 1000 \quad (2)$$

where DW_0 and DW_t are the biomass concentration (g L^{-1}) at time t_0(d) and time t(d) (Chen et al., 2017).

Glycerol and Nitrogen Concentrations

Glycerol concentration (g L^{-1}) was determined by M-100 Biosensors (SIEMAN, China). The TN concentration (TN, mg L^{-1}) was determined by DR2700 spectrophotometer (HACH, United States) with reagent No. 2714100 (Qin et al., 2018). The nutrient consumption rate (CR, mg L^{-1} d^{-1}) and biomass yield per TN consumed ($Y_{X/TN}$, mg mg^{-1}) were calculated by following formula:

$$\text{Glycerol CR}\left(mg\ L^{-1}\ d^{-1}\right) = (GC_0 - GC_t)/(t - t_0) \times 1000 \quad (3)$$

where GC_t and GC_0 are the glycerol concentration (g L^{-1}) at time t (d) and time t_0 (d).

$$\text{TN CR}\left(mg\ L^{-1}\ d^{-1}\right) = (NC_0 - NC_1 + NC_2 - NC_t)/(t - t_0) \quad (4)$$

where NC_t and NC_0 are the TN concentration (mg L^{-1}) at time t(d) and time t_0(d), NC_1 and NC_2 are the TN concentration (mg L^{-1}) before and after the nitrogen addition on the 6th day in two-phase cultivation.

$$Y_{X/TN}(mg\ mg^{-1}) = (DW_t - DW_0)/(NC_0 - NC_1 + NC_2 - NC_t) \times 1000 \quad (5)$$

where DW_0 and DW_t are the biomass concentration (g L^{-1}) at time t_0 (d) and time t (d), NC_t and NC_0 are the TN concentration (mg L^{-1}) at time t (d) and time t_0 (d), NC_1 and NC_2 are the TN concentration (mg L^{-1}) before and after the nitrogen addition on the 6th day in two-phase cultivation.

Pigments

Natural pigments were extracted by organic solvents, and the qualitative and quantitative analysis were carried out by high performance liquid chromatography (HPLC) method modified from references (Zang et al., 2015; Chen et al., 2017). Briefly, 10-mg freeze-dried algal powder was mixed with ceramic bead and the mixture of acetone: methanol (1:1, v/v) precooled at 0–10°C in 15-mL tube, and then disrupted using the grinder (Tissuelyser-24, JINGXIN, China) at 70 Hz for 30 s. The supernatant was collected by centrifugation at 5900 × g for 3 min after freezing in the liquid nitrogen for 30 s. The disruption and centrifugation process were repeated until the pellet was colorless, and then dried all supernatants by nitrogen flow gas. 1-mL methanol: methyl tert-butyl ether solution (1:1, v/v) was added to dissolve the residue and filtered through a 0.22 μm nylon membrane for further pigments detection.

High performance liquid chromatography system (DIONEX P680, Thermo Scientific, Waltham, MA, United States) equipped with PDA detector and YMC^{TM} Carotenoids column (150 mm × 4.6 mm, 3 μm) was employed for pigments analysis. The column temperature was maintained at 30°C, the flow rate was 0.8 mL min^{-1}, and detection wavelength was 440 nm. Methanol and methyl tert-butyl ether were employed as mobile phases A and B, respectively. The gradient program was as followed: 0∼6 min, A: 95%→80%, B: 5%→20%; 6∼12 min, A: 80%→60%, B: 20%→40%; 12∼19 min, A: 60%→55%, B: 40%→45%; 19∼20 min, A: 55%→95%, B: 45%→5%; 20∼23 min, A: 95%, B: 5%. The peaks of pigments were characterized according to the retention time of Fx and Chl *a* standards (Sigma-Aldrich Chemical Co., St. Louis, MO, United States), and external standard curve was used for quantification. The volumetric concentration of Fx (VC_{Fx}, mg L^{-1}), intracellular amount of Fx (CC_{Fx}, pg cell^{-1}) and Fx productivity (P_{Fx}, mg L^{-1} d^{-1}) were calculated by the following formula:

$$VC_{Fx}(\text{mg L}^{-1}) = \text{Biomass}(\text{g L}^{-1}) \times \text{Fx content (mg g}^{-1}\text{, DW)} \quad (6)$$

$$CC_{Fx}(\text{pg cell}^{-1}) = VC_{Fx}(\text{mg L}^{-1})/\text{Cell density}(\text{cells mL}^{-1}) \quad (7)$$

$$P_{Fx}(\text{mg L}^{-1}\text{ d}^{-1}) = (VC_{Fxt} - VC_{Fx0})/(t - t_0) \quad (8)$$

where VC_{Fx0} and VC_{Fxt} are the volumetric concentration of Fx (mg L^{-1}) at time t_0(d) and time t(d).

Quantitative Real Time PCR

To evaluate the expression levels of key genes in Fx biosynthesis pathway during two-phase culture, total RNA isolation from cells under R:B light (5:1) group on the 6th, 8th, 10th, 12th day was carried out using Plant RNA Kit (Omega, America) for qRT-PCR analysis. Evo M-MLV RT Kit for gDNA clean and SYBR Green Premix *Pro Taq* HS qPCR Kit (Accurate biotechnology, China) were employed. qRT-PCR analysis was performed on CFX96 Touch^{TM} Deep Well Real-Time PCR Detection System (Bio-rad, America). The gene coding β-actin was selected as an internal control (Xie et al., 2014). The sequences of target genes were obtained from KEGG database[1] and primers shown in **Table 1** were designed by NCBI.[2] Total RNA samples were performed in triplicates. The relative expression levels of target gene transcripts were normalized using β-*actin* as reference gene by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

TABLE 1 | Primer sequence of key genes in fucoxanthin biosynthesis pathway.

Gene	Primer (5′-3′)	Gene	Primer (5′-3′)
β-*actin*	GACTCCACCTTCCAGACCATTA	*LCYB*	GCATTGCGACGTACATGGTC
	GACCCTCCAATCCAAACAGAG		TCGTCGAGCTTCACTCTTGG
DXS	AGCCAATTCTGGACTCGGTG	*ZEP1*	GGCACTCGAACGCATCAATC
	GCAAGGCAACAGTGAGTTCG		TCGAAGCGTACCAACCAGTC
PSY	CCACGCCGAACATGCTTTAG	*ZEP2*	ATACACCGTCTTTGCGGGAG
	GACTTCTTGCACTTGTGCCG		CCATCACCGACATCACTCGT
PDS1	TTCTCCACGACACTCAAGGC	*ZEP3*	CGGTTTTTCTGTGCTGGGTG
	CCGGTTTCGATCCAGTCTCC		AGTCTTGAATGGCGGCAGAA
PDS2	GTGTTCTCGGTGGCAGTCTT	*VDE*	TTCCATCAAGGCGCAAAAGC
	GAGCCGACGCTAGAGAAGTC		GCTGGGAGGTTTCTCGTTCA
ZDS	TTGGACTCGATGGAAGGTGC	*FCPb*	AGCACCGCTTGGATTCTACG
	CCGCTTTCCTCTTTCGCTTG		TGCCAAGTATCCAGCAACGG

Statistical Analysis

All data were performed in biological triplicates and presented as mean ± standard deviation (SD). Origin V9.0 software was used to plot figures. The statistical analysis was performed by one-way analysis of variance (ANOVA) and LDS *t*-test with SPSS V22.0 software. Significant differentiation level were set at $^{*}p < 0.05$ and $^{**}p < 0.01$ by compared with control group.

RESULTS AND DISCUSSION

Effects of Initial Cell Density and Light Intensity

The effects of initial cell density was shown in **Figure 1A**. The rapid increase of cell growth were observed in the first 8 days but slowed down in the next 6 days. With the increase of initial cell density, the average specific growth rate of cells was significantly decreased ($p < 0.01$) (**Table 2**). The highest final cell density (6.72×10^7 cells mL^{-1}) and the maximal biomass concentration (3.73 g L^{-1}) were achieved at the highest initial cell density (1×10^7 cells mL^{-1}) (**Figures 1A,C**). Even though the highest content (18.61 mg g^{-1}) and intercellular amount of Fx (1.23 pg cell^{-1}) were attained at 4×10^6 cells mL^{-1} (**Figure 1C** and **Table 2**), the highest volumetric concentration of Fx reached to 59.66 mg L^{-1} at the highest initial cell density (1×10^7 cells mL^{-1}) (**Table 2**). Therefore, for improving biomass and Fx production in a shorter time, the highest initial cell density was the option in the following experiments.

To further promote the cell growth under high cell density, the effects of light intensity were evaluated. Interestingly, the increase of light intensity had no obviously negative effect on

[1]https://www.kegg.jp/kegg-bin/show_organism

[2]https://www.ncbi.nlm.nih.gov/tools/primer-blast/

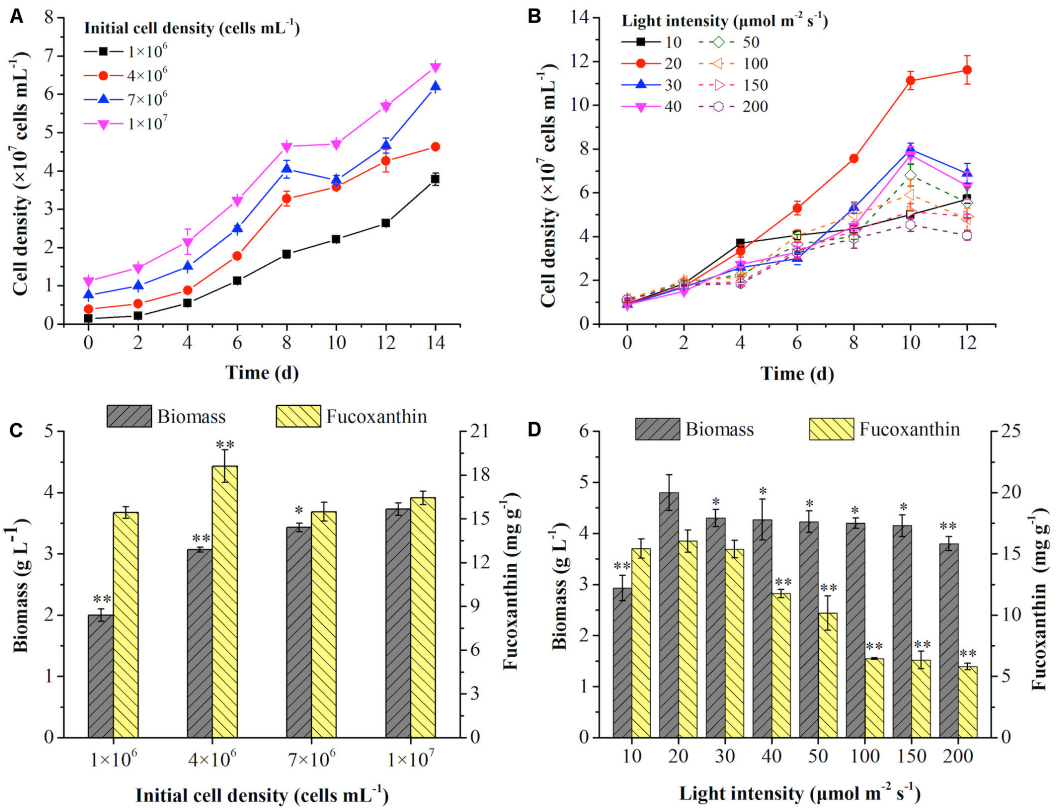

FIGURE 1 | Effects of initial cell density and light intensity on cell growth **(A,B)**, biomass concentration and fucoxanthin content **(C,D)** in the mixotrophic growth of *P. tricornutum*. Significant differentiation level was set at * $p < 0.05$ and ** $p < 0.01$ compared with initial cell density of 1×10^7 cells mL^{-1} and light intensity of 20 μmol m^{-2} s^{-1}, respectively.

TABLE 2 | Effects of initial cell density and light intensity on cell growth, fucoxanthin production and nutrient consumption in the mixotrophic growth of *P. tricornutum*.

	μ (d^{-1})	Volumetric Fx concentration (mg L^{-1})	Consumption rate (mg L^{-1} d^{-1})		Intercellular Fx amount (pg $cell^{-1}$)	Chl *a* (mg g^{-1})	Fx/Chl *a*
			Glycerol	TN			
Initial cell density (cells mL^{-1})							
1×10^6	0.24 ± 0.00**	30.84 ± 0.98**	164.98 ± 5.42**	4.50 ± 0.33	0.81 ± 0.01**	NA	NA
4×10^6	0.18 ± 0.01**	57.07 ± 1.66	188.87 ± 6.35**	7.33 ± 0.33**	1.23 ± 0.04**	NA	NA
7×10^6	0.15 ± 0.00**	53.06 ± 1.46*	199.87 ± 3.18*	7.00 ± 0.17**	0.86 ± 0.02	NA	NA
1×10^7	0.13 ± 0.00	59.66 ± 2.40	220.04 ± 9.53	9.50 ± 0.17	0.89 ± 0.04	NA	NA
Light intensity (μmol m^{-2} s^{-1})							
10	0.15 ± 0.00**	45.11 ± 2.23**	136.11 ± 4.81**	8.71 ± 0.38**	0.79 ± 0.02**	24.63 ± 0.67*	0.63 ± 0.04**
20	0.21 ± 0.01	76.78 ± 4.26	227.78 ± 9.62	15.88 ± 0.13	0.66 ± 0.01	28.63 ± 1.55	0.56 ± 0.01
30	0.17 ± 0.00*	66.09 ± 1.97**	166.67 ± 8.33**	13.00 ± 1.00**	0.96 ± 0.04**	25.50 ± 0.66*	0.60 ± 0.02*
40	0.16 ± 0.01**	50.13 ± 4.35**	155.56 ± 4.81**	9.32 ± 0.57**	0.79 ± 0.03**	16.49 ± 0.40**	0.71 ± 0.00**
50	0.14 ± 0.01**	42.81 ± 4.62**	119.44 ± 9.62**	8.17 ± 0.17**	0.77 ± 0.02**	14.94 ± 1.33**	0.68 ± 0.04**
100	0.12 ± 0.01**	27.04 ± 0.89**	141.67 ± 8.33**	8.50 ± 0.50**	0.57 ± 0.04*	8.99 ± 0.16**	0.72 ± 0.02**
150	0.13 ± 0.01**	26.20 ± 2.13*	122.22 ± 4.81**	7.83 ± 0.50**	0.53 ± 0.03**	7.84 ± 0.14**	0.81 ± 0.08**
200	0.11 ± 0.00**	22.01 ± 0.46**	144.44 ± 4.81**	7.59 ± 0.25**	0.55 ± 0.03**	7.53 ± 0.42**	0.77 ± 0.01**

*Data are expressed as mean ± SD of three replicates. NA-No detected. Significant differentiation level with * $p < 0.05$ and ** $p < 0.01$ compared with initial cell density 1×10^7 cells mL^{-1} and light intensity 20 μmol m^{-2} s^{-1}, respectively.*

biomass production from 30 to 200 μmol m^{-2} s^{-1} (**Figure 1D**), but an inhibiting effect on cell density was observed (**Figure 1B**). The highest biomass concentration (4.80 g L^{-1}) and Fx content (16.03 mg g^{-1}) could be reached at light intensity of 20 μmol m^{-2} s^{-1} (**Figure 1D**). The content and intercellular amount of Fx significantly decreased when the light intensity exceeded

30 μmol m^{-2} s^{-1}, which was consist with the previous studies. For instance, the Fx content reached the maximal value at 13.5 μmol m^{-2} s^{-1} (Gomez-Loredo et al., 2016) and dropped from 7.50 mg g^{-1} to 1.10 mg g^{-1} when the light intensity increased from 30 to 180 μmol m^{-2} s^{-1} in *P. tricornutum* (Wang H. et al., 2018). However, the cell growth and biomass production were both decreased with the increase of light intensity under autotrophic condition, which may be explained by the photo-inhibition (Wang H. et al., 2018). Besides, Fx and Chl *a* contents were significantly reduced but fucoxanthin-to-chlorophyll *a* ratio (Fx/Chl *a*) was significantly increased from 20 μmol m^{-2} s^{-1} in this study ($p < 0.05$) (**Table 2**). Since Chl *a* exists not only in FCP, but also in free Chl *a* (Nymark et al., 2009), the increase of Fx/Chl *a* ratio indicated that free Chl *a* degraded more than that in FCP, resulting in higher degradation of Chl *a* in total Chl *a* than Fx when cells exposed to high light. Similarly, both of Fx and Chl *a* contents decreased sharply with the increase of light intensity in autotrophic *P. tricornutum* (Nur et al., 2019; Conceicao et al., 2020). The transcriptome analysis indicated that the genes encoding enzymes in biosynthesis of GGPP, which is the precursor of both Chl *a* and Fx, were downregulated upon high light intensity in *P. tricornutum*, leading to the reduction of Chl *a* and Fx (Nymark et al., 2009). When cells were exposed to high light, NPQ was activated to convert excess light energy into heat energy (Nymark et al., 2009). In diatoms, Ddx cycle plays a critical role in NPQ, which protects cell from high-light damage (Hao et al., 2018). Under high light, the contents of Ddx and Fx were lower than low light (Conceicao et al., 2020), which due to the upregulation of VDE promoted the conversion of Ddx to Dtx and violaxanthin to zeaxanthin, leading to the decline of Fx (Nymark et al., 2009). Therefore, the dim light at 20 μmol m^{-2} s^{-1} is favorable for cell growth and Fx accumulation under mixotrophic condition without photo inhibition.

Effects of Light Quality

The light spectrums of WL and various red: blue lights (R: B, 0:1, 6:1, 1:1, 1:2, 1:0) were shown in **Figure 2A**. The wavelength of WL was ranged from 402 nm to 760 nm, and the peak wavelength of blue and red light was 452 nm and 636 nm, respectively. As shown in **Figure 3A**, the cells grew rapidly under red: blue lights and the highest biomass concentration (5.53 g L^{-1}) with productivity (351.39 mg L^{-1} d^{-1}) could be achieved at R: B light (6:1), which was 1.22- and 1.38-fold higher than WL ($p < 0.01$) (**Figure 3B** and **Table 3**). Even though the biomass productivity in R: B light (6:1) and R: B light (1:2) were similar (**Table 3**), which might due to the nutritional limitation in the late phase of culture, the former in biomass productivity was 4.12% higher than the later. Similarly, the previous study indicated that the biomass was significantly higher at the mix of red and blue light than the fluorescent WL in autotrophic culture of *P. tricornutum*, but it was similar in the group between R:B (70:30) and R:B (30:70), which was consist with this study (Sirisuk et al., 2018). The increase of biomass in the first 6 days (**Figure 3B**) was due to the prior uptake of tryptone and urea as carbon and nitrogen source, which explained the slow consumption of glycerol in this period (**Figure 3C**). The maximum average glycerol consumption rate during 12-days cultivation reached to 327.78 mg L^{-1} d^{-1}, while the highest biomass yield per TN consumed ($Y_{X/TN}$) reached to 28.49 mg mg^{-1} at R: B light (6:1) (**Table 3**). Moreover, the highest Fx content (16.28 mg g^{-1}) and Chl *a* content (32.68 mg g^{-1}) were attained under BL (**Figure 3D** and **Table 3**), which were 12% and 36% higher than WL ($p < 0.01$), respectively. These results were similar with that the Fx and Chl *a* contents were significantly higher under BL than WL in autotrophic *P. tricornutum* and *Coscinodiscus granii* (Sirisuk et al., 2018; Su, 2019). However, the biomass concentration (3.63 g L^{-1}) under BL was the lowest one (**Figure 3B**) in this study. Compared to WL, the results indicated that R: B light (6:1) could promote the cell growth and biomass production, but BL has positive effect on Fx accumulation.

The similar reports could be found that *P. tricornutum* produced more biomass under higher proportion of red light in mixture light under autotrophic condition (Sirisuk et al., 2018), but higher contents of xanthophyll cycle pigments (including Fx) were obtained under BL (Jungandreas et al., 2014). The light qualities, red, green, and blue light, played a vital role in regulation of carbon flow distribution. For example, the shift from BL to red light increased the intermediates of glycolysis and promoted the accumulation of carbohydrates. On the contrary, the shift of red light to BL led to the accumulation of amino acids and tricarboxylic acid (TCA) cycle intermediates, as well as biosynthesis of proteins (Jungandreas et al., 2014). The previous study indicated that the expression of photosynthesis-related nuclear genes were light quality-independent, while the energy transfer efficiency, photo protection and PSII repair related genes were highly dependent on light quality, especially BL (Valle et al., 2014). Similar results were observed in another research, in which compared to red light, BL performed larger pool size of xanthophyll cycle pigments and higher value of NPQ and de-epoxidation state [DES = Ddt/(Ddx + Ddt)], which meant BL showed more capacity of photo protection (Costa et al., 2013). BL not only influenced the genes expression to regulate metabolism, but also directly regulate the activities of specific enzymes, like nitrate reductase (Azuara and Aparicio, 1983). The significant reduction of C/N ratio in cells under BL indicated that it performed higher nitrogen assimilation in cells compared to red light (Jungandreas et al., 2014). This phenomenon was similar in our study, the nitrogen consumption rate was increased with higher proportion of BL in the mixed light except R:B (1:1) (**Table 3**). For the reason why TN consumption rate at R:B (1:1) was higher than at (1:2), it might be due to that the nitrogen source used in this study was the mixture of tryptone and urea. Perhaps more TN was used as carbon source at R: B (1:1), resulting in the higher TN consumption rate with the lower consumption rate of glycerol in this group. Interestingly, when cells were exposed to WL, BL, and red light, respectively, after dark treatment, the transcript levels of *PSY*, *PDS*, *ZEP*, and *FCPb* increased immediately upon blue and WL, while the expression levels were much lower in response to red light (Coesel et al., 2008). Additionally, transcriptome analysis indicated that the transcripts under BL were enriched in Fx-related expressed sequence tag (EST) database (Coesel et al., 2008), and the expression of *PSY*, *PDS*, *ZDS*, and *ZEP3* were enhanced, resulting in the promotion of Fx production

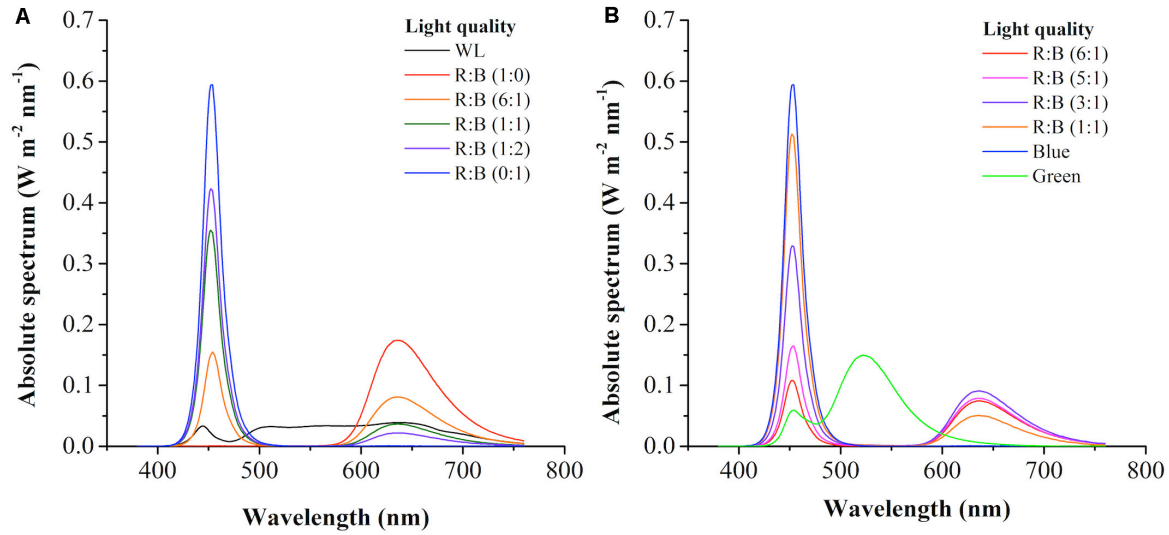

FIGURE 2 | The light spectrum of white light (WL) and various red: blue lights (1:0, 6:1, 1:1, 1:2, 0:1) **(A)** and light regimes in the two-phase culture **(B)**.

(Costa et al., 2013; Valle et al., 2014). Therefore, choosing R: B light (6:1) as the optimal light in phase 1 and BL as inducing light in phase 2 were carried out in further experiments.

Effects of Light Shift and Nitrogen Supplementation in Two-Phase Culture

Feeding substrates in batch culture is a common approach in microalgal cultivation which benefits biomass and bioactive compounds production (Ceron-Garcia et al., 2013; Lu et al., 2018). Fx exists in FCP, and sufficient nitrogen is essential not only for biomass production, but also for Fx accumulation (McClure et al., 2018; Wang H. et al., 2018; Nur et al., 2019). Thus, the effects of light shift and nitrogen supplementation in phase 2 were evaluated in two batch cultures.

In the batch 1, the mixotrophic cells were cultivated at R:B light (6:1) for 6 days in phase 1, then light shifted to pure BL for induction with or without supplementation of nitrogen [tryptone, T; urea, U; or the mixture, T:U = 1:1 (N mol)] in different groups in phase 2. As shown in **Figure 4A**, the culture groups of BL + T or BL+ the mixture in phase 2 could observe the promotion of cell growth compared to BL group, but BL + U inhibited the cell growth as well as Fx production (**Figure 4B**). The final cell density and biomass concentration reached to the highest value of 1.30×10^8 cells mL^{-1} and 5.80 g L^{-1} by tryptone addition (**Figures 4A,B**), which were 23% and 58% higher than the control group (BL) ($p < 0.05$), respectively. The highest biomass productivity achieved 373.61 mg L^{-1} d^{-1} under tryptone addition, which was significant higher than BL + U and the control group (**Table 4**). In contrast, the previous study showed that successive supplementation of 0.01 mol L^{-1} urea in mixotrophic growth of *P. tricornutum* led to 5.37-fold higher biomass, while the biomass was reduced when urea concentration exceeded 0.01 mol L^{-1} with 0.10 mol L^{-1} glycerol (Garcia et al., 2005). This phenomenon was caused by that the cells prioritized to utilize amino acids in tryptone as carbon and nitrogen source in phase 1, while the higher urea residual after urea addition in phase 2 exceed the optimum concentration, leading to the negative impact on cell division and Fx production in this study. As shown in **Figure 4B** and **Table 4**, the highest content (13.21 mg g^{-1}) and volumetric concentration (76.58 mg L^{-1}) of Fx were attained by tryptone addition in phase 2, with the consumption rate of glycerol (233.33 mg L^{-1} d^{-1}) and TN (13.69 mg L^{-1} d^{-1}). Besides, the intercellular amount of Fx was significantly increased up to 0.61 pg $cell^{-1}$ in BL + T group in phase 2, which was 1.42-fold higher than the control group (BL) (**Table 4**). Compared to the group of 12-days cultivation under R: B light (6:1) (**Figures 3B,D**), the biomass, Fx content and volumetric concentration were increased by 5%, 7%, and 12% under the two-phase culture in batch 1 (**Figure 4B**), respectively.

In the batch 2, to verify the inducing effect on Fx production, after cultivated at R: B light (6:1) for 6 days in phase 1, various light shifts were evaluated in phase 2 compared with the control group (BL + T). Among them, BL was enhanced solely to form R: B lights (5:1, 3:1, 1:1), or light shifted to pure green light (GL). All of the groups were combined with tryptone addition in phase 2. The light spectrums were shown in **Figure 2B**. Interestingly, the cell growth showed obvious difference from the 6th day in phase 2 but reached to a similar level of final cell density (**Figure 4C**), leading to a same specific growth rate in all groups (**Table 4**). However, the nutrition consumption rates were different (**Table 4**), which affected the metabolic flux of nutrients converted to intercellular components (carbohydrates, proteins, lipids etc.) in different groups, resulting in the difference of biomass concentrations. The highest biomass concentration (6.52 g L^{-1}), which was 17% higher than the control group (BL + T) ($p < 0.01$), was achieved with the maximum productivity (402.78 mg g^{-1} L^{-1}) in R: B (5:1) + T group in phase 2 (**Figure 4D** and **Table 4**). Glycerol consumption rate and $Y_{X/TN}$ were consistently increased with the increasing biomass, and reached to the highest value in R: B (5:1) + T group

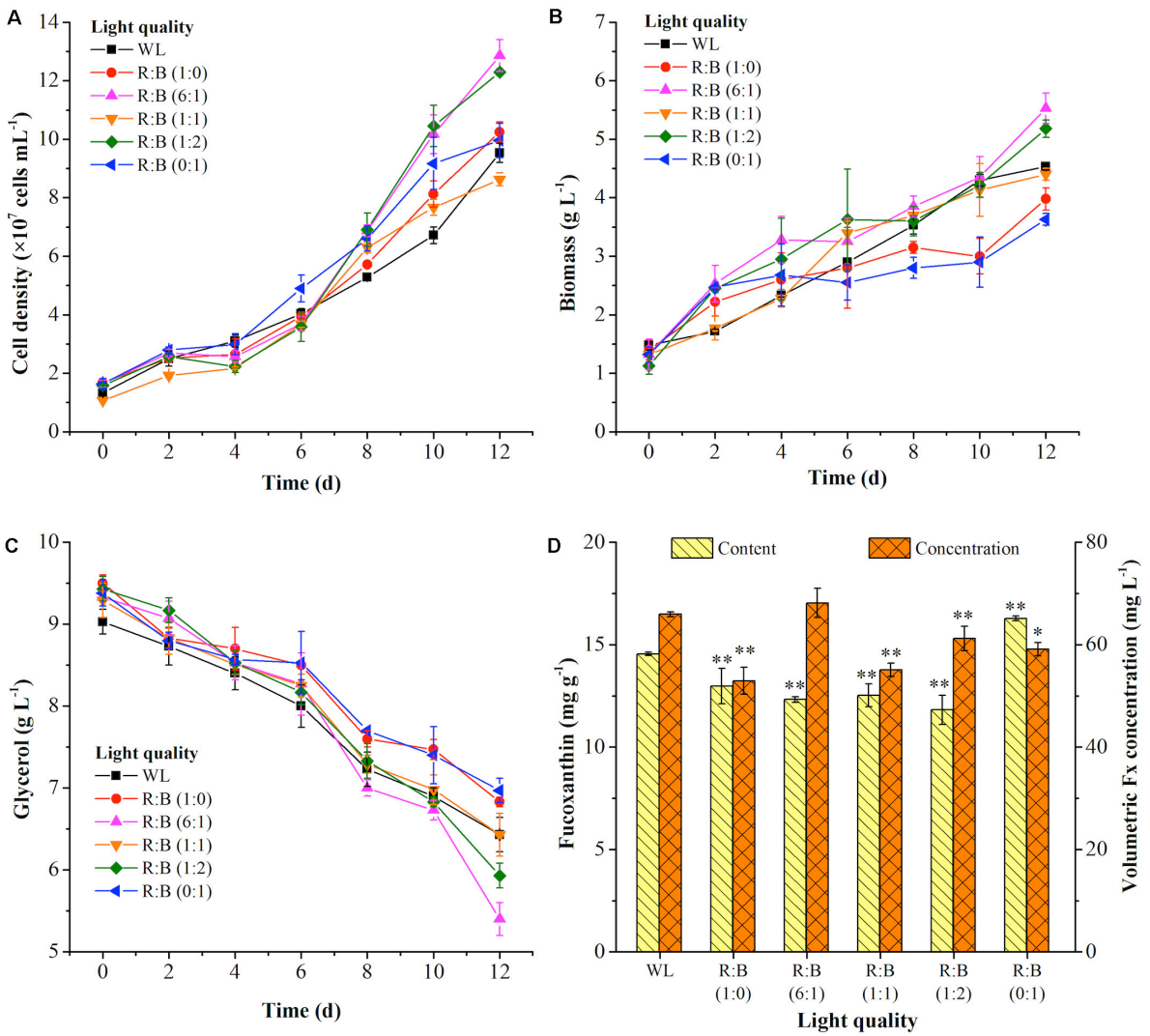

FIGURE 3 | The cell growth **(A)**, biomass **(B)** and glycerol concentration **(C)**, fucoxanthin production **(D)** at white light (WL) and various red: blue lights (1:0, 6:1, 1:1, 1:2, 0:1). Significant differentiation level was set at * $p < 0.05$ and ** $p < 0.01$ compared with white light (WL).

TABLE 3 | Nutrients consumption, biomass and Fx production at white light and various R: B lights under the mixotrophic mode.

Light quality	μ (d^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Consumption rate (mg L^{-1} d^{-1})		$Y_{X/TN}$ (mg mg^{-1})	Chl *a* (mg g^{-1})
			Glycerol	TN		
White light (WL)	0.16 ± 0.00	254.17 ± 7.22	216.67 ± 8.33	11.18 ± 0.16	22.73 ± 0.82	23.97 ± 1.85
R:B (1:0)	0.15 ± 0.00*	223.61 ± 2.41**	222.22 ± 9.62	11.83 ± 0.17**	18.90 ± 0.14**	29.28 ± 0.87*
R:B (6:1)	0.17 ± 0.00*	351.39 ± 9.62**	327.78 ± 4.81**	12.33 ± 0.17**	28.49 ± 0.48**	20.53 ± 0.95*
R:B (1:1)	0.17 ± 0.00*	256.94 ± 2.41	238.00 ± 6.77*	13.75 ± 0.25**	18.69 ± 0.50**	22.26 ± 1.39
R:B (1:2)	0.17 ± 0.00*	337.50 ± 8.33**	291.67 ± 8.33**	12.50 ± 0.17**	26.45 ± 0.86**	23.76 ± 0.46
R:B (0:1)	0.15 ± 0.01*	193.06 ± 2.41**	201.39 ± 6.36	13.17 ± 0.50**	14.68 ± 0.58**	32.68 ± 0.95**

*TN, total nitrogen; $Y_{X/TN}$, biomass yield per total nitrogen consumed. Data are expressed as mean ± SD with three replicates. Significant differentiation level with *$p < 0.05$ and **$p < 0.01$ compared with white light (WL).*

in phase 2 (**Table 4**), respectively. Compared to the control group (BL + T) in phase 2, even though the Fx content was lower in R:B (5:1) + T group ($p < 0.05$), the intercellular amount of Fx was similar in the two groups (**Table 4**) but the volumetric concentration of Fx (84.48 mg L^{-1}) achieved the highest level ($p < 0.05$) (**Figure 4D**). Therefore, the shift to R: B (5:1) with

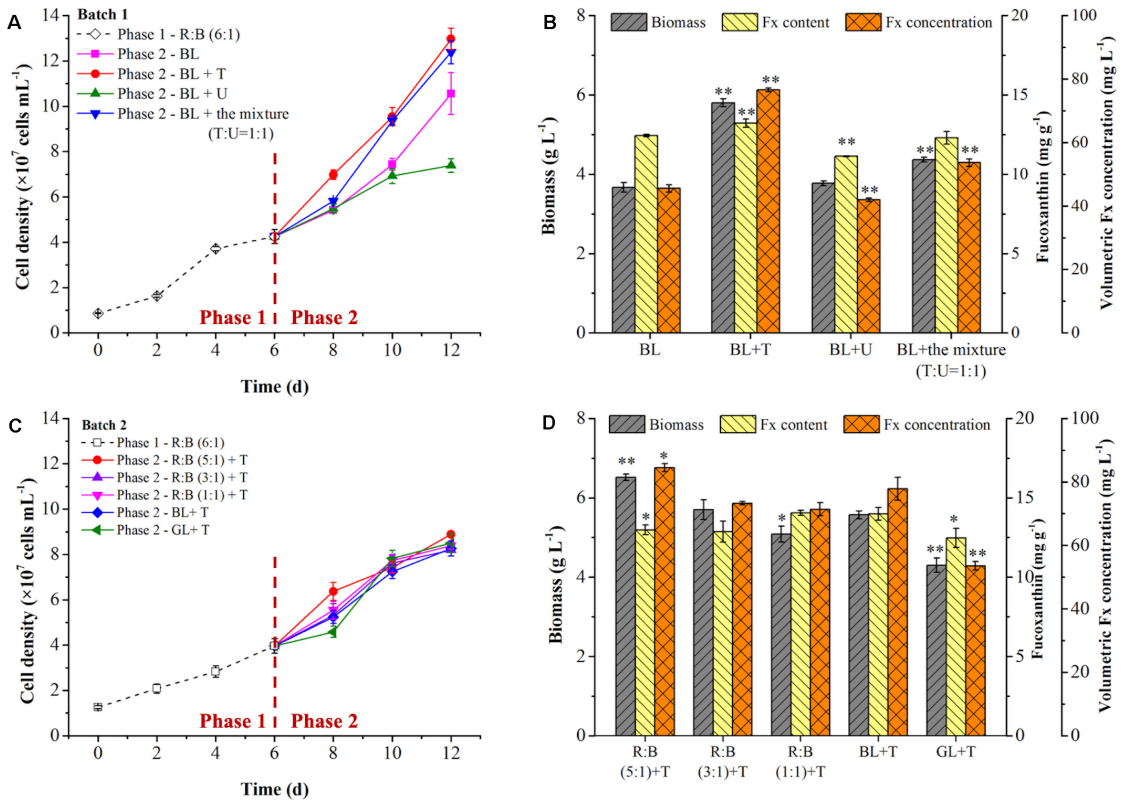

FIGURE 4 | Effects of light shift and nitrogen supplementation on cell growth **(A,C)** and fucoxanthin production **(B,D)** in two phase of two batch cultivation. In batch 1 **(A,B)**, the mixotrophic cells grew at R: B light (6:1) for 6 days in phase 1, then light shifted to pure blue light (BL) with (+) nitrogen supplementation in phase 2. Nitrogen source was presented as urea (U), tryptone (T) and their mixture at a ratio (T:U = 1:1, N mol). Significant differentiation level was set at ** $p < 0.01$ compared with blue light (BL) shift. In batch 2 **(C,D)**, blue light was strengthened solely to form R: B lights (5:1, 3:1, 1:1), or shifted to pure blue light (BL) or pure green light (GL), respectively, with (+) tryptone (T) addition in phase 2. Significant differentiation level was set at * $p < 0.05$ and ** $p < 0.01$ compared with blue light shift with tryptone addition (BL + T) in batch 2.

TABLE 4 | Effects of LED light shift and nitrogen supplementation in two-phase culture strategy.

Strategy	μ (d^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Consumption rate (mg L^{-1} d^{-1})		$Y_{X/TN}$ (mg mg^{-1})	Intercellular Fx amount (pg $cell^{-1}$)	Fx/Chl *a*
			Glycerol	TN			
Batch 1: blue light shift with nitrogen supplementation in phase 2							
BL	0.21 ± 0.00	208.33 ± 4.17	144.44 ± 4.81	11.75 ± 0.08	17.73 ± 0.23	0.43 ± 0.02	0.65 ± 0.03
BL + T	0.22 ± 0.00**	373.61 ± 6.36**	233.33 ± 14.43**	13.69 ± 1.21*	27.41 ± 2.12**	0.61 ± 0.03**	0.63 ± 0.02
BL + U	0.18 ± 0.00**	201.39 ± 8.67	180.56 ± 4.81**	10.81 ± 0.41*	18.64 ± 0.14**	0.57 ± 0.02**	0.67 ± 0.02
BL + the mixture (T:U = 1:1)	0.22 ± 0.00**	240.28 ± 4.81**	238 ± 17.35**	12.22 ± 0.77	19.70 ± 1.10*	0.43 ± 0.01	0.68 ± 0.01
Batch 2: light shift with tryptone addition in phase 2							
R:B (5:1) + T	0.16 ± 0.00	402.78 ± 6.36**	386.11 ± 9.62**	9.33 ± 0.67	43.27 ± 2.42**	0.95 ± 0.02	0.45 ± 0.05
R:B (3:1) + T	0.16 ± 0.00	338.89 ± 13.39	366.67 ± 14.43**	9.67 ± 0.67	35.11 ± 1.27	0.89 ± 0.00*	0.45 ± 0.01
R:B (1:1) + T	0.16 ± 0.00	286.11 ± 6.36**	297.22 ± 4.81*	9.33 ± 0.67	30.73 ± 1.52	0.85 ± 0.01**	0.43 ± 0.01
BL + T	0.16 ± 0.00	338.89 ± 6.36	275.00 ± 8.33	10.11 ± 0.51	33.55 ± 1.04	0.94 ± 0.02	0.45 ± 0.04
GL + T	0.16 ± 0.00	273.61 ± 9.62**	247.22 ± 4.81**	8.78 ± 0.35*	31.18 ± 0.61*	0.63 ± 0.02**	0.62 ± 0.05*

*N, nitrogen; TN, total nitrogen; $Y_{X/TN}$, biomass yield per total nitrogen consumed; Fx, fucoxanthin. U, urea; T, tryptone; T:U = 1:1, the mixture of tryptone and urea; BL, pure blue light; GL, pure green light. Data are expressed as mean ± SD in three replicates. Significant differentiation level with *$p < 0.05$ and **$p < 0.01$ compared with the control groups [blue light shift (BL) in batch 1, blue light shift with tryptone addition (BL + T) in batch 2], respectively.*

tryptone addition in phase 2 was the best option to encourage more biomass production and accumulation of intercellular Fx, leading to an increasing volumetric concentration of Fx.

It is noteworthy that the cell density was increased rapidly in GL + T in phase 2 (**Figure 4C**), but the lowest biomass concentration was observed (**Figure 4D**). Similarly in the

previous report, the biomass entered stable phase and the lipid content increased by 53% and 29% when light shifted from R:B (50:50) (Sirisuk et al., 2018) and BL (Jung et al., 2019) to green light, respectively. Additionally, the content and intercellular amount of Fx were the lowest in GL + T compared to other groups, resulting in the lowest volumetric concentration of Fx (**Figure 4D** and **Table 4**). It was reported that carotenoids content was reduced after 3-days green light exposure (Jung et al., 2019), and the genes encoding LCYB, ZEP1, and ZEP 2 performed high initial transcription levels, then balanced out the difference after 24 h exposure to green light (Valle et al., 2014), which explained the reduction of Fx under green light. Additionally, the previous study indicated that Chl *a* was more inhibited compared to carotenoids under green light induction (Jung et al., 2019). These results explained the increase of Fx/Chl *a* ratio under green light in **Table 4**. Therefore, shifting to green light was neither the option for biomass nor for Fx production.

Bioprocess Analysis of R: B (5:1) + T Group in the Batch 2

To further understand the physiological response and regulation mechanism of cells in R: B (5:1) + T group in the batch 2, the biomass and Fx production, nutrient consumption were analyzed in the two-phase culture. The cell samples were taken from the culture in phase 2 at four time points (6th, 8th, 10th, and 12th days) to evaluate the Fx content and key genes expression in Fx biosynthesis pathway.

As shown in **Figure 5A**, the biomass increased slowly with low biomass productivity (208.33 mg L^{-1} d^{-1}) in phase 1, and then the cells utilized glycerol more quickly for rapid growth from 4th day, which might be due to the exhaustion of available amino acids in tryptone as carbon source of prior utilization. With the rapid consumption of glycerol in phase 2, the biomass productivity reached to 597.22 mg L^{-1} d^{-1}, which was 2.87-fold higher than phase 1 ($p < 0.01$) (**Figures 5A,B**). The glycerol consumption rate achieved 533.33 mg L^{-1} d^{-1} in phase 2, resulting the 223% increase in the final biomass concentration (**Figures 5A,B**). However, the consumption rate of TN was lower in phase 2 (**Figure 5B**), which might be caused by a slow urea consumption in the medium since 6th day. The addition of tryptone provided sufficient organic carbon and nitrogen for biomass production in phase 2, while the Fx content and intercellular amount reached to the highest level (13.26 mg g^{-1} and 0.95 pg $cell^{-1}$) on the 8th and 12th day, respectively (**Figure 5C**). At the end of cultivation, the

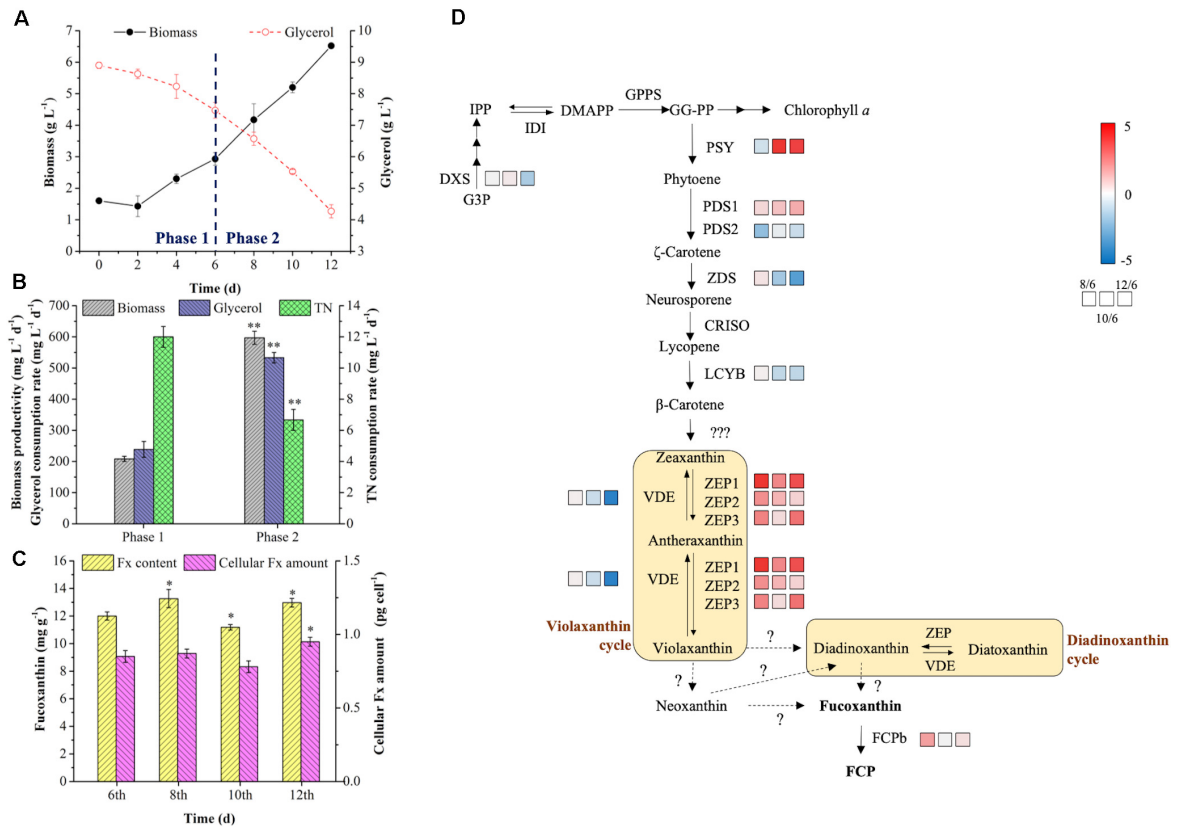

FIGURE 5 | Bioprocess analysis of R: B (5:1) + T group in the batch 2. Production and nutrient consumption **(A,B)** in two-phase culture, fucoxanthin content and intercellular amount **(C)**, genes expression levels in fucoxanthin biosynthesis pathway on the 6th, 8th, 10th, 12th days in phase 2 **(D)**. Significant differentiation level was set with * $p < 0.05$ and ** $p < 0.01$ compared with that at 6th day in phase 1, respectively. The color boxes indicate the expression values of log_2FC (8th/6th), log_2FC (10th/6th), and log_2FC (12th/6th).

TABLE 5 | Biomass, fucoxanthin content and productivity from *P. tricornutum* in this study compared to the previous literatures.

Strain	Trophic mode	Carbon source	Nitrogen source	Light strategy	Biomass (g L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Fucoxanthin		References
							Productivity (mg L^{-1} d^{-1})	Content (% DW)	
P. tricornutum CCMP 1327	M	0.1 mol L^{-1} glycerol	0.02 mol N L^{-1}, mixture (T:U = 1:1), with T addition	Batch 2*	6.52	597.22 (Phase 2)	8.22 (Phase 2)	1.30	This study
P. tricornutum SAG 1090-6	M	Spruce hydrolysates (contain 2 g L^{-1} glucose)	Yeast extract (C/N,60)	100 μmol m^{-2} s^{-1}, L:D, 14:10 h	3.31	254#	–	0.51 (carotenoids)	Patel et al., 2019
P. tricornutum UTEX 640	M	0.1 mol L^{-1} glycerol, Semi-continuous culture	0.85 g L^{-1} $NaNO_3$	465 μmol m^{-2} s^{-1}	12.08	1008	–	0.70 (carotenoids)	Ceron-Garcia et al., 2013
P. tricornutum UTEX 640	M	0.1 mol L^{-1} glycerol	0.01 mol L^{-1} urea	165 μmol m^{-2} s^{-1}	2.87	396.24#	–	0.49 (carotenoids)	Garcia et al., 2005
		0.1 mol L^{-1} glycerol	0.01 mol L^{-1} urea successive implementation	165 μmol m^{-2} s^{-1}	15.40	1524#	–	–	
P. tricornutum CCAP1055/1	A	0.5% CO_2	0.075 g L^{-1} $NaNO_3$	150 μmol m^{-2} s^{-1}	<0.2	–	–	2.68	Conceicao et al., 2020
P. tricornutum CS-29	A	1% CO_2	0.75 g L^{-1} $NaNO_3$	150 μmol m^{-2} s^{-1}	0.37	–	2.16	5.92 ± 2.28	McClure et al., 2018
P. tricornutum	A	1% CO_2	1.45 g L^{-1} KNO_3	300 μmol m^{-2} s^{-1}	4.05 (Day 9)	–	4.73# (Day 6)	1.03 (Day 3) 0.66 (Day 12)	Gao et al., 2017

M-Mixotrophy; A-autotrophy; T-tryptone. Batch 2- Phase 1: R:B light (6:1) at 20 μmol m^{-2} s^{-1} for 6 days; Phase 2: R:B light (5:1) at 25 μmol m^{-2} s^{-1} for 6 days. #-maximum biomass/fucoxanthin productivity during the cultivation.*

content and intercellular amount of Fx increased by 8% and 12% compared to day 6 ($p < 0.05$) (**Figure 5C**). Through enhancing biomass concentration and Fx content in phase 2, the Fx productivity increased to 8.22 mg L^{-1} d^{-1}, which was the highest level ever reported so far (**Table 5**).

It is known that biosynthesis of Fx involved in methylerythritol phosphate (MEP) pathway, IPP pathway and Fx formation (Bertrand, 2010). However, the final steps of Fx formation were not know completely so far (Lohr and Wilhelm, 2001; Dambek et al., 2012; **Figure 5D**). The DXS and PSY are two key rate-limiting enzymes to control the biosynthesis of Fx. The previous study indicated that the overexpression of *DXS* and *PSY* could raise the content of Fx by 2.40- and 1.80-fold in *P. tricornutum*, respectively (Eilers et al., 2016). Under the mixotrophic condition, glycerol consumption could supply abundant G3P (Villanova et al., 2017), which is the substrate of DXS and carbon skeleton of carotenoids biosynthesis. In this study, the expression of *DXS* was almost stable from 6th to 10th day, but decreased 1.46-fold on the 12th day compared to the 6th day (**Figure 5D**), suggesting that the BL strengthening with tryptone addition induced more carbon flux to TCA cycle and protein biosynthesis rather than pigments formation (Jungandreas et al., 2014). In contrast, the expression of *PSY* was 0.70-fold downregulated on the 8th day and significantly upregulated ($|\log_2$fold change$| > 2, p < 0.01$) from the 10th day. Similarly, the previous report indicated that BL induction was proved to upregulate the expression of *PSY* under autotrophic condition in *P. tricornutum* (Coesel et al., 2008). One possible reason for the delay of *PSY* response was that the cells need time to adapt to light shift (Jungandreas et al., 2014), the other possibly reason relate to the expression of genes encoding ZEP. There were three types of *ZEP* identified in *P. tricornutum* (Bowler et al., 2008), and the expression levels of *ZEP1*, *ZEP2*, and *ZEP3* were 4.87-, 2.06-, and 2.28-fold upregulated on the 8th day compared to 6th day, which not only contributed to the improvement of Fx content on the 8th day, but also accelerated the conversion from zeaxanthin to violaxanthin, leading to the promotion of *PSY* expression level from 10th day. Additionally, the expression level of *FCPb* was 1.70-fold higher on the 8th day than the 6th day but returned to the initial level from 10th day. However, the expression of *VDE* performed an opposite pattern, in which *VDE* transcript level changed slightly from 6th to 10th day and was 4.29-fold decreased on the 12th day ($p < 0.01$). In the previous report, the accumulation of Fx was not synchronized with the abundance of *PSY* transcripts, while the Fx content at the stationary phase was correlated with the amounts of *PSY* transcripts at the exponential phase (Kadono et al., 2015). Therefore, even though the intercellular amount of Fx increased slightly on the 8th day, the Fx content on the 8th day in phase 2 was significantly improved which depended on the upregulation of *ZEP*s and *FCPb*. And the reduction of intercellular amount and content of Fx on the 10th day might be due to the downregulation of *PSY* on the 8th day and to initial transcript level of *FCPb* on the 10th day. More importantly, the continued upregulation of *PSY* and *ZEP*s with downregulation of *VDE* contributed to the final Fx accumulation (both of intercellular amount and content) on the 12th day (**Figure 5C**), which proved that the option of R: B (5:1) + T in phase 2 was beneficial for enhancing Fx production in two-phase culture in the batch 2.

It was noteworthy that the expression of *ZEP*s were significantly upregulated ($|\log_2$fold change$| > 2, p < 0.01$) when the culture shifted to phase 2. Among them, *ZEP1* was the most sensitive gene in response to the BL induction (**Figure 5D**). A similar phenomenon was observed in the previous study, in which the increase of *ZEP1* transcript level was over 50-fold higher than *ZEP2* and *ZEP3* under BL induction after dark treatment (Coesel et al., 2008). Since violaxanthin cycle and Ddx cycle were two xanthophyll cycles in diatoms participated in NPQ (Lavaud et al., 2003), BL and light intensity play vital roles in NPQ (Bertrand, 2010; Costa et al., 2013). One possible explanation was that *P. tricornutum* did not have the specific enzymes of Dtx epoxidase/diadinoxanthin de-epoxidase (DEP/DDE) in Ddx cycle, and the enzymes of ZEP/VDE in violaxanthin cycle played the same role instead (Bowler et al., 2008). So the *ZEP1* regulated the transformation from zeaxanthin to violaxanthin and *ZEP3* regulated the conversion from Dtx to Ddx (Nymark et al., 2009), leading to different response pattern of the *ZEP1* and *ZEP3* under BL. The another possible explanation was that *ZEP1* and *ZEP2* were suggested to be classified into category that contained light-harvesting complex and enzymes for pigments synthesis, while *ZEP3* was classified as enzyme involved in photo-protection (Nymark et al., 2013; Valle et al., 2014). In this study, the high cell density (**Figure 4C**) resulted in less light exposure to individual cell during the 6th to 12th day, and *ZEP3* might drive the conversion of violaxanthin to zeaxanthin and Ddx to Dtx in low light.

In a word, the expression levels of key genes involving Fx biosynthesis (*PSY*, *ZEP*s, *FCPb*, and *VDE*) were significantly regulated by BL strengthening and tryptone addition, which had positive effects on Fx accumulation, leading to the a great improvement of Fx production.

CONCLUSION

In this study, the combination of red: blue light at a favorable ratio in phase 1 and light shift with tryptone addition in phase 2 was employed to significantly improve Fx production by the mixotrophic *P. tricornutum*, which achieved the highest level of ever reported so far. The analysis of gene expression levels involving Fx biosynthesis revealed that *PSY*, *ZEP*s, and *FCPb* were upregulated while *VDE* was downregulated under BL strengthening and tryptone addition, indicating the possible regulatory mechanism on the enhanced Fx production in phase 2. This study developed a novel approach of two-phase culture to produce Fx efficiently by the mixotrophic *P. tricornutum*, which facilitate the scale-up production of Fx by photo fermentation in the future.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

AUTHOR CONTRIBUTIONS

RY and DW conceived and designed the experiments and drafted the manuscript. RY performed the experiments and analyzed the data. DW contributed to the funding. All authors contributed to the article and approved the submitted version.

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REVIEW
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Bioengineering of Microalgae: Recent Advances, Perspectives, and Regulatory Challenges for Industrial Application

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Microalgae, due to their complex metabolic capacity, are being continuously explored for nutraceuticals, pharmaceuticals, and other industrially important bioactives. However, suboptimal yield and productivity of the bioactive of interest in local and robust wild-type strains are of perennial concerns for their industrial applications. To overcome such limitations, strain improvement through genetic engineering could play a decisive role. Though the advanced tools for genetic engineering have emerged at a greater pace, they still remain underused for microalgae as compared to other microorganisms. Pertaining to this, we reviewed the progress made so far in the development of molecular tools and techniques, and their deployment for microalgae strain improvement through genetic engineering. The recent availability of genome sequences and other omics datasets form diverse microalgae species have remarkable potential to guide strategic momentum in microalgae strain improvement program. This review focuses on the recent and significant improvements in the omics resources, mutant libraries, and high throughput screening methodologies helpful to augment research in the model and non-model microalgae. Authors have also summarized the case studies on genetically engineered microalgae and highlight the opportunities and challenges that are emerging from the current progress in the application of genome-editing to facilitate microalgal strain improvement. Toward the end, the regulatory and biosafety issues in the use of genetically engineered microalgae in commercial applications are described.

Keywords: microalgae, genetic engineering, *omics*, genome editing, regulatory issues

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INTRODUCTION

The proficient photosynthetic microorganisms including green microalgae, diatoms, and cyanobacteria offer remarkable advantage over the terrestrial plants as a rich source of various biomolecules to be used for food, feed, and fuel applications. In addition to the faster growth rate, higher biomass productivity, and ability to synthesize complex metabolites with minimal resources are some of their key advantages. The wide taxonomic and inherent biochemical diversity among the microalgal species makes them suitable resource of abundant biomolecules with industrial and biomedical importance. Owing to this, microalgae have been continuously exploited for the production of biomolecules such as lipids, proteins, and carbohydrates. Apart from the

production of secondary metabolites, microalgae have also been targeted for various applications in nutraceuticals, pharmaceuticals, dietary supplements, and personal care products. Microalgae are also utilized for concomitant CO_2 sequestration, wastewater treatment, and biomass production for high-volume low-value products (Yadav et al., 2014; Mehar et al., 2019). In the last few years, owing to the high lipid content in microalgae (20–70% of dry cell weight), various start-up companies in the sector of clean energy production have attempted for commercialization of microalgae derived biofuels (Mata et al., 2010; Chisti, 2013). According to a global market research, the market for algal products across various segments is expected to grow at a compound annual growth rate of 4.2% from 2018 to 2025 and will have a total market value of more than 3.4 billion USD (https://www.alliedmarketresearch.com/algae-products-market).

Even though the commercial potential of microalgae along with its market portfolio is well-known, challenges pertaining to its economic feasibility still remain to be addressed. High biomass production along with the desired metabolite(s), cost-efficient dewatering and harvesting of biomass, green and efficient process for product extraction are some of the broad challenges to further improve the microalgal process economics. Among all these, the robust and highly efficient strain with desired characteristics can substantially improve the economics of upstream processing. Though various nutritional-, environmental-, and physiological-alteration-based cultivation have been attempted for improved microalgal productivities, commercial success remains limited (Pierobon et al., 2018). This is mainly due to the fact that these biotechnological amendments in the cultivation processes could not enhance the inherent metabolic capacity of the microalgae to hyperaccumulate the desired metabolite(s). For example, triggering the lipid accumulation in microalgae through nutrient deprivation inevitably lowers the cell division, thereby making it difficult to simultaneously achieve high lipid accumulation and high growth rate, thus decreasing the final lipid productivity (Lenka et al., 2016).

In this context, the genetic engineering of microalgae can help to overcome the inherent limitation of metabolic capacity for higher accumulation of desired biomolecules, thus eventually improving the economic feasibility of the production process. Though the wide taxonomic and genetic diversity among the microalgae offer several opportunities for genetic modifications, the scarcity of genomic resources and genetic tools limits the progress in algal bioengineering. For instance, the information of genome sequence, metabolic pathway maps, and the other genetic resources that are the key to identify target gene(s) is available only for the limited (mostly model) microalgal strains. However, despite the available genome sequence information, the annotation, and the gene functional studies related to the microalgae are still very limited. Since many of the microalgal genome sequences will be studied in near future, the computational biology and the bioinformatics may play an important role in precise genome assembly and its annotation. In addition, the multiomics datasets for microalgae can also be explored to improve the biorefinery capabilities and the quality of the microalgal bioproducts (Fayyaz et al., 2020). Moreover, the functional genetic screening through genome scale mutant libraries and their high-throughput screening may help to make robust strategies for microalgal strain improvement. Therefore, such information is extremely essential for purpose-specific bioengineering of microalgal strains. The typical strategic path from the integration of different datasets to the microalgal strain improvement is illustrated in **Figure 1**. In the process of genetic-engineering-based strain improvement, the molecular tools for stable transformation, selective screening, and precise gene targeting are extremely important to accomplish the genetic modification. Unlike other microorganisms, such as bacteria, yeast, and fungi, the microalgal bioengineering suffer the lack of efficient genetic tools and techniques.

Considering these shortcomings, in this review, we have thoroughly mapped the information regarding the evolution of genetic modification strategies from the conventional to the emerging genome-editing tools and their implication in microalgae bioengineering. Although the bioengineering of microalgae holds the great potential to improve process economics, the risk assessment, biosafety, and regulatory issues pertaining to the use of genetically engineered microalgae must be considered and are summarized in this review. We attempt to comprehensively describe the resources for microalgae bioengineering, including omics resources, mutant resources, and their high throughput screening methodologies, transformation methods, selective markers, and precise gene-editing tools. We have also illustrated the applications of genetic engineering in the key areas of microalgal research, such as production of biomass, lipids, and bioactive molecules with the help of case studies along with the strategies used till date for the improvement of algal strains.

ADVANCEMENT IN THE RESOURCES FOR MICROALGAL RESEARCH

Omics Resources

Genomic and Transcriptomic Resources

Until 2008, only three microalgal species, namely *Chlamydomonas reinhardtii*, *Thalassiosira pseudonana*, and *Phaeodactylum tricornutum*, had been sequenced (Fu et al., 2019). In the last decade, revolution in "next-generation sequencing" technologies has led to the swift increase in the available number of draft as well as completed genomes of algal species (**Table 1**). Recently, Fu et al. (2019) have reviewed the efforts to sequence the genome of diverse group of microalgal species. The three sequencing projects, including one transcriptome sequencing and two genome sequencing projects, have been undertaken to generate the genetic resource for algal species. The transcriptome sequencing project named *Marine Microbial Eukaryote Transcriptome Sequencing Project* aimed to sequence nearly 700 marine microbial species of 17 phyla (Keeling et al., 2014). The sequence information of this dataset is available at iMicrobe Project (www.imicrobe.us/#/projects/104) and Sequence Read Archive (SRA) (BioProject PRJNA231566). Among the other sequenced transcriptomes, 140 are of marine microalgae species. Most

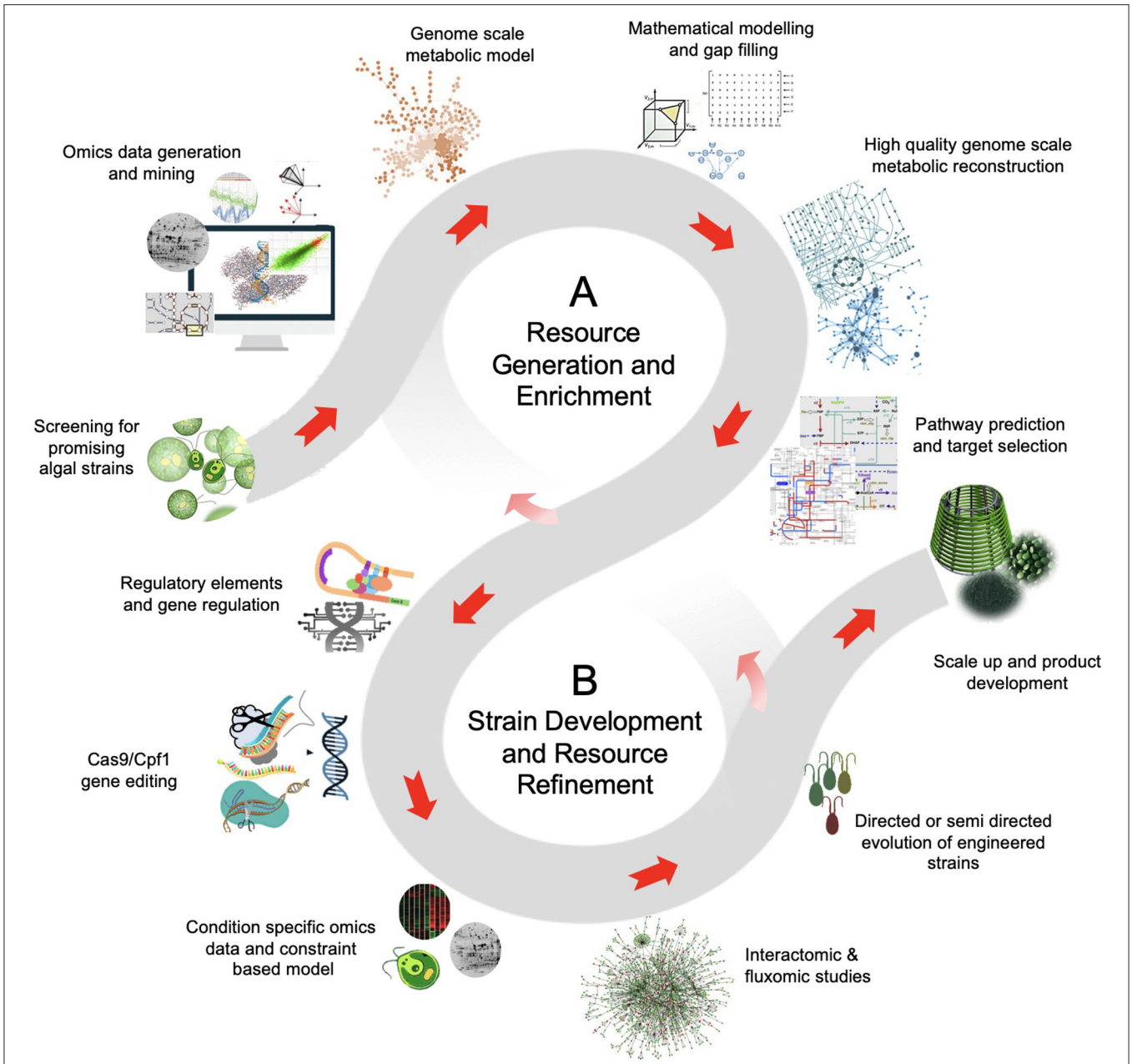

FIGURE 1 | The schematic of data and resource driven strategy for microalgal bioengineering. **(A)** *Resource generation and enrichment:* The high-throughput technologies, intense computation and bioinformatic analysis, and the extensive research interest on microalgae can generate high-quality curated data. The genomic and transcriptomic data of model organisms provides a basic understanding of the biosynthetic pathway. This imperative information is aided by proteomics and metabolomics that offers functional insights for bioproduct discovery in microalgae. Also, the metabolomic data can be implemented to novel microbial isolates with limited genomic and transcriptomic information. **(B)** *Strain development and resource refinement:* The leads from metabolic models and the use of state-of-the-art technologies, such as genome-editing and high-throughput variant selection can be used for microalgae strain development. Often the metabolic flux shifts of the mutants implies an organism's evolution to optimize flux rearrangement. The objective of the flux balance shift can be biomass production or enhanced production of desired product. Moreover, the information obtained from fine-tuned modeling and genomic-editing experiments create resource avenues for further discoveries.

of these sequenced species are culturable and taxonomically well-defined. Therefore, unambiguously the dataset has bias toward the gene prediction of relatively selected group of culturable isolates. Indeed, this transcriptomic data is still very helpful because it provides the extensive reference dataset for novel gene discovery and construction of computation-based metabolic models. One of the two genome sequencing projects, the *ALG-ALL-CODE*, was launched at NYU Abu Dhabi (lassb.abudhabi.nyu.edu/algallcode.php) and aimed at sequencing over 120 genomes of algal isolates belonging

to several evolutionarily distinct phylum. Till date, the draft genome assemblies for 21 isolates are available in public domain, while the draft genome assemblies for 106 isolates will be available in near future. The other recently launched genome sequencing project is the 10KP, which aimed to generate genomic resource for 10,000 plants and eukaryotic microbes. Among the 10,000 genomes, at least 1,000 green algae (microalgae and macroalgae), and 3,000 photosynthetic and heterotrophic protists (majority will be of microalgae) are expected to be sequenced in 10KP genome sequencing initiative (Cheng et al., 2018). At present, around 60 algal accessions have been sequenced and their complete or draft genomes are available at "*Phytozome*" (phytozome.jgi.doe.gov) and "*The Greenhouse*" (greenhouse.lanl.gov). The complete or near to complete genome sequences for microalgae are summarized in **Table 1**. Altogether, these genome sequencing projects will generate a huge genetic resource for the microalgal species, which remained untapped due to the lack of information of their metabolic pathways, regulatory networks, and genetic potentials. In addition, there are three web-based resources available for algal genomics. The first database, pico-PLAZA, contains the genome information and other intuitive tools for functional genomics of 16 photosynthetic algal species (http://bioinformatics.psb.ugent.be/pico-plaza/) (Vandepoele et al., 2013). The second database is AlgaePath (http://algaepath.itps.ncku.edu.tw) that provides the details of gene expression based metabolic pathway prediction in *Chlamydomonas reinhardtii* and *Neodesmus sp.* UTEX 2219-4 (Zheng et al., 2014). The third one holds the information of gene co-expression data for two algal species (*Chlamydomonas reinhardtii* and *Cyanidioschyzon merolae*) and is available at ALCOdb (http://alcodb.jp) (Aoki et al., 2016). In addition, the random information of complete and draft genome sequence is available at JGI Genome Portal (https://genome.jgi.doe.gov) and Phytozome (https://phytozome.jgi.doe.gov). Besides the availability of robust computational methods, the complementation of the genome datasets with other omics datasets is indeed required for rational use of synthetic biology approach. For instance, the advantage of different omics datasets (genomics, proteomics, and metabolomics) and their integration for biological research is recently exemplified by sulfur-metabolic capacity of 14 diverse and representative strains of microalgae from different clades and habitats (Nelson et al., 2019).

Proteomic Resources

The quantitative data of protein expression under different experimental conditions is advantageous for better understanding of regulatory pathways, which differ at the post-transcriptional level. Since, several studies failed to give a high correlation between transcriptomic and proteomic data (Haider and Pal, 2013), the availability of quantitative proteomic and transcriptomic data under defined experimental condition will provide strategic insights for strain improvement in microalgae. In particular, several analyses have been performed to identify the proteome dynamics and the corresponding transcriptome analysis. However, this was mainly focused to understand the lipid metabolism in model and/or oleaginous microalgae with potential of biofuel production (**Table 1**). The literature mining shows that the majority of proteomics studies were performed under experimental conditions, including nitrogen starvation, copper deprivation, light intensity regimes, heterotrophic cultivation, and salt stress (**Table 1**). The majority of differentially abundant proteins were found to have functions in metabolic pathways related to fatty acid and lipid metabolism, carbohydrate metabolism, photosynthesis, and cell structure integrity and maintenance. In addition, the large numbers of algal proteins have been predicted through genomic sequence analysis and the information is available at the Uniprot (https://www.uniprot.org) and Protein Data Bank archive (https://www.rcsb.org). In an attempt to comprehensively cumulate the structural, physicochemical, and functional information of algal proteome, the non-redundant protein database of 31 algal species was developed and is available at Algal Protein Annotation Suite (Alga-PrAS) (Kurotani et al., 2017).

Metabolomics and Metabolic Models

The metabolites are the intermediate or end products of the cellular regulatory processes that are implicated through the transcriptome and proteome, and thus represent the cellular response to the stimulus. Some metabolites are also involved in the regulation of cellular responses by regulating the activity of enzymes involved (Wegner et al., 2015). Thus, information of metabolic profile in response to the experimental conditions may help to target the processes or pathways, which could be helpful in metabolic-engineering of microalgal strains. The quantitative and qualitative analysis of metabolites is now fairly possible even though they have a wide variation in chemical properties, such as polarity, charge, solubility, volatility, and molecular weight. This has become possible due to the advances in non-targeted metabolite profiling and its platforms, such as capillary electrophoresis-mass spectrometry, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, Fourier transform ion cyclotron resonance-mass spectrometry, and nuclear magnetic resonance spectroscopy. Similar to the transcriptome and proteomic studies in microalgae, the majority of metabolomic (though only few untargeted metabolic studies reported) studies were also focused on lipid metabolism under various environmental conditions (**Table 1**). Recently, the potential of single-probe mass spectrometry technology has been demonstrated for near *in-situ* analyses of single cell of *Scrippsiella trochoidea* under nitrogen starving and light vs. dark conditions to analyze the lipid content and lipid profile (Sun et al., 2018). This single-cell-targeted metabolomics may prove to be instrumental in the future algal research, since it reduces the chances of experimental artifacts and confounds, thereby minimizing the cell to cell metabolic variability. Unlike genome and transcriptome databases, unfortunately, no dedicated database is available for the microalgal metabolomics. Although the attempts were made to reconstruct genome-scale metabolic models at system level, they are based on the information of the genome, transcriptome, and scarcely available experimental data. For organisms like *C. reinhardtii*, *Chlorella* spp., *P. tricornutum*, and some blue-green algae (cyanobacteria), the genome-scale metabolic models are available. The core metabolic models and genome-scale system-level metabolic networks available for

TABLE 1 | List of microalgae and diatoms with complete or near to complete genome, and the overview of reported omics studies.

Organism (strain used for genome sequencing)	Genome size (Mb)	Conditions or aim of *omics* studies			Focus	Accession numbers and references
		Transcriptomic studies	Proteomic studies	Metabolomic studies/ metabolic models		
Auxenochlorella protothecoides (0710)	22.92	Response to temperature and phosphate stress; trophic growth conditions; oil accumulation	Response to temperature, nitrogen and phosphorus starvation, ionizing radiation; trophic growth conditions, oil accumulation,	Response to temperature and, phosphate and nitrogen starvation, copper stress; oil accumulation, glycome profiling, trophic growth conditions / Genome scale and core metabolic model	Biofuel	PRJNA428835, PRJNA484804 (Li et al., 2013, 2014b; Gao et al., 2014a; Sibi et al., 2014; Wu et al., 2015; Park and Choi, 2018; Park et al., 2018; Vogler et al., 2018; Xing et al., 2018)
Bathycoccus prasinos (RCC 1105)	15.07	Normal growth conditions	-	-	Comparative analysis	PRJNA231566, https://www.imicrobe.us/#/projects/104
Bigelowiella natans (CCMP2755)	91.41	High light stress and small RNA profiling	Profiling of proteins targeted to plastid and peri-plastid space	-	Model Organism	GSE124831, GSE115762 (Hopkins et al., 2012)
Botryococcus braunii (Showa)	184.32	Response to nitrogen deprivation, high salt, cobalt enrichment, $NaHCO_3$, salicylic acid, methyl jasmonate, and acetic acid	-	Response to different nutrients, growth phases; tetraterpenoid and hydrocarbons analysis / Genome scale metabolic model	Hydrocarbons and biofuels	FY358876, GES71296, SRP161189, GSE96585 (Molnar et al., 2012; Cornejo-Corona et al., 2016; Thapa et al., 2016; Blifernez-Klassen et al., 2018)
Chlamydomonas debaryana (NIES-2212)	120.36	-	-	Oxylipin analysis, lipid profiling in response to different light and CO_2 levels		de los Reyes et al., 2014; Toyoshima and Sato, 2015, 2018; Yoshitomi et al., 2019
Chlamydomonas reinhardtii (CC-503 cw92 mt+)	120.4	Response to nutrient starvation, oxidative and heat stress, high light intensity, diurnal cycle; ciliogenesis; lipid accumulation	Response to nitrogen and sulfur starvation; exposure to high salinity, high CO_2, dark and anoxic conditions; lipid mutant, lipid droplet proteins	Response to nitrogen starvation, dark and anoxic conditions / Genome scale and core metabolic model	Model organism	GSE17970, PRJNA379963 (May et al., 2009; Chen et al., 2010; Baba et al., 2011; Nguyen et al., 2011; Longworth et al., 2012; Mastrobuoni et al., 2012; Choi et al., 2013; Chaiboonchoe et al., 2014; Wase et al., 2014; Sithtisarn et al., 2017; Salguero et al., 2019)
Chlorella pyrenoidosa (FACHB-9)	56.99	Response to CO_2 deprivation, bisphenol A, salt stress, high light stress, glucose starvation and hydroxyl radical; trophic growth conditions	Dried biomass, exposure to inhibitor of mitochondrial respiratory electron transport	Lipid profiling under copper stress and different nitrate levels / Core metabolic model	Biofuels	SRX399080, GSE40028, GSE69816, PRJNA292642, PRJNA526277 (Yang et al., 2000; Sibi et al., 2014; Liu et al., 2018b; Wan et al., 2018; Zhang et al., 2018; Duan et al., 2019)
Chlorella sorokiniana (1230)	58.53	Response to nitrogen deprivation, different pH, and high CO_2	Response to inoculum sizes, light intensity and glucose concentrations, nitrogen starvation; bioactive peptide analysis	Response to high-density cultivation and UV radiation; fatty acid profiling	Biofuels	GAPD00000000, GSE98781, GCUV00000000 (Lu et al., 2013; Ma et al., 2013; Rosenberg et al., 2014; Li et al., 2015a; Chen et al., 2017; Kumar et al., 2018; Tejano et al., 2019)

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TABLE 1 | Continued

Organism (strain used for genome sequencing)	Genome size (Mb)	Conditions or aim of *omics* studies			Focus	Accession numbers and references
		Transcriptomic studies	**Proteomic studies**	**Metabolomic studies/ metabolic models**		
Chlorella variabilis (NC64A)	46.16	Response to early phase of *Chlorella* virus-1 infection	-	Nitrogen deprivation and long-chain alkenes/Genome scale metabolic model	Biofuels	SRP026413 (Juneja et al., 2016; Sorigue et al., 2016)
Chlorella vulgaris (NJ-7)	39.08	Response to nitrogen starvation and salt stress	Response to nitrogen depletion and repletion, heterotrophic and Na induced lipid accumulation, S-nitrosylated proteome in nitrogen deplete and replete condition	Lipid profiling under copper stress, effect of graphene oxide nanomaterial, N-glycan profiling / Core metabolic model	Biofuels	LDKB00000000 (Guarnieri et al., 2011, 2013; Sibi et al., 2014; Li et al., 2015b; Ouyang et al., 2015; Henard et al., 2017; Zuñiga et al., 2018; Mocsai et al., 2019)
Chloroidium sp. (CF)	54.31	-		Normal growth conditions / Genome scale metabolic model	Ecological importance	Nelson et al., 2017, 2019
Chromochloris zofingiensis (SAG 211-14)	58	Response to nitrogen deprivation, high light; heterotrophic conditions, different growth conditions	Lipid droplets analysis	Lipid and carotenoid profiling in response to glucose	Carotenoids and fatty acids	SRP067324, GSE92515 (Wang et al., 2019c; Zhang et al., 2019)
Coccomyxa sp. (LA000219)	48.54	Response to arsenic treatment	-	Response to arsenic treatment	Model organism and biofuels	Koechler et al., 2016
Coccomyxa subellipsoidea (C-169)	48.83	Response to CO_2 supplementation; miRNA profiling	-	Response to nitric oxide, cadmium stress, carbon source, nitrogen starvation, phytohormones	Biofuels	GSE76638 PRJNA428141 (Kováčik et al., 2015; Allen et al., 2017; Liu et al., 2018a; Wang et al., 2019e)
Cyanidioschyzon merolae (10D)	16.55	Response to diurnal cycle, different CO_2 level, blue and red light, UV irradiance	Response to low temperature acclimatization; photosystem II proteins	Response to different CO_2 level, diurnal cycle; hydrocarbon and lipid profiling in response to cyanobacterial Acyl-ACP Reductase overexpression	Model organism	GSE37673, GSE83828, GSE100372 (Krupnik et al., 2013; Rademacher et al., 2016; Nikolova et al., 2017; Miyagishima et al., 2019)
Dunaliella salina (CCAP 19/18)	343.7	Response to osmotic and oxidative stress, nitrogen depletion, salinity, high light; different growth phases	Response to arsenate, high salinity, high light and high bicarbonate ion level; flagella composition	Response to nitrogen starvation	Halophile, Biofuels, β-carotene and glycerol production	Katz et al., 2007; Jia et al., 2009, 2016; Gu et al., 2014; Ge et al., 2016; Lv et al., 2016; Zhao et al., 2016; Wei et al., 2017b; Wang et al., 2019d
Emiliana huxleyi (CCMP1516)	167.68	Response to nitrogen, sulfate and phosphorus starvation, calcium concentrations, elevated temperature and CO_2	Response to different calcium concentration	Response to host-virus (*E. huxleyi* virus) interaction, phosphorus and nitrogen starvation; lipidomic	Coccolithophore	GSE24341, E-MTAB-2274, SRP017794, SRX756940 (Benner et al., 2013; Rokitta et al., 2014; Hunter et al., 2015; McKew et al., 2015; Wördenweber et al., 2018)
Fistulifera solaris (JPCC DA0580)	49.74	Response of nutrient depleted and replete conditions on lipid accumulation and its degradation	Lipid droplet proteins	-	Biofuels	DRA002404 (Nonoyama et al., 2019)

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TABLE 1 | Continued

Organism (strain used for genome sequencing)	Genome size (Mb)	Conditions or aim of *omics* studies			Focus	Accession numbers and references
		Transcriptomic studies	**Proteomic studies**	**Metabolomic studies/ metabolic models**		
Fragilariopsis cylindrus (CCMP1102)	80.54	Response to temperature, high CO_2, prolonged darkness, and nitrogen and iron limitation; small RNA profiling	Response to temperature, salinity stress, prolonged darkness, high CO_2, iron starvation	Response to different growth phases	Psychrophile	E-MTAB-5024, GSE57987 (Lyon et al., 2011; Boroujerdi et al., 2012; Kennedy et al., 2019)
Galdieria sulphuraria (074W)	13.71	Response to cold acclimation	Photosystem-II analysis	-	Extremophile	PRJNA487158, GSE89169 (Thangaraj et al., 2010)
Guillardia theta (CCMP2712)	87.15	Small RNA profiling under light and dark conditions, mRNA splicing analysis	Response to different light intensities	-	Eukaryote endosymbiosis	GSE124831, SRR747855 (Kieselbach et al., 2018)
Haematococcus pluvialis (SAG 192.80)	365.78	Response to high light, salinity, iron, acetate, salicylic acid and jasmonic acid, nitrogen depletion and repletion, photooxidative stress; distinct growth phases	Cell wall protein, astaxanthin accumulation, response to high light stress, salicylic acid, and jasmonic acid	Lipid analysis, pigments and protein profiling, live single-cell analysis	Carotenoids	Wang et al., 2004; Tran et al., 2009; Peled et al., 2011; Gu et al., 2014; Recht et al., 2014; Su et al., 2014; Gao et al., 2016; Baumeister et al., 2019; Luo et al., 2019
Helicosporidium sp. (ATCC 50920)	12.37	Transition from free-living organism to obligate intracellular parasite	-	-	Parasite	Pombert et al., 2014
Klebsormidium nitens (NIES-2285)	104.21	Response to auxin treatment and cold stress	-	Response to cold stress	Tolerance to UV and harsh conditions	PRJDB4958, PRJNA500592 (Nagao et al., 2008)
Micromonas commoda (RCC299)	21.11	Response to different light regimes and ultra-violet light stress	Response to chronic phosphate limitation and subsequent relief, high light and UV-radiation	-	Marine phytoplankton	Cuvelier et al., 2017; Guo et al., 2018
Micromonas pusilla (CCMP1545)	21.96	Response to phycodnavirus MpV-SP1 infection, phosphate deplete and replete, day-night cycle	Phosphate deplete and replete condition, day-night cycle	Response to phosphate deplete and replete condition; different growth phases,	Marine phytoplankton	PRJNA422663 (van Baren et al., 2016; Waltman et al., 2016; Kujawinski et al., 2017)
Micromonas sp. (ASP10-01a)	19.58	Normal growth conditions	-	-	Marine phytoplankton	van Baren et al., 2016
Monoraphidium neglectum (SAG 48.87)	69.71	Nitrogen deprivation	-	-	Biofuels	PRJNA221625 (Jaeger et al., 2017)
Nannochloropsis gaditana (CCMP1894)	30.86	Response to light intensity regimes and nitrogen replete and deplete condition	Fresh and atomized biomass	Response to light intensity regimes and nitrogen deprivation / Genome scale metabolic model	Biofuels	Radakovits et al., 2012; Sorigue et al., 2016; Ajjawi et al., 2017; Shah et al., 2017; Fernandez-Acero et al., 2019; Patelou et al., 2020
Nannochloropsis limnetica (CCMP505)	33.51	-	-	Nitrogen deprivation	Biofuels	Sorigue et al., 2016

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TABLE 1 | Continued

Organism (strain used for genome sequencing)	Genome size (Mb)	Conditions or aim of *omics* studies			Focus	Accession numbers and references
		Transcriptomic studies	**Proteomic studies**	**Metabolomic studies/ metabolic models**		
Nannochloropsis oceanica (LAMB2011)	29.26	Response to different CO_2 levels, phosphorus and nitrogen limitation, light and dark cycle, fresh water acclimation; transition from quiescence to autotrophy	Response to long-term nitrogen starvation, low CO_2; single-cell-level phenotypic heterogeneity	Response to osmotic downshift and nitrogen depletion	Biofuels	Dong et al., 2013; Pal et al., 2013; Sorigue et al., 2016; Poliner et al., 2018; Chen et al., 2019; Wei et al., 2019
Nannochloropsis oculate (CCMP525)	26.27	-	Nitrogen deprivation, cadmium stress	Nitrogen deprivation	Lipids and protein content	Kim et al., 2005; Sorigue et al., 2016; Tran et al., 2016
Ostreococcus lucimarinus (CCE9901)	13.2	-	-	Genome scale metabolic model	Small genome	Krumholz et al., 2012
Ostreococcus tauri (RCC4221)	13.03	Response to OtV5 virus infection, light and dark cycle, iron limitation, and high light; life cycle stages	Phosphoproteome in response to casein kinase 2, light dark cycle	Glycerolipid profiling under nutrient deprived condition, diurnal variations, nitrogen deprivation / Genome Scale metabolic model	Small genome	Krumholz et al., 2012; Martin et al., 2012; Hindle et al., 2014; Le Bihan et al., 2015; Lelandais et al., 2016; Sorigue et al., 2016; Degraeve-Guilbault et al., 2017; Hirth et al., 2017
Parachlorella kessleri (NIES-2152)	59.18	Response to salt stress and sulfur deplete and replete	Salt stress	Nitrogen, sulfur and phosphorus deprivation		Ota et al., 2016a,b; Shaikh et al., 2019; You et al., 2019
Phaeodactylum tricornutum (CCAP 1055/1)	27.45	Response to nitrogen, iron, carbon and phosphorus deprivation, cadmium stress, mixotrophic growth, grazing stress, different light intensities, and regimes, salicylic acid; non-coding microRNA	Response to nitrogen limitation, oxidative and dark stress; phosphoproteomics under high light, nitrogen, and iron deficiency	Response to blue and red light, nitrogen and phosphorus deprivation; glycerolipid profile; mixotrophic growth / Genome scale and core metabolic model	Model organism	PRJEB11970, SRX648639 (Chen et al., 2014; Ge et al., 2014; Jungandreas et al., 2014; Rosenwasser et al., 2014; Yang et al., 2014; Abida et al., 2015; Alipanah et al., 2015; Feng et al., 2015; Bai et al., 2016; Longworth et al., 2016; Sorigue et al., 2016; Yoneda et al., 2016; Villanova et al., 2017; Remmers et al., 2018; Smith et al., 2019)
Picochlorum sp. (SENEW3 / DOE 101)	13.39 / 15.25	Response to salinity stress and high temperature	-	-	Biofuels	PRJNA245752, PRJNA389600
Scenedesmus sp. (ARA3, ARA)	93.24	Response to phosphorus and nitrogen starvation, lipid accumulation	Response to salinity stress; lipid accumulation	Response to salinity and arsenic stress; lipid accumulation	Biofuels	PRJNA428298 (Chu et al., 2011; Arora et al., 2018, 2019; Wang et al., 2019b)
Scenedesmus obliquus (UTEX393)	107.72	Response to diurnal changes and nC_{60}; wild type and starch less mutant comparison	Thylakoid membrane proteome, toxicity of silver nanoclusters	Response to nC_{60} and silver nanoparticles; different photoperiod and growth phases	Lipid and biomass	E-MTAB-7009 (Kantzilakis et al., 2007; Du et al., 2017; Zhang et al., 2017; Vendruscolo et al., 2019; Wang et al., 2019a)

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TABLE 1 | Continued

Organism (strain used for genome sequencing)	Genome size (Mb)	Conditions or aim of *omics* studies			Focus	Accession numbers and references
		Transcriptomic studies	**Proteomic studies**	**Metabolomic studies/ metabolic models**		
Symbiodinium minutum (Mf 1.05b.01)	609.48	Diurnal cycle, cultured, and freshly isolated cells	-	Response to acidification	Coral symbiont	PRJNA544863 (Jiang and Lu, 2019)
Symbiodinium microadriaticum (CCMP2467)	808.2	Response to different temperature, dark, and salinity stress; normal growth conditions, miRNA profiling	-	Response to environmental variation	Coral symbiont	GSE47373, GSE47372 (Klueter et al., 2015; Aranda et al., 2016)
Tetraselmis striata (LANL1001)	227.95	Normal growth	-	-		PRJNA231566, https://www.imicrobe.us/#/projects/104
Thalassiosira oceanica (CCMP1005)	92.18	Response to iron and copper	Response to iron and copper; extracellular superoxide production	-	Model organism	PRJNA382002, SRA045825 (Lommer et al., 2012; Diaz et al., 2019)
Thalassiosira pseudonana (CCMP1335)	32.44	Response to nitrogen and phosphorus deprivation, salinity, light intensity, triphenyltin chloride, silicon, CO_2 levels, source of light, and nitrogen	Response to nitrogen and phosphorus starvation, light intensity, salinity, triphenyltin chloride, CO_2 levels, silicon, micronutrients deficiency, benzo(a)pyrene, *K. brevis* allelopathy; composition of nano- and micropatterned biosilica cell wall, mitochondrial and plastid proteome	Response to phosphate deplete and replete condition, cobalamin scarcity; *K. brevis* allelopathy	Model organism	Carvalho and Lettieri, 2011; Dyhrman et al., 2012; Du et al., 2014; Kettles et al., 2014; Kustka et al., 2014; Luo et al., 2014; Poulson-Ellestad et al., 2014; Yi et al., 2014; Jian et al., 2017; Kujawinski et al., 2017; Chen et al., 2018; Heal et al., 2019; Schober et al., 2019
Trebouxia gelatinosa (LA000220)	61.73	Response to dehydration and subsequent rehydration	-	-	Colonization through symbiosis	PRJNA213702
Volvox carteri f. *magariensis* (Eve)	137.68	Response to low dose of UV-B radiation; somatic and reproductive cells	-	-	Multicellular alga, model organism	E-MTAB-5691 and GSE104835
Yamagishiella unicocca (NIES-3982)	134.24	Normal growth condition	-	-	Multicellular alga, model organism	PRJNA532307

different microalgal species are given in **Table 1**. In addition, some databases, such as KEGG (https://www.genome.jp/kegg/pathway.html), Reactome (https://reactome.org), and Metacyc (https://metacyc.org) contain predicted and experimentally proven metabolic network information and can be explored for predictive and integrative biology in microalgae. The information available through the genetic characterization of cellular pathways, and high throughput genome-scale studies under different experimental conditions, is contributing toward the refinement of metabolic models for system-level analysis of biological processes.

Mutant Resources for Microalgae

The mutant library for an organism is the best available tool to accelerate the functional characterization of enormous set of uncharacterized genes for better understanding of fundamental biological processes. The potential of such mutant libraries has been exemplified by those that are available for organisms such as *Saccharomyces cerevisiae* (www.sequence.stanford.edu/group/yeast_deletion_project/deletions3) and *Arabidopsis thaliana* (www.arabidopsis.org/portals/mutants/index.jsp). The mutant libraries are instrumental in the reverse genetic studies. However, generating such libraries for microalgae is limited by the lack of efficient transformation and genetic manipulation protocols (discussed in later sections). The insertional mutagenesis through random non-homologous end-joining is the method of choice to generate the mutant libraries. Till date, only two genomewide random insertion mutant libraries have been generated for *C. reinhardtii* using the insertional mutagenesis approach. The first collaborative project, Chlamydomonas Library Project (CLiP) was launched in 2010 by Jonikas (now at Princeton University, USA) and Grossman at Carnegie Institution for science (USA), Fitz-Gibbon (University of California Los Angeles, USA), and Lefebvre (University of Minnesota, USA) to generate the genome-scale insertional mutant library for *C. reinhardtii*. The mutants from this library have been released for the research community and other stakeholders on periodic basis. The complete library featuring more than 62,000 mutants that covers 83% of nuclear protein-encoding genes is now available at Chlamydomonas Resource Center (www.chlamycollection.org/products/clip-strains). Importantly, the mutants in this library are fully mapped for insertion sites and indexed with unique DNA barcode for high-throughput screening of pooled mutants for a particular trait or biological process (Li et al., 2016b, 2019). Similarly, the Huang group at Institute of Hydrobiology, China, generated another insertional mutant library of *C. reinhardtii* with ∼150,000 insertional mutants (Cheng et al., 2017). Although this library contains higher number of mutants than that of CLiP, the list of mutants and their mapping information is not available in public domain. In addition, a non-indexed insertional mutant library of *C. reinhardtii* with ∼49,000 mutants was also developed and is available for the scientific community at Chlamydomonas Resource Center (http://chlamycollection.org). The potential utility of these mutant libraries can be attributed to the discovery of novel candidate genes involved in biological and physiological processes, such as photosynthesis, lipid biosynthesis, and intraflagellar transport in microalgae (Dent et al., 2015; Li et al., 2016b, 2019).

In addition to the insertional mutagenesis, the mutagenic agents are being regularly used to generate the mutant strains with desired traits. Several attempts have been made using forward genetic approach to characterize the genes involved in the molecular pathways targeting a desired trait. Since the *C. reinhardtii* is considered as premier reference organism for understanding the basic algal metabolism and biological processes, most of the forward genetic screens have been performed in this model organism. These forward genetic screening in *C. reinhardtii* and some other model microalgae have been performed mostly to identify the genetic factors responsible for desirable traits, such as higher biomass and cell culture density (Thung et al., 2018), enhanced lipid content (Cagnon et al., 2013; Lee et al., 2014), or to understand the basic cellular processes such as photosynthesis (Dent et al., 2015; Li et al., 2019), non-photochemical quenching (Schierenbeck et al., 2015), lipid metabolism (Li et al., 2016b; Schulz-Raffelt et al., 2016; Cheng et al., 2017), and flagellar responses (Hilton et al., 2016; Cheng et al., 2017). In an integrative approach, the *P. tricornutum* mutants with enhanced carotenoid biosynthesis were subjected to genome-scale metabolic network simulation to identify the metabolic reactions that are highly correlated with the carotenoid biosynthesis (Yi et al., 2018). This study exemplified the use of system-biology approach to target the key pathway(s) that should be considered during bioengineering in diatoms. Recently, using a modified approach named as bulked mutant analysis and bulked mutant RNA sequencing, the single nucleotide polymorphisms and indels were identified that are associated to the growth-related genes in *Nannochloropsis oceanica* (Liang et al., 2019). These methods of forward genetic screen have the potential to facilitate the genetic investigation of diverse microalgae with various desirable traits.

High-Throughput Screening Methodologies for Microalgae

The previous section reviewed various genomic and mutant resources that are available for the microalgae research. The resources for microalgal forward genetics have the potential to revolutionize the identification of mutants with desired traits, however limited to availability of the rapid screening methods. Moreover, the screening of microalgae natural pools to identify functional components is low due to the lack of effective rapid and high-throughput analysis tools (Lee et al., 2013). In addition, this also limits our capacity for the real-time monitoring of process for target compound production using microalgae. To enrich the mutants capable of accumulating high lipid content, Sharma et al. (2015) developed and validated a high-throughput work flow strategy based on *in-situ* analysis of lipid bodies using confocal Raman microscopy combined with fluorescence activated cell sorting (FACS). A precise and efficient Raman platform was developed to distinguish the contrasting features of lipids such as chain length and saturation level in lipid-expressing cells generated through UV mutagenesis. Terashima et al. (2015) introduced another high-throughput advanced

technique, named *Chlamydomonas* high-lipid sorting (CHiLiS), which enables to isolate mutants with high lipid content. CHiLiS is based on the fact that Nile Red (lipid detecting dye)-stained lipid pools were enriched by using FACS. In this method, the staining extent was raised to a certain level for increasing the enrichment tendency without interfering with the cell's viability. These high-throughput methods have the potential of selecting the mutant strains that can be used either for the understanding of molecular basis of high lipid accumulation or engineering of microalgae for maximizing the production of lipids. Based on the staining of lipid bodies with fluorescent dyes, several high throughput systems are available commercially. Semi-automated QPix^{TM} 400 Series system from Molecular Devices is one such example (https://www.moleculardevices.com/sites/default/files/en/assets/brochures/biologics/qpix-400-systems). The Fourier transform infrared spectroscopy also demonstrated its sensitivity to screen mutants of *C. reinhardtii* for variation in their lipid and carbohydrate profile under specific nutrient stress conditions (Bajhaiya et al., 2016). Based on this screening, nutrient starvation response genes (*PSR1*, *SNRK2*.1, and *SNRK2*.2) with possible role in lipid and starch accumulation were identified.

In an another approach, to isolate the algal cells with superior photosynthetic activity, the high-throughput microfluidics were used in the microalgal selection process (Kim et al., 2016). This system used the strong positive relationship between phototaxis and photosynthetic efficiency, where the competitive phototactic response was employed to isolate the highly photosynthetic efficient strains at the single-cell level using a microfluidic system. Also, the putative candidate genes related to the transcriptional regulation (JGI Chlre4, protein ID: 525919, 516641, 513996), cellular metabolism (519327, 523869, 515661) signal transduction (516786), flagellar function (518826), and membrane transport (protein ID; 516748, 516786, 513005, 520695, 512634) were identified, that might have some role in enhanced photosynthetic activity and phototactic response in mutant strains. The putative candidate genes identified in this study may be cataloged for their use in microalgal strain engineering strategies. Even after the identification of photosynthetic efficient microalgal strains, optimization of the light conditions remains critical to augment system efficiency. Recently, a novel high-throughput screening system was developed by Sivakaminathan et al. (2018), which simulates fluctuating light regimes in mass cultures. This high-throughput miniaturized light system is capable of screening up to 18 different combinations of light regime and up to 1,728 conditions to evaluate species-specific light conditions for maximum photosynthetic efficiency and productivity.

For the screening of biopigments accumulation, a 96-well microplate-based high-throughput assay was developed to identify *P. tricornutum* mutants with high carotenoid content (Yi et al., 2018). The assay was based on the fact that fluorescence intensity of chlorophyll a and neutral lipids (stained with fluorescence dye) has a significant correlation with the carotenoid content during exponential growth phase of *P. tricornutum*. Generally, the *in-situ* optical detection-based methods fail to provide detailed information on the pigment composition in microalgae because of the possible overlapping of absorbance and emission spectra of various pigments. In such cases, the extraction and subsequent detection is the only method of choice. However, the extraction of a particular pigment type is a time-consuming multi-step process that also required a suitable extraction solvent to effectively extract the pigment. A rapid and reliable microwave-assisted extraction and subsequent detection of microalgal pigment using relevant method could be helpful in developing high-throughput screening platform for microalgal pigments (Pasquet et al., 2011). An enzyme-linked immunosorbent assay (ELISA) on microtiter platform was developed by Jirásková et al. (2009) to detect the presence of phytohormones, such as abscisic acid, indole-3-acetic acid, cis- and trans-zeatin, and isopentenyladenosine in microalgae. This high-throughput application of ELISA-based microtiter platform can be extrapolated to the other bioactive compounds if suitable antibodies and/or antigens are available. Likewise, a simple and inexpensive high-throughput bioassay was developed to screen the algal mutants or isolates producing high H_2 under saturating light intensity (Wecker and Ghirardi, 2014). The screening assay used the agar overlay of *Rhodobacter capsulatus* bacteria carrying a green fluorescent protein that responds to H_2 produced by single algal colony. Among the other high-throughput screening methods, the phenotype microarray technologies have also shown promise to screen-defined metabolic activities in response to array of different drugs, chemicals, and metabolites (www.biolog.com).

Genetic Engineering in Microalgae

Transformation Technologies and Selectable Markers

The first nuclear transformation of *C. reinhardtii* using polyethylene glycol or poly-L-ornithine was demonstrated in early 1980's. Here, the complementation of arginine-requiring, cell-wall deficient mutant was performed through successful integration of yeast *arg4* locus (Rochaix and Dillewijn, 1982). In the late 1980's, the successful stable nuclear transformation in *C. reinhardtii* was demonstrated using the biolistic transformation approach to deliver the native genes to complement auxotrophic growth in mutants (Debuchy et al., 1989; Kindle et al., 1989; Mayfield and Kindle, 1990). Later in the 1990's, the success of glass bead agitation and electroporation were demonstrated, where the later was found to be the most efficient method to transform the nuclear genome of *C. reinhardtii* (Kindle, 1990). The droplet electroporation on microfluidic chip was found to have threefold higher transformation efficiency than the electroporation cuvettes (Qu et al., 2012). In addition, the use of other methods by employing silicon carbide whiskers (Dunahay, 1993), *Agrobacterium tumefaciens* (Kumar et al., 2004), and nanoparticles (Kim et al., 2014) have been also demonstrated to successfully transform the nuclear genome of *C. reinhardtii*. The methods for the nuclear transformation in other microalgal species such as *Phaeodactylum*, *Nannochloropsis*, *Dunaliella*, and *Haematococcus* are available (**Table 2**). The various transformation techniques and the selectable markers used for the screening of transformants, and mainly includes the use of antibiotic, herbicide resistance, and auxotrophic markers are listed in **Table 2**. The evolutionary divergence of the cellular machinery in microalgae, however, limits the use of existing plant

TABLE 2 | List of microalgae and the molecular tool and techniques available for their genetic engineering.

Organism	Genetic tools and techniques			References
	Transformation	Genetic manipulation	Selectable markers	
Botryococcus braunii	Electroporation	Gene integration and expression	Antibiotic: *aphVIII*	Berrios et al., 2016
Chlamydomonas reinhardtii	Biolistic, glass bead agitation, electroporation, and agrobacterium-mediated	Gene expression, RNA interference, gene-editing using ZFNs and CRISPR	Antibiotic: *aphVIII*, *aphVII*, *nptII*, *addA*, *tetX*, *hph,* and *ble.* Autotrophic: *arg* and *trp*. Herbicide: 2-fluoroadenin resistance	Kim and Cerutti, 2009; Greiner et al., 2017; Mini et al., 2018
Chlorella pyrenoidosa	Electroporation	Gene integration and expression	Antibiotic: *nptII*	Run et al., 2016
Chlorella sorokiniana	Biolistic	Gene integration and expression	Autotrophic: *nr*	Dawson et al., 1997
Chlorella vulgaris	Electroporation, glass bead agitation, and agrobacterium-mediated,	Gene integration and expression	Antibiotic: *nptII* and *aphVII*	Cha et al., 2012; Muñoz et al., 2018
Chromochloris zofingiensis	Biolistic	Gene integration and expression	Herbicide: norflurazon-resistance	Liu et al., 2014
Coccomyxa sp.	Biolistic and electroporation	Gene integration and expression, gene-editing using CRISPR	Autotrophic: *umps*	Kasai et al., 2018; Yoshimitsu et al., 2018
Coccomyxa subellipsoidea	Electroporation	Gene integration and expression	Antibiotic: *hptII*	Kania et al., 2019
Cyanidioschyzon merolae	PEG-mediated	Gene integration and expression, RNAi	Antibiotic: *cat* Auxotrophic: *ura*	Ohnuma et al., 2009; Sumiya et al., 2015; Fujiwara et al., 2017
Dunaliella salina	Electroporation, biolistic, glass beads agitation, and agrobacterium-mediated	Gene integration and expression	Antibiotic: *aphVII* and *nptII* Herbicide: *bar* Auxotrophic: *nr*	Li et al., 2007; Radakovits et al., 2010; Srinivasan and Gothandam, 2016
Fistulifera solaris	Biolistic	Gene integration and expression	Antibiotic: *nptII*	Muto et al., 2013
Gonium pectorale	Biolistic	Gene integration and expression	Antibiotic: *aphVIII*	Lerche and Hallmann, 2009
Haematococcus pluvialis	Biolistic	Gene integration and expression	Antibiotic: *aadA* Herbicide: norflurazon resistance	Steinbrenner and Sandmann, 2006; Yuan et al., 2019
Monoraphidium neglectum	Electroporation	Gene integration and expression	Antibiotic: *aphVII*	Jaeger et al., 2017
Nannochloropsis gaditana	Electroporation	Gene integration and expression, gene-editing using CRISPR	Antibiotic: *aphVII, nptII* and *BSD*	Ajjawi et al., 2017
Nannochloropsis limnetica	Electroporation	Gene integration and expression	Antibiotic:*aphVII* and *nptII*	Chen and Hu, 2019
Nannochloropsis oceanica	Electroporation	Gene integration and expression, RNAi, gene-editing using CRISPR	Antibiotic: *sh ble* and *nptII*	Li et al., 2014a; Poliner et al., 2018; Osorio et al., 2019
Nannochloropsis oculata	Electroporation	Gene integration and expression	Antibiotic: *sh ble*	Li et al., 2014a
Ostreococcus tauri	Electroporation and PEG-based	Gene integration and expression	Antibiotic:*nptII* and *neo*	van Ooijen et al., 2012; Sanchez et al., 2019
Parachlorella kessleri	Biolistic and agrobacterium-mediated	Gene integration and expression	Antibiotic: *nptII* and *aadA*	Rathod et al., 2013
Phaeodactylum tricornutum	Biolistic, electroporation, and bacterial conjugation	Gene integration and expression, gene-editing using MNs, CRISPR, and TAELNs	Antibiotic: *nat*, *sat-1*, *addA*, *sh ble* and *cat* Autotrophic: *ura* Herbicide: 5-fluoroorotic acid and 2-fluoroadenine resistance	Daboussi et al., 2014; Serif et al., 2018; Sharma et al., 2018
Scenedesmus obliquus	Electroporation	Gene integration and expression	Antibiotic: *cat*	Guo et al., 2013
Symbiodinium microadriaticum	silicon carbide whiskers	Gene integration and expression	Antibiotic:*nptII* and *hpt*	Te et al., 1998
Thalassiosira pseudonana	Biolistic and bacterial conjugation	Gene integration and expression, gene-editing using CRISPR	Antibiotic: *nat* and *sat-1*	Karas et al., 2015
Volvox carteri f. *magariensis*	Biolistic	Gene integration and expression, gene-editing using CRISPR	Antibiotic: *hpt* and *BSD* Autotrophic: *nr*	Ortega-Escalante et al., 2019a,b

or other microbe-based selectable markers for selection purposes. For instance, the trait stacking in the industrially important microalgae "*Nannochloropsis*" through genetic engineering is mostly limited by the availability of selectable markers (Verruto et al., 2018). The use of auxotrophic selection marker is mostly a desirable trait; however, a pre-requisite that the

strain to be transformed must be auxotrophic mutant for the selectable marker, which may sometimes interfere with the experimental setup.

Despite the recent advancement in the transformation technologies, the microalgae transformation is still facing the problem of low efficiency, except in *Chlamydomonas* when compared to the plant system. In an advancement, the development of nuclear episomal vector to transform diatoms *via* conjugation-based method that directly deliver the vector from *E. coli* to diatom provides an efficient method for diatom transformation (Karas et al., 2015). This method offers several advantages over the conventional transformation methods such as capacity to deliver large DNA fragments (may be multiple genes from a pathway), stable self-replication of episomal vector (due to presence of yeast-derived regulatory sequences, CEN/ARS), loss of transgene upon removal of selection pressure, and low possibility of positional or epigenetic effects (Doron et al., 2016). Recently, the application of conjugation-based method in CRISPR/cas9 mediated genome-editing of *Pt MYBR1* gene in *P. tricornutum* (Sharma et al., 2018) and nitrate reductase gene (*NR*) in *Nannochloropsis oceanica* (Poliner et al., 2019) has been demonstrated to generate transgene-free mutants. Although 20–100 times higher transformation efficiency and rapid transformant appearance was observed in the conjugation-based method, there was a significant delay in the appearance of mutants in the positive transformants (Sharma et al., 2018). The plausible explanation for this delayed mutant appearance was attributed to the lower Cas9 expression due to higher rate of cell division in conjugatively transformed cells. In addition, the episomal vector system adapted for diatom was able to transform the green oleaginous microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* through bacterial conjugation (Muñoz et al., 2019). Although the transformation efficiency was sufficiently higher as compared to the biolistic-based vector delivery system, this application of diatom adapted episomal vector system in other microalgae has some limitations that are discussed in the following section.

Genome-Editing

Over the years, significant progress has been made to improve the catalog of available tools for genetic engineering in microalgae, with the ultimate aim to improve the feasibility of microalgae as a model organism for scientific and/or industrial applications. In the past decade, the gene-editing tools such as zinc-finger nucleases (ZFNs), meganucleases (MNs), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) have been emerged as the efficient tools for genome-editing in many organisms (Razzaq et al., 2019). All these tools are able to introduce a double-strand break at targeted DNA sequence that can be further repaired *via* either non-homologous end-joining (may disrupt gene through mutations) or homologous recombination (may insert or replace gene with exogenous donor DNA) (Jeon et al., 2017). The CRISPR is often used interchangeably to the term genome-editing; however, the ZFNs and TALENs were among the first molecular tools available for the genome-editing. The applicability of these tools largely depends on the factors such as cost, complexity, and ability to cause multiple edits simultaneously. Among the others, the CRISPR/cas9-mediated genome-editing system now became the state-of-the-art tool due to its simplicity and versatility.

The initial reports of gene-editing in microalgae were from ZFN-mediated genome-editing. Sizova et al. (2013) and Greiner et al. (2017) used engineered ZFNs to target the *COP3* and *COP4* genes in *C. reinhardtii*. However, the efficiency of the ZFNs was only observed in the tailored model strain of *C. reinhardtii*. In addition, it was also suggested that ZFNs prefer the homology-directed repair when supplied with larger donor DNA (>750 bp) for the clean and predictable gene modification. Beside the recent developments in the ZFN technology, the most challenging task is to create unique ZFNs with high specificity and affinity toward the target sites. This requires the validation of ZFNs using gene targeting selection system before conducting the actual experiment (Sizova et al., 2013). Meanwhile, the use of MNs and TALENs was also demonstrated to target the *uridyl diphosphate* (*UDP*)-*glucose pyrophosphorylase* in *P. tricornutum* for enhanced lipid accumulation (Daboussi et al., 2014). The use of TALENs for the disruption of urease gene through homologous recombination has been successfully achieved in *P. tricornutum* (Weyman et al., 2015). Similarly, in an attempt to evaluate the use of uridine monophosphate (UMP) synthase as an endogenous positive selectable marker for DNA-free genome editing, Serif et al. (2018) used TALEN to generate knock-out mutants of *UMP synthase* gene in *P. tricornutum*. Although the efficiency of the gene disruption using TALENs was quite low (only 16%), the applicability of TALENs for gene-editing in microalgae has been established. However, though the use of TALENs for gene-editing has been exemplified in several organisms, no report has been observed till date in *Chlamydomonas*. The functioning of transcription activator-like effectors (TALEs) has been established in *Chlamydomonas* to induce the expression of endogenous genes, *ARS1* and *ARS2* through the binding of gene-specific artificially designed TALEs to the promoter region of the targeted genes (Gao et al., 2014b). This study indicates that the TALEs coupled to nuclease(s) can (TALENs) be used as one of the approaches to target the gene-editing in *Chlamydomonas*.

The successful use of CRISPR/cas9 system in microalgae species was first demonstrated by Jiang et al. in *C. reinhardtii* (Jiang et al., 2014). In this study, four genes were successfully edited through the expression of codon-optimized *Cas9* gene and corresponding single guide RNA (sgRNA). However, the constitutive expression of Cas9 shows cytotoxic effect in *C. reinhardtii* that reduce the cell viability of transformants (Ng et al., 2020). Therefore, the transient delivery of *in-vitro* assembled Cas9/sg RNA ribonucleoprotein (RNP) complex *via* electroporation is a promising methodology to efficiently edit genes in *C. reinhardtii* without the cytotoxic effect of Cas9, and this approach was established recently (Shin et al., 2016a; Baek et al., 2016a). The use of Cas9/sgRNA-RNP-complex-mediated approach could exempt the genome-edited microalgae from the regulations of genetically modified organism (GMO) regulations, since it does not involve the integration of foreign DNA (*cas9* gene) in the host genome. In addition, the Cas9/sgRNA

RNP complex further reduces the off-target effects and is less cytotoxic to the cells because of transient expression of cas9, thus improving the efficiency of gene-editing. In an effort to improve the efficiency of CRISPR/Cas9 system in *C. reinhardtii*, Jiang and Weeks (2017) employed gene-within-a-gene methodology that uses hybrid *Cas9* gene containing an artificial intron having sgRNA gene. Although the hybrid cas9 system was functional in *Chlamydomonas*, the improvement in the efficiency of gene editing was only marginal. A higher editing-efficiency of up to 9 and 3.3% in *Chlamydomonas* was observed by Greiner et al. (2017) after using a *Cas9* gene from *Staphylococcus aureus* and *S. pyogenes*, respectively. Recently, Guzmán-Zapata et al. (2019) used transient expression of *S. pyogenes* cas9 to disrupt the *atp9* gene in *Chlamydomonas* with efficiency of up to 30% on preselected 2-fluoroadenine resistant colonies. This approach of pre-selection based on the editing of selectable marker gene could also be used for the multiplexed editing. In another approach, an ortholog of cas9, Cpf1 was used in single-step co-delivery of CRISPR/Cpf1 RNP complex along with single-stranded DNA repair template, and this approach resulted in ∼10% efficiency for precise gene-editing in *C. reinhardtii* (Ferenczi et al., 2017). Using dcas9 (dead cas9, nuclease defense), the functioning of a variant of CRISPR, named as CRISPRi (CRISPR interference) was also established in *C. reinhardtii* through downregulation of *PEPC1* expression to enhance the lipid content (Kao and Ng, 2017).

Besides *Chlamydomonas*, the adaptability of the CRISPR system was also successfully demonstrated for another model marine microalgae *P. tricornutum*. Using codon-optimized *S. pyogenes cas9*, the disruption of *P. tricornutum CpSRP54* gene with 31% efficiency indicates that, unlike *Chlamydomonas*, the Cas9 constitutive expression is not likely to be toxic for diatoms (Nymark et al., 2016). Recently, Sharma et al. (2018) compared the effect of constitutive and transient expression of cas9 on editing frequency and stability of mutant lines generated through biolistic and bacterial conjugation, respectively. Although the efficiency of CRISPR-induced targeted mutations were similar for both methods, the use of conjugation-based episomal CRISPR/Cas9 system is capable of avoiding re-editing of mutant lines caused by constitutive expression of Cas9 in the progeny (Sharma et al., 2018; Slattery et al., 2018). Intriguingly, the simultaneous knock-out of multiple genes has also been demonstrated in *P. tricornutum* through the delivery of Cas9/sgRNA RNP complex (Serif et al., 2018). In addition, the CRISPR/cas9 system was also able to edit urease gene in another diatom, *T. pseudonana* with up to more than 60% of disruption efficiency (Hopes et al., 2016). The application of CRISPR system on industrially important oleaginous marine microalgae *N. oceanica* was first demonstrated through the disruption of nitrate reductase gene (Wang et al., 2016), however with a very low efficiency of nearly 1%. Later, the cas9 editor line of *N. gaditana* was developed that constitutively expressed the cas9 and was used for editing of targeted transcription factor genes with high efficiency range of up to 78% (Ajjawi et al., 2017). In the recent past, various strategies have been successfully applied for gene-editing in several microalgal species. However, the above literature shows the inconsistency in the editing-efficiency of CRISPR system across the microalgal species and is still a concern. Thus, the identification of novel or optimized nucleases that may prove to be useful in gene-editing in microalgae is required. Moreover, the constitutive expression of Cas9 (or other nucleases) may sometime induce the undesired re-editing of the mutant lines (Slattery et al., 2018). In this context, the episomal-vector system has the advantage of transient Cas9 (or other nucleases) expression that can prevent re-editing of mutant lines, which is a common complication associated with the constitutive expression of Cas9. Moreover, the elimination of episomal CRISPR/Cas9 vector from the host upon removal of selection pressure makes the mutant lines be considered as non-transgenic. In contrast to diatom, using similar vector system, recently Muñoz et al. (2019) were not able to rescue the episomal plasmids from positive transformants of green oleaginous microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans*. Moreover, the continuous subculturing in the selection-free medium was not sufficient to remove the episomal vector. This indicates the possible chromosomal integration event even in the bacterial conjugation-based episomal vector delivery. Therefore, the episomal maintenance of delivered plasmids in microalgae other than diatoms through diatom-adapted, yeast-derived centromeric sequences (CEN/ARS) is not possible yet. Rather, episomal maintenance is a function of species-specific adaptation of yeast centromeric regions that should be optimized before the wider application of episomal vectors in microalgal bioengineering.

CASE STUDIES FOR GENETIC ENGINEERING IN MICROALGAE

The previous sections reviewed key resources that can augment the bioengineering in microalgae. This section describes the various algal bioengineering research such as: the enhancement of (1) photosynthesis and biomass production, (2) lipid production, (3) the production of biomolecules, and other value-added products.

Photosynthetic Efficiency and Biomass Production

Enhanced CO_2 fixation through augmentation of photosynthetic efficiency is the key process to improve microalgae biomass production, a pre-requisite to develop microalgae as the next-generation feed-stock. The carbon fixation is dependent on multiple factors, where selectivity and velocity of RuBisCo enzyme remains one of the major factors. RuBisCo is capable to fix CO_2 as well as O_2 into 3-phosphoglycerate and 2-phosphoglycolate, where 2-phosphoglycolate is undesirable and toxic to the cells. The phenomenon called photorespiration occurs in the mitochondrion and peroxisome, which uses 2-phosphoglycolate to release CO_2. These futile side reactions ultimately hamper the photosynthetic activity. Therefore, attempts have been made to simultaneously improve the selectivity and catalytic rate of RuBisCO through genetic engineering, though with limited success (Du et al., 2003; Spreitzer et al., 2005). Alternatively, the problem of RuBisCO selectivity can be mitigated by controlling the design

consideration of cultivation system to enrich the CO_2 supply. Nevertheless, in order to improve the catalytic rate of RuBisCO, its genetic modification is preferable mode than to select efficient RuBisCO from the diverse pool of natural variants. In one such effort, the small subunit of RuBisCO enzyme of *Chlamydomonas* has been replaced with that of Arabidopsis, spinach, and sunflower to enhance the carboxylation catalytic efficiency and CO_2/O_2 specificity (Genkov et al., 2010). Although the hybrid RuBisCO enzyme had 3–11% increase in specificity, the velocity of the enzyme remained same. Likewise, several amino-acid residues have been identified in the conserved region of small subunit of the RuBisCO that can be the potential target for engineering RuBisCo to improve its catalytic efficiency (Du et al., 2000; Spreitzer et al., 2001; Genkov et al., 2006; Genkov and Spreitzer, 2009). In another approach through regulation of RuBisCo activity, the photosynthetic biomass production in *N. oceanica* was substantially enhanced upon overexpression of RuBisCO activase (Wei et al., 2017a). Beside RuBisCO the other relatively low abundant enzymes of Calvin cycle regeneration phase, such as fructose−1,6-bisphosphatase (FBPase), fructose 1,6-bisphosphate aldolase (FBA), and sedoheptulose 1,7-bisphosphatase (SBPase) are the prime target to manipulate the photosynthetic activity. Recently the engineering of Calvin cycle through the overexpression of cyanobacterial FBA was found to enhance the photosynthetic capacity of *C. vulgaris* (Yang et al., 2017). Similarly, the overexpression of *Chlamydomonas* SBPase was reported to improve the photosynthetic activity in *Dunaliella bardawil* (Fang et al., 2012). The FBPase was found to enhance the photosynthetic efficiency upon overexpression in higher plants (Tamoi et al., 2006). However, its overexpression in *Chlamydomonas* had detrimental effect on growth and photosynthetic activity under high CO_2 photoautotrophic conditions. This was mainly due to the reduced amount of glyceraldehyde−3-phaspahte because there was enhanced conversion of fructose−1,6-bisphosphate into fructose−6-phosphate (Dejtisakdi and Miller, 2016). This indicates that in microalgae the reaction catalyzed by the FBPase is not a rate limiting one that can be targeted to improve the photosynthetic efficiency and concomitant biomass accumulation.

The photosynthetically efficient microorganisms operate the CO_2-concentrating mechanisms (CCMs) to increase the CO_2 concentration in the proximity of RuBisCO, which eventually reduce the photorespiration and promote carboxylation. In comparison to the terrestrial plants, the green microalgae have efficient CCM because of sequestration of the enzymes of photosynthetic machinery in the pyrenoid or peroxisome (Mackinder, 2018; Hennacy and Jonikas, 2020). Several functional and regulatory factors have been identified, which are responsible to facilitate the carboxylation reaction of RuBisCO through CCM. Among these factors *CIA5*, transporter of inorganic carbon (Ci) and carbonic anhydrases (CA) are considered as the targets for manipulation to increase the photosynthetic performance and eventually biomass yield (Moroney et al., 2011; Wang et al., 2015; Yamano et al., 2015; Gee and Niyogi, 2017). However, there are no such reports on successful engineering of CCM components in microalgae, and thus it remains a challenge to enhance the carbon fixation process.

On the other hand, the cultivation of microalgae at high cell density often encounters a problem of photo-limitation because of light shading. The high light intensity at the surface cell layers saturates the photosynthetic process and causes photoinhibition, whereas excess energy is dissipated through non-photochemical quenching. Meanwhile, the low-light intensity at the lower layer of cells compels them to perform photorespiration instead of photosynthesis. This uneven distribution of the light intensity results in suboptimum photosynthetic efficiency that eventually reduces the biomass yield. Reducing the size of antenna or light-harvesting complex is one of the approaches that has the potential to improve the light transmission and light absorption capacity. For instance, the reduction of chlorophyll *b* content and consequent reduction of antenna size in *Chlamydomonas* through RNAi-mediated silencing of *chlorophyllide a oxygenase*, resulted in enhanced photosynthetic activity and higher growth rate as compared to chlorophyll *b* mutant under saturating light conditions (Perrine et al., 2012). Similarly, the *C. vulgaris* mutant with truncated antenna size and reduced chlorophyll *a* and *b* content, generated through random mutagenesis of chloroplast signal recognition particle (*CpSRP43*), exhibit enhanced photosynthetic efficiency associated with reduced non-photochemical quenching and higher biomass yield (Shin et al., 2016b, 2017). The engineering of photosystem II protein D1 isoform in *Chlamydomonas* showed enhanced photosynthetic efficiency under saturating light conditions (Vinyard et al., 2014). In another novel approach, the diatom *P. tricornutum* was engineered to establish a concept of intracellular spectral recompositioning for improved light absorption and consequent higher biomass production (Fu et al., 2017). In this case, the overexpressed green fluorescent protein absorbs the excess blue light energy from incident light and subsequently emits energy as green light that can be harvested by accessory pigments. Thus, spectral recompositioning eventually improves the light absorption and reduces the non-photochemical quenching and may mitigate the problem of photoinhibition at high cell density cultures through deeper penetration of emitted green light. A similar ecological mechanism has been observed in the coral-algae symbionts to acclimatize deep water light environment by facilitating homogenous distribution of available light energy (Smith et al., 2017). Although significant information is availed through genetic engineering to get insight into the photosynthetic efficiency, most of these leads are from the model algal systems. Moreover, this information is yet to be applied for large scale applications. In addition, the design consideration of cultivation system has significant effect on the photosynthetic efficiency and eventually on productivity. Therefore, there is a need of more comprehensive and cumulative approach, such as fine tuning the flux balance of Calvin cycle toward enhanced CO_2 fixation or perturbation of multiple targets at once to get a synergistic effect. The various strategies to improve photosynthetic efficiency and biomass production are illustrated in **Figure 2**.

Lipid Production

Lipids from microalgae are at the center of attention due to their yield and nutraceutical importance. The quantity, quality, and the type of lipids synthesized by microalgae not only

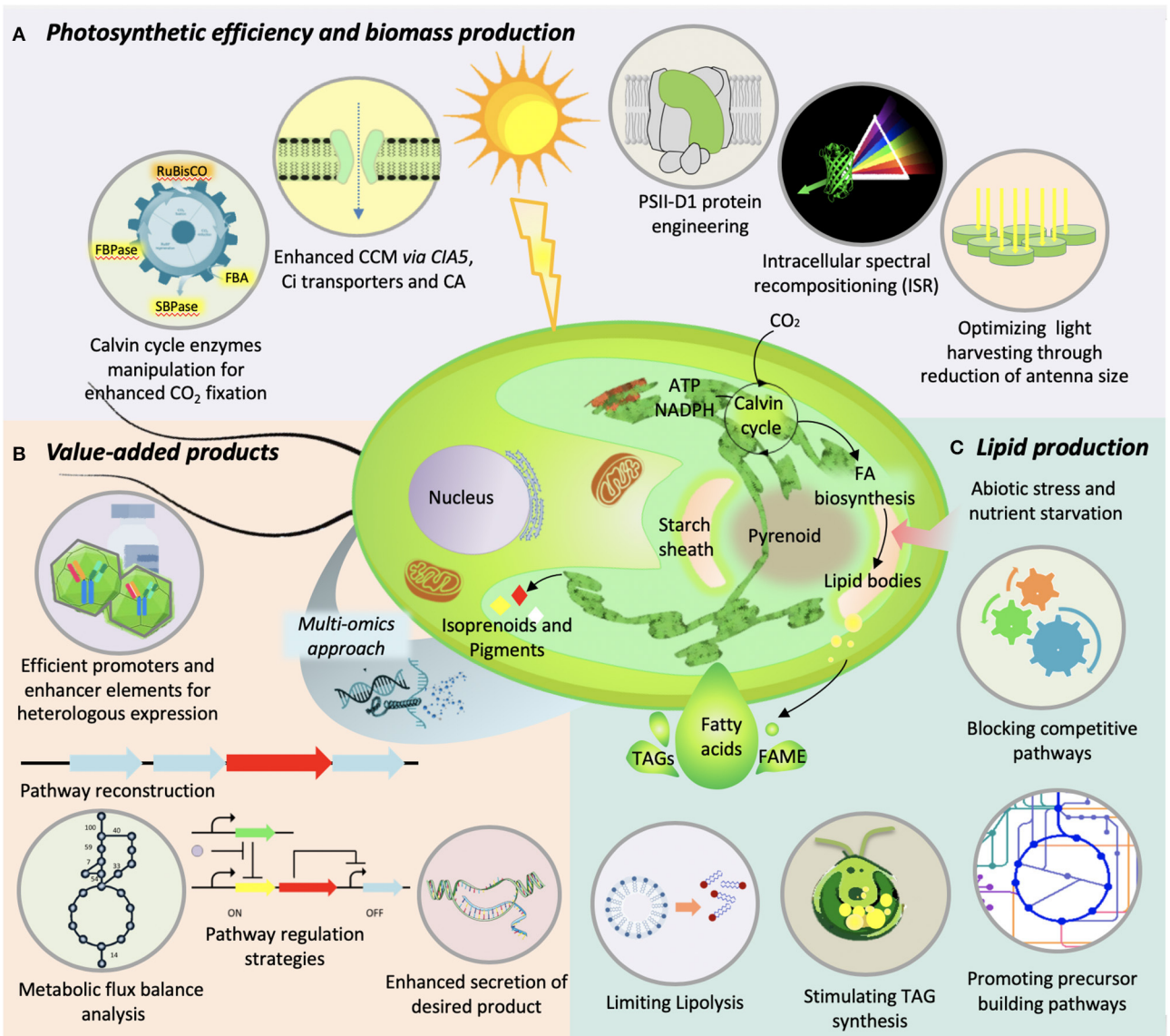

FIGURE 2 | Illustration of various genetic-engineering strategies used in microalgae to improve **(A)** photosynthetic efficiency and biomass production, **(B)** value-added product synthesis, and **(C)** lipid production.

help in diversifying their application but influence the biodiesel properties if chosen for the fuel purpose (Shekh et al., 2016). For researchers working in this area, lipid productivity remains a key parameter for strain selection. In fact, the kind of lipids a microalga accumulates plays a key role in its commercial utilization for food, feed, or fuel purpose. Over the years, a trade-off between enhancing microalgal lipid content by various means without compromising the lipid productivity was targeted. Various augmentations in environmental, nutritional, and physiological conditions for cultivation of microalgae, as well as genetic manipulations, have been attempted for enhanced lipid production (**Figure 2**). However, genetic engineering of the robust strains for enhanced lipid production remains one of the most viable options to improve the process economics. In the recent past, various genes involved in lipid biosynthesis were knocked-out or overexpressed to examine their effects on lipid accumulation. *Acetyl-CoA Carboxylase* (*ACCase*), which encodes enzyme for fatty acid synthesis, was overexpressed for the first time in 1996 by Dunahay et al. (1996). Even though the overexpression of ACCase was characterized by 2- to 3-fold increase in ACCase activity, it could not lead to increased lipid accumulation (Sheehan et al., 1998). However, upregulation of ACCase in tandem with malic enzyme, which catalyzes malate to pyruvate conversion, was effective in enhanced lipid accumulation in *D. salina* (Talebi et al., 2014). Overexpression of diacyl glycerol acyl transferase, which catalyzes the final step in TAG synthesis, is often-used strategy, which also resulted in lipid enhancement (Niu et al.,

2013; Iwai et al., 2014; Li et al., 2016a). Also, the enhanced expression of pyruvate dehydrogenase, acetyl-CoA synthase, phosphoenolpyruvate carboxylase, NAD(H) kinase, and glycerol kinase has resulted in hyperaccumulation of lipids in various microalgal species. Simultaneous expression of multiple acyl transferases from *S. cerevisiae* and *Yarrowia lipolytica* in *Chlorella minutissima* resulted in twofold lipid accumulation (Hsieh et al., 2012). Overexpression of RuBisCO activase in *N. oceanica* has resulted in an increase in the productivity, thereby increasing lipid accumulation (Wei et al., 2017a). Inhibiting the expression of a multifunctional lipase/phospholipase/acyltransferase in *T. pseudonana* resulted in enhanced lipid accumulation without compromising the growth (Trentacoste et al., 2013). On the other hand, it is known that the transcriptional regulation can influence metabolomic flux of the system as transcription factors can target multiple regulatory points in a metabolic pathway. Overexpression/knockdown of transcription factors targeting the upregulation of lipid biosynthesis genes may accumulate higher lipids. In one of the efforts, knockdown of a single transcription regulator *ZnCys* in *N. gaditana* resulted in twofold increase in lipid content (Ajjawi et al., 2017). Strategies to prevent the degradation of synthesized lipids were also studied to improve lipid yields. A knock-out mutant of the phospholipase A2 gene (*C. reinhardtii*) had the total lipid content increased up to 64.25% (Shin et al., 2019). In another study, a 10-fold increase in TAG was reported upon silencing the *cht7* gene encoding a TAG lipase (Tsai et al., 2014). Most recently, CRISPR/Cas9-based technology for gene manipulation in *C. vulgaris* was used wherein a fragment of Cas9 with sgRNA designed on omega-3 fatty acid desaturase (*fad3*) gene was constructed. This has resulted in 46% (w/w) higher accumulation of lipid content (Lin and Ng, 2020). Even though various studies to genetically engineer microalgae for enhanced lipid accumulation have been attempted, they are mostly restricted to model and/or selected microalgae strains. The recent advancements in gene-editing technologies especially CRISPR/Cas9 may allow the gene manipulations in commercially important oleaginous strains so as to improve the process economics.

Biomolecules and Value-Added Products

Beside lipids, microalgae are rich in biomolecules such as carotenoids with potential application in human health. The accumulation of biopigments in microalgae is known to be affected by various biotic and abiotic factors, the details of which have been recently reviewed by Saini et al. (2020). Since the carotenoid biosynthesis pathway has been extensively studied, the metabolic engineering, in addition to the mutant screening, has been applied to enhance the production of carotenoids in microalgae. Perturbing the pathway enzymes such as phytoene synthase and phytoene desaturase, microalgae have known to enhance the production of carotenoids (Steinbrenner and Sandmann, 2006; Couso et al., 2011; Dambek et al., 2012; Tran et al., 2012; Liu et al., 2014; Eilers et al., 2016; Galarza et al., 2018). In addition, several other enzymes involved in the subsequent steps of the carotenoid pathways have also been targeted. For instance, the overexpression of *Haematococus pluvialis* gene encoding β-carotene ketolase in *Dunaliella salina* resulted in production of astaxanthin (Anila et al., 2016). The downregulation of squalene epoxidase through RNAi in *Chlamydomonas* was found to accumulate squalene (Kajikawa et al., 2015). Similarly, the knock-out mutant of *zeaxanthin epoxidase* in *Chlamydomonas* had significantly higher zeaxanthin content than the wild type (Baek et al., 2018). However, diverting the flux toward desired metabolites is not that simple and may require perturbation of multiple genes of a pathway. In one such recent example, the overexpression of three exogenous enzymes, namely oxidosqualene cyclase (from *Lotus japonicus*) and cytochrome P450 along with its native reductase (from *Medicago truncatula*) in *P. tricornutum*, leads to the production of triterpenoids *viz.* lupeol and botulin (D'Adamo et al., 2019). Similarly, the production of sesquiterpenoids and diterpenoids through genetic engineering of *Chlamydomonas* has also been reported (Lauersen et al., 2016, 2018; Wichmann et al., 2018). The introduction of additional copy of gene encoding gateway enzyme of terpenoid pathway, 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*), resulted in enhanced accumulation of fucoxanthin in *P. tricornutum* (Eilers et al., 2016). However, the hyperaccumulation of carotenoids or any other secondary metabolites sometimes causes feedback inhibition. Therefore, generating an additional metabolic sink (in a place other than the site of production) or expressing the flux controlling enzyme(s) that can resist the feedback inhibition could be the possible strategies. However, this strategy may get limited by the lack of information on transporters or the flux controlling enzymes. Here the genomic information can substantially improve the scenario of metabolic engineering in microalgae.

The algal nuclear or chloroplast engineering has been extensively carried out using synthetic biology approach for the production of recombinant proteins having therapeutic properties. Some of the inherent features of algae such as lack of infectious agents or toxins, efficient folding of complex proteins and scope for the development of whole algae as low-cost oral vaccine, makes them ideal platform for heterologous production and offer several advantages over the better established microbial and mammalian systems. Although, most of the chloroplast transformation attempts have been made in the model microalgae *Chlamydomonas*, the successful chloroplast engineering has also been demonstrated in few other microalgal species [reviewed by Siddiqui et al. (2020)]. It was reported that over 100 different recombinant proteins have been successfully expressed in algal chloroplast. Among these recombinant proteins, the vaccines, antibodies and immunotoxins, and therapeutic proteins are the major targets (Dyo and Purton, 2018). The production of whole algal cells as oral vaccines specially for farm animals, where the fusion of protein adjuvant (cholera toxin B subunit: CTB) to the N-terminus of the antigen facilitates the antigen absorption through gut epithelium, provided an alternative low-cost vaccination strategy. Moreover, this bioencapsulation of therapeutic proteins has advantages of long-term storage at room temperature and also protects them from the degradation in animal stomach (Dreesen et al., 2010; Gregory et al., 2013). Besides all these advantages, the yield of the recombinant proteins is still a major concern to adopt algae as protein production platform. Although

several recombinant proteins have been successfully produced through genetic engineering of nuclear genome, a much lower success rate with production yield of only up to 0.25% of total soluble proteins was reported (Scranton et al., 2016). In comparison, the production of proteins through chloroplast engineering may reach up to 0.1–5% of total soluble protein (Dyo and Purton, 2018). Nevertheless, the nuclear expression of the protein offers some interesting features such as ability to target the protein to secretory pathway or to the specific organelles that may also allow the post-translational modification of the proteins (Lauersen et al., 2015). The various signal peptides have been used to target the proteins either to secretory pathway or to an organelle. Recently, the performance of two *in-silico* identified signal peptides (1,3-α-glucosidase and SAD1p derived) to efficiently secrete expressed reporter protein in *C. reinhardtii* has been successfully demonstrated (Molino et al., 2018). The different promoters and their respective 5' UTRs as well as their synthetic variants have been used to derive the expression of transgene in order to mitigate the constrains of inefficient transgene expression in microalgae (Coragliotti et al., 2011; Specht and Mayfield, 2013; Gimpel et al., 2015). For example, the use of strong promoter such as 16S ribosomal RNA fused to 5'UTRs of endogenous photosynthetic genes can be used to enhance the expression of transgene to some extent (Rasala et al., 2011). However, the performance of the endogenous 5′UTRs to translate the gene of interest is still the major constrain. The intrinsic features of photosynthetic genes derived 5′UTRs are also responsible for feedback regulation of translation. It does so through "control by epistasis of synthesis" that prevent overaccumulation of protein subunit in the absence of other subunits of the protein assembly (Coragliotti et al., 2011). In addition, the constitutive expression of the transgene negatively impacts the growth of the transgenic algae as an extra metabolic burden. Therefore, the use of inducible promoter to tightly regulate the expression of transgene could be the better strategy to improve the growth efficiency, and hence the productivity of desired product (Fajardo et al., 2020). The various promoters used so far in the microalgal research are given in **Table 3**. The advancement in the synthetic biology and our understanding on the regulation of protein synthesis in microalgae will enable us to improve the protein expression level in microalgae so as to make microalgae a feasible host system for commercial application. The various strategies to improve production of bioactive of interest in microalgae are illustrated in **Figure 2**.

RISK ASSESSMENT, BIOSAFETY, AND REGULATORY ISSUES

Though genetic engineering is considered as one of the most potent tools to augment production of commercially valuable metabolites in microalgae, it inevitably invites varying opinions on the safe use of genetically modified (GM) algae for consumption and environment. On the contrary, several algal performance-improvement strategies, which could have environmental and ecological threats, are in use without much debate. In many parts of the world, strict laws/policies require transgenic/recombinant algae to undergo regulatory compliances. When research and policy complement each other, technological advances move at a rapid pace. In this case, even if various researchers across the globe are working on strain improvement for enhanced microalgae performance through genetic modifications, their commercial use is restricted. Reports indicate that the Florida-based biotechnology company named Algenol was given approval for use of GM cyanobacteria for cultivation in outdoor closed-photobioreactor. At the same time, the secretariat of the Convention on Biological Diversity in its 2015 report has raised the concerns over strict physical containment of these GM microorganisms by the company (https://www.cbd.int/ts/cbd-ts-82-en.pdf). It is arguably said that the U.S. Environmental Protection Agency (US-EPA) relies upon a regulatory regime-Toxic Substances Control Act (TSCA), which has become outdated and is incapable of assessing the novel risks arising out of the new biotechnological inventions. Under TSCA, companies are only required to file a Microbial Commercial Activity Notice for commercialization of a new GM microorganism. Till date, no outdoor cultivation of GM microalgae is reported probably due to various predictable and unexpected risks associated with its open cultivation (Nethravathy et al., 2019). Cultivation of GM microalgae possesses several risks, which includes spills that may become uncontrollable. These algae upon proliferation compete with natural species and may outgrow them. In fact, the genetically modified traits of the organisms may provide them the competitive advantage in natural ecosystem. Risks also exist for genetic contamination /interbreeding with wild-type or sexually compatible strains. Threats of harmful algal blooms, negative impacts on ecosystem, increased selection pressure, horizontal gene transfer, health and environmental impacts, unpredictable future of GM traits, loss of management control, and ethical concerns are some of the major concerns associated with cultivation of GM algae (Nethravathy et al., 2019). Apart from regulations for the use of GM algae, strict biosecurity laws are required to safeguard the importation of foreign species (GM and/or wild-type) to the local environment. Though the import and use of foreign algae strains, which are non-native to local environment, have a very little regulatory control, the associated risk of these strains dominating the local species must be seriously considered (Campbell, 2011). The concrete environmental risk due to algal spills must not only be limited to the GM aspect of the strains. Further, assessment needs to be carried out considering fitness of invading foreign species in comparison with local algal community along with intricacies and population stability characteristics of the ecological system in question (Henley et al., 2013). To further improve the situations for the use of GM algae, in-depth cost-benefit analysis of GM microalgae to society and environment must be carried out. Strict monitoring of the handling and cultivation process with health and environmental risk assessment analysis are integral to design the biosafety regulations for GM microalgae. Since GM algae are considered as one of the solutions to overcome techno-economic challenges in algal industry, it is imperative that various stakeholders including business promoters and policy makers collectively reach to a consensus on a road map for the

TABLE 3 | List of endogenous and heterologous promoters used in microalgae research.

Target species	Promoters	Nuclear (*N*)/ chloroplast (*C*) expression	Salient features	References
Ankistrodesmus convolutus	AcRbcS promoter	*N*	Light-regulated promoter	Thanh et al., 2012
C. reinhardtii	ARG7 promoter	*N*	Strong promoter	Specht et al., 2015
	β-TUB2 promoter	*N*	Constitutive promoter	Crozet et al., 2018
	CABII-1	*N*	Light-dependent promoter	Doron et al., 2016
	Cyc6 and Cpx1 promoter	*N*	Copper- and oxygen-dependent promoter	Quinn et al., 2000
	CrGPDH3 promoter	*N*	Salt inducible promoter	Beltran-Aguilar et al., 2019
	Fea1 promoter	*N*	Iron-responsive promoter	Barjona do Nascimento Coutinho et al., 2019
	HSP70A-RBCS2 promoter	*N*	Strong hybrid promoter	Lauersen et al., 2015
	HSP70A promoter	*N*	Strong promoter activity	Schroda et al., 2000
	psaD promoter	*N*	Light-responsive constitutive promoter	Crozet et al., 2018
	sap11 promoter	*N*	Synthetic strong promoter	Scranton et al., 2016
	RBCS2 promoter	*N*	Strong promoter activity	Lumbreras et al., 1998
	psaA promoter	*C*	Light-responsive strong promoter	Michelet et al., 2011
	psbA promoter	*C*	Light-responsive strong promoter	Rasala et al., 2011
	psbD promoter	*C*	Light-responsive strong promoter	Rasala et al., 2011
	atpA promoter	*C*	Constitutive promoter activity	Rasala et al., 2011
	16S promoter-psbA 5' UTR	*C*	Strong promoter	Rasala et al., 2011
	rbcL promoter	*C*	Light-responsive strong constitutive promoter	Rasala et al., 2011
Chaetoceros gracilis	Lhcr5 promoter	*N*	Constitutive promoter	Ifuku et al., 2015
C. vulgaris	CaMV35S promoter	*N*	Constitutive promoter	Chow and Tung, 1999
	CvpsaD promoter	*N*	Light-responsive promoter	Kim et al., 2018
Chlorella ellipsoida	Ubi1- **Ω** promoter	*N*	Strong constitutive expression	Chen et al., 2001
Cyclotella cryptica	ACCase promoter	*N*	Constitutive promoter	Dunahay et al., 1996
Cylindrotheca fusiformis	fruα3 promoter	*N*	Strong constitutive expression	Fischer et al., 1999
D. salina	LIP promoter	*N*	Light-inducible promoter	Baek et al., 2016b
	GAPDH promoter	*N*	Constitutive promoter	Doron et al., 2016
Fistulifera sp.	fcpB promoter	*N*	Constitutive promoter	Muto et al., 2013
	H4 promoter	*N*	Constitutive promoter	Muto et al., 2013
H. pluvialis	CaMV 35S	*N*	Constitutive promoter	Kathiresan et al., 2009
	Ptub promoter	*N*	Strong promoter	Yuan et al., 2019
	rbcL promoter	*C*	Light-responsive strong constitutive promoter	Gutiérrez et al., 2012
P. tricornutum	CaMV 35S promoter	*N*	Constitutive promoter	Chow and Tung, 1999
	U6 promoter	*N*	Constitutive promoter	Serif et al., 2018; Stukenberg et al., 2018
	Lhcf promoter	*N*	Light-dependent promoter	Lepetit et al., 2010
	NIT promoter	*N*	Ammonium inducible promoter	Chu et al., 2016
	pPhAP1 promoter	*N*	Strong promoter	Lin et al., 2017
	Pt211 promoter	*N*	Strong constitutive promoter	Zou et al., 2018
	fcp promoter	*N*	Constitutive promoter	Watanabe et al., 2018
	V-ATPase promoter	*N*	Strong constitutive promoter	Watanabe et al., 2018
	ef2 promoter	*N*	Constitutive promoter	Seo et al., 2015
	HASP1 promoter	*N*	Strong constitutive promoter	Erdene-Ochir et al., 2019
	rbcL promoter	*C*	Light-responsive strong constitutive promoter	Xie et al., 2014
N. oceanica	β-tubulin promoter	*N*	Constitutive promoter	Li et al., 2014a
	CMV viral promoter	*N*	Constitutive promoter	Osorio et al., 2019

(Continued)

TABLE 3 | Continued

Target species	Promoters	Nuclear (*N*)/ chloroplast (*C*) expression	Salient features	References
	ef promoter	*N*	Constitutive promoter	Poliner et al., 2018
	Ribi promoter	*N*	Bidirectional strong constitutive promoter	Poliner et al., 2018
	EM7 promoter	*N*	Constitutive promoter	Osorio et al., 2019
	NIT promoter	*N*	Ammonium inducible promoter	Jackson et al., 2019
	VCP promoter	*N*	Constitutive promoter	Li et al., 2014a
	rbcL promoter	*C*	Light-responsive strong constitutive promoter	Gan et al., 2018
N. gaditana	TCT promoter	*N*	Constitutive promoter	Ajjawi et al., 2017
	RPL24 promoter	*N*	Constitutive promoter	Ajjawi et al., 2017
	4ALL promoter	*N*	Constitutive promoter	Ajjawi et al., 2017
	EIF3 promoter	*N*	Constitutive promoter	Ajjawi et al., 2017
N. oculata	HSP70A-RBCS2 promoter	*N*	Strong hybrid promoter	Shih et al., 2015
N. salina	TUB promoter	*N*	Constitutive promoter	Koh et al., 2019
	UEP promoter	*N*	Constitutive promoter	Koh et al., 2019
T. pseudonana	Lcfs9 promoter	*N*	Constitutive promoter	Poulsen et al., 2006
	NIT promoter	*N*	Nitrate inducible promoter	Poulsen et al., 2006
Volvox carteri	LHCBM1 promoter	*N*	Constitutive promoter	Tian et al., 2018
	nitA promoter	*N*	Nitrate inducible promoter	von der Heyde et al., 2015
	ISG promoter	*N*	Developmental stage (embryonic inversion) specific promoter	Hallmann and Sumper, 1994
	Arylsulfate promoter	*N*	Sulfur starvation inducible promoter	Hallmann and Sumper, 1994

use of GM algae in future. Various federal governments across the globe must bring in place the policies and regulations that govern the safe use of GM algae for human and environmental benefit.

CONCLUSION AND FUTURE PROSPECTS

Currently, economically feasible, environmentally sustainable, and replicable microalgal processes with higher technology readiness levels are required for ease of doing algal business. To improve the economic feasibility of the algal processes, the genetic engineering of microalgae is at forefront for development of robust microalgal strains. Advances in the high-throughput technologies and molecular biology tools have facilitated the biotechnological approach to engineer the microalgal strains for performance improvement. The synergy of microalgal multi-omics datasets and the advanced molecular tools offer a rapid and predictable strategic path for the strain improvement. In this review, various microalgal resources such as genome sequence, mutant libraries, high-throughput screening methodologies, and genetic tools and techniques were summarized that holds the potential for the development of microalgae as a next-generation renewable resource. In addition, the catalog of various *omics* study under different conditions across the diverse microalgal species is generated (**Table 1**). Despite the variation in the inter- and intraspecies *omics* datasets, the several conserved factors can be mined to predict the biological outcomes with the comprehensive use of system biology approach. Various omics-based approaches must aim to enhance microalgal capacities to produce high value metabolites. Future research may focus on developing purpose-specific robust bioengineered strains for high photosynthetic efficiency, high CO_2 fixation, and high biomass productivities. Also, targeted enhancement of low-volume, high-value metabolites of biomedical applications from microalgae must be considered using genetic engineering.

Though the genetic engineering of microalgae holds great potential to improve process economics, it is limited mainly due to the unavailability of the genetic information for robust and commercially suitable strains. In recent times, rapid advances in DNA synthesis, genetic manipulation tools and techniques, availability of functional genomes have improved the chances to better engineer microalgae with complex functions. However, the lack of genetic strain design principles is still hurting the progress in this area. Further, once the genetically improved strains are developed, safety to human health and environment will define its commercial success. Therefore, it is recommended that strict regulations and monitoring should be in place to evaluate the environmental and human health risk of using GM microalgae particularly in outdoor cultivation. Here, the recent development in precise genome editing technologies such as non-transgenic and marker-free CRISPR has the potential to revolutionize the microalgal bioengineering for the production of non-GMO algal products. The non-GMO tag to the bioengineered microalgae is expected to improve the biosafety and alleviate the regulatory issues associated with the usage of GM microalgae. In view of uncertainty within the academic and industrial community regarding the regulations for the use of GM strains, and the

inadequacy of current regulations for the use of GM algae, a clear road map for regulatory regime covering the commercial use of GM microalgae is urgently required. Since the robustness of non-model microalgae species has advantages in commercial and industrial applications over model species, there is a need to develop advanced research tools for the non-model microalgal species. Moreover, to improve the economic competitiveness of algal-derived products, the development of efficient extraction methods or the use of whole cells is needed. Indeed, beside all the developments, bio-prospection for novel and robust microalgae with industrial viability must continue.

AUTHOR CONTRIBUTIONS

GK: conceptualization, writing—original draft preparation, writing—reviewing, editing, and supervision. AS: conceptualization, writing—original draft preparation, writing—reviewing, and editing. SJ: investigation—data collection and writing—original draft preparation. YS: visualization and writing—original draft preparation. RK: writing—original draft preparation. TS: writing—reviewing, editing, and supervision. All authors contributed to the article and approved the submitted version.

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Targeted Metabolomic and Biochemical Changes During Nitrogen Stress Mediated Lipid Accumulation in *Scenedesmus quadricauda* CASA CC202

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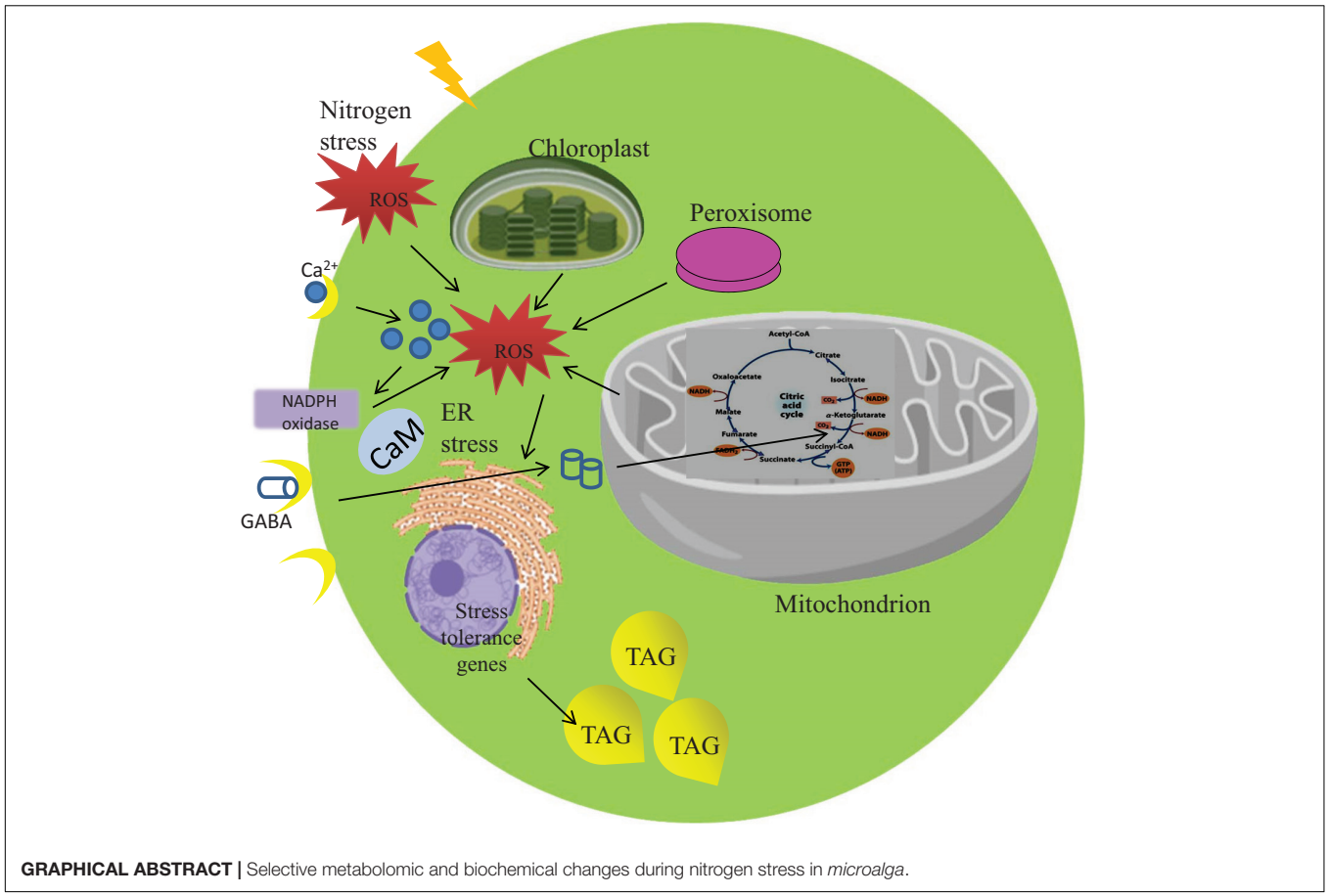
Scenedesmus quadricauda CASA CC202, a potent freshwater microalga is being used as a biofuel feedstock, which accumulates 2.27 fold lipid during nitrogen stress induction. Upon nitrogen starvation, *S. quadricauda* undergoes biochemical and metabolic changes that perturb the cell to cope up the stress condition. The nitrogen stress-induced biochemical changes in mitochondrion exhibits due to the oxidative stress-induced Reactive Oxygen species (ROS) generation at high membrane potential ($\Delta\psi_m$). The predominant ROS generated during nitrogen starvation was H_2O_2, OH^-, $O_2\cdot^-$ and to suppress them, scavenging enzymes such as peroxidase and catalase increased to about 23.16 and 0.79 U/ml as compared to control (20.2, 0.19 U/ml). The targeted metabolic analysis showed, stress-related non-proteinogenic amino acids and energy equivalents elevated during the initial hours of nitrogen starvation. The nitrogen stress-triggered biochemical and metabolic changes along with other cellular events eventually lead to lipid accumulation in *S. quadricauda*.

Keywords: microalgae, biofuel, nitrogen stress, Reactive Oxygen species, mitochondria, metabolic changes

INTRODUCTION

Microalgae are renowned as biofuel feedstock as it has the potential to meet current energy requirements. It has been well addressed that they accumulate increased lipid content per cell. The storage lipids in microalgae were synthesized in two steps such as *de novo* synthesis of fatty acids in plastids and triacylglycerol (TAG) biosynthesis in endoplasmic reticulum (ER). The Acetyl CoA from Calvin cycle is converted into Malonyl ACP and by the action of fatty acid synthase complex fatty acids were synthesized and free fatty acids were released in the plastid. Further the free fatty acids were entering into the ER and TAG synthesis by Kennedy pathway occurs (Wang et al., 2018). When an oleaginous (oil-producing) microalgae exposed to nitrogen stress which accumulates more lipid as an energy reserve (Lim et al., 2012). One such microalga, *Scenedesmus quadricauda* CASA CC202 which accumulates about 2.27 fold lipid during nitrogen starvation (Anand and Arumugam, 2015). In order to adapt the harsh environmental stimuli, several stress-responsive changes were occurring in the cell. Primarily the biomolecules such as lipid and carbohydrate



GRAPHICAL ABSTRACT | Selective metabolomic and biochemical changes during nitrogen stress in *microalga*.

level increase with a reduction in protein and photosynthetic pigments under nitrogen starvation (Msanne et al., 2012; Anand and Arumugam, 2015).

The actual biochemical and metabolic events in nitrogen stress mediated lipid accumulation and other abiotic stress is poorly addressed. It is complex cascade of reactions resulting in an increased lipid accumulation. An array of cellular events that switches on the lipid biosynthesis pathway to maintain the C/N homeostasis of the cell. The initial stress markers and signaling molecules lead to a tremendous rearrangement of metabolic pathways. The byproduct of these metabolic rearrangements is mainly activating the energy-saving shunt pathways and its associated reactions (Sweetlove et al., 2002; Bolton, 2009; Recht et al., 2014). Also, these stimulate an increased carbon channeling into fatty acid synthesis under nitrogen starvation. Therefore, a selective metabolomic study, is envisioned to understand the stress mediated lipid accumulation in *S. quadricauda*.

As mentioned above metabolic rearrangements of cells during nitrogen starvation is induced by various stress signaling molecules. The stress signaling molecules like Reactive Oxygen species (ROS), Ca^{2+}, Melatonin, Abscisic acid, etc helps the cell to sense the unfavorable environment. It eventually activates cascade of signal transductions to initiate a series of counter-reactions, which will lead to tolerance of the stressed cell. Reactive Oxygen species which are formed by redox reactions of the reactive forms of molecular Oxygen including H_2O_2, $O_2\cdot^-$ or OH^- radicals during abiotic stress are recognized as signals to activate the defense response (Vranova et al., 2002) and also as a second messenger to activate several signaling cascades (Shi and Collins, 2017). The increased ROS accumulation during prolonged nitrogen starvation, leads to an oxidative damage and eventually promotes neutral lipid accumulation in *Dunaliella salina*. An increase in both ROS production and lipid peroxidation were observed under nitrogen starvation in association with increased lipid accumulation (Yilancioglu et al., 2014). Thus ROS have direct effects on neutral lipid accumulation in microalgae under nitrogen starvation. Therefore, the understanding of an initial biochemical changes under nitrogen starvation need to be unraveled.

Along with the metabolic and biochemical changes, nitrogen stress also induces alterations in an internal bimolecular pattern and morphology. As a preliminary study, the morphological variation in *S. quadricauda* was characterized in the present study. *Scenedesmus* is a pleomorphic strain which changes its morphology during nitrogen starvation as unicells or coenobia (Anusree et al., 2017). As the microalga is having these peculiar characteristics the nitrogen stress-driven morphological variation in a population of *S. quadricauda* has not been completely studied.

Finally, the stress responses may be activating the genes of lipidomic, carbon and other metabolic pathways leading to neutral lipid accumulation is remain unexplored. The major evidence for the query covers different omics approaches like transcriptomics, proteomics, lipidomics and metabolomics which explains the key regulators and proteins for TAG accumulation under nitrogen starvation (Miller et al., 2010; Boyle et al., 2012; Park et al., 2015; Javee et al., 2016). The monogenic approach may not reveal a complex reactions of stress mediated lipid accumulation. Thus the present study aims to study the initial stress associated morphological, biochemical, and metabolic changes in nitrogen stress-mediated lipid accumulation in a comprehensive manner.

MATERIALS AND METHODS

Culturing and Induction of Nitrogen Stress

Scenedesmus quadricauda CASA CC202 were cultivated on Bold Basal Medium (BBM; Nichols and Bold, 1965), in a fabricated algal culture rack placed in an air conditioned Algal culture room maintained at 25°C. Philips Fluorescent tubes were used for illumination with a light intensity of 40 μmol/m^2/s (Apogee Full spectrum Quatum meter – MQ 500) and the light–dark period was regulated by automated timer. The composition of media wherein (g/l): $NaNO_3$ – 25; $CaCl_2.2H_2O$ – 2.5; $MgSO_4.7H_2O$ – 7.5; K_2HPO_4 – 7.5 KH_2PO_4 17.5; NaCl – 2.5, trace elements are (mg/l) $FeCl_3.6H_2O$ – 97; $MnCl_2.4H_2O$ – 41; $ZnCl_2.6H_2O$ – 5; $CoCl_2.6H_2O$ – 2; $Na_2MoO_4.2H_2O$ – 0.75, and the media also contain vitamins (g/l) Biotin 0.1; vitamin B12 1; Thiamine 0.2. The pH of the media was adjusted to 6.8–7. The nitrogen starved medium (N^-) completely lacks the $NaNO_3$ as nitrogen source.

The nitrogen stress induction was performed in two-stage cultivation processes; in the first stage, the microalgae are grown in control (N^+) media to obtain maximum cell density (240×10^6 cells/ml). Further, in the second stage, the harvested biomass was washed twice with distilled water and re-inoculated to the nitrogen starved (N^-) medium (Anand and Arumugam, 2015; Minhas et al., 2016; Sulochana and Arumugam, 2016).

Effect of Nitrogen Stress Induction in the Morphology of *S. quadricauda*

Morphological Variation

Twenty microliter of the sample was dropped into a clean microscopic slide covered with a coverslip and the slide was allowed to stand for a few minutes. After that, the slide was observed under a light microscope (Di LEICA DM 200).

Morphological Variation in a Population of *S. quadricauda* and Detection of Mitochondrial Membrane Potential by Flow Cytometry Analysis

The same samples were used for detecting the mitochondrial membrane potential of nitrogen stressed *S.quadricauda*. The sample of 1 ml taken from nitrogen stress-induced (N^-) and respective control (N^+) in the microcentrifuge tube from the three experimental replicates ($n = 3$). From that 100 μl sample was taken and diluted with 900 μl autoclaved distilled water and the pellet was obtained by centrifugation at 10,000 rpm for 10 min. Then the pellet was collected and washed twice with phosphate buffer saline (PBS) (pH 7.4). The washed pellet was fixed with 2.5% glutaraldehyde in PBS of 50 μl for 5 min. The pellet was then collected by centrifugation at 10,000 rpm for 5 min and washed with 1 ml PBS and again pelleted by centrifugation. The washed pellet was resuspended in 1 ml of Rhodamine 123 dye (SIGMA-ALDRICH, CAS No. 62669-70-9) (1 mg/ml ethanol stock) of 10 μl diluted with 990 μl of distilled water and incubated for 5 min at 20°C. After incubation, the excess dye was washed away by centrifugation and was resuspended pellet in 1 ml of PBS. Then the fluorescent intensity was analyzed by flow cytometry BD FACS Aria™ II excitation at 505 nm and emission at 534 nm using software BD FACS Diva™ (Morris et al., 1985; Baracca et al., 2003). The mean values of three independent replicates showing morphological variation (cell size) were plotted as a graph with standard deviation as an error bars.

Quantification of Reactive Oxygen Species and Antioxidant Enzymes During Nitrogen Starvation

Measurement of H_2O_2

The control (N^+) and nitrogen starved (N^-) algal cells from three experimental replicates were harvested by centrifugation and resuspended in 0.1% w/v Trichloro Acetic Acid (TCA) solution for sonication. The total cell lysate was collected by centrifugation at 13000 rpm for 10 min. After that 0.5 ml of the supernatant was taken into fresh tubes and added 0.5 ml of 10 mM phosphate buffer (pH 7.0). To that add 1 ml of 1 M potassium iodide and mix the contents well. The absorbance of the solution was read at 390 nm. A standard curve was plotted using known concentrations of H_2O_2 and from that, the H_2O_2 concentration (μmol H_2O_2/gFW) of the sample was calculated (Velikova et al., 2000). The mean values of three independent of H_2O_2 concentration were plotted as a graph with standard deviation as an error bars.

Quantification of $O_2\cdot^-$

The control (N^+) and nitrogen starved (N^-) algal cells of three experimental replicates ($n = 3$) were harvested by centrifugation, sonicated with 5 ml of 65 mM potassium phosphate buffer (pH 7.8). The cell lysate was collected by centrifugation at 12,000 rpm for 5 min. From that 1 ml of supernatant was taken into a fresh tube and mixed with 0.9 ml of 65 mM potassium phosphate buffer (pH 7.8). About 0.1 ml of 10 mM hydroxyl ammonium chloride was added to the mixture and incubated at 25°C for 20 min. After the incubation, 1 ml of 17 mM sulphanilic acid, and 1 ml of 7 mM α-naphthylamine were added to the mixture. Again the tubes were incubated for 20 min and the absorbance was read at 530 nm. The mean values of three independent replicates of $O_2\cdot^-$ concentration were plotted as a graph with standard deviation as

an error bars. Sodium nitrite was used to plot the standard curve from that the production of $O_2 \cdot^-$ was calculated (Liu et al., 2010).

Measurement of OH^-

The control (N^+) and nitrogen starved (N^-) algal cells of three experimental replicates ($n = 3$) were harvested by centrifugation and sonicated with 2 ml of 50 mM potassium phosphate buffer (pH 7.0). Then the homogenate was centrifuged at 12,000 rpm for 5 min. From that 0.5 ml of supernatant was taken into a fresh tube and added 0.5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2.5 mM of 2-deoxy ribose. The tubes were kept at 35°C in dark for 1 h. After the incubation 1 ml of 1% Thiobarbituric acid (TBA) in 0.5 M sodium hydroxide and 1 ml of acetic acid were added and mixed well. The tubes were boiled for 30 min and immediately cooled on ice. Further, the absorbance was read at 532 nm and the OH^- content was expressed as absorbance units per gram of Fresh Weight (FW; Halliwell, 2006). The mean values of three independent replicates of OH^- concentration were plotted as a graph with standard deviation as an error bars.

Lipid Peroxidation

Microalgal cells of three independent replicates from N^+ and N^- samples were harvested by centrifugation at 8000 rpm for 10 min. Then the cells were sonicated in 2 ml of 80:20 (v/v) ethanol: water and the lysate were collected by centrifugation at13,000 rpm for 10 min. Further, 1 ml of the supernatant was taken into fresh test tubes and added 1 ml of TBA solution [20% (w/v) TCA, 0.01% butylated hydroxytoluene and 0.65% TBA]. The samples were mixed well and heated at 95°C for 25 min and cooled. The contents were centrifuged at 13,000 rpm for 10 min and the absorbance of the supernatant was read at 450, 532, and 660 nm (Hodges et al., 1999). The mean values of three independent replicates were plotted as a graph with standard deviation as an error bars.

Malondialdehyde (MDA) (μmol/gFW) = $[6.45 \times (A_{532} - A_{600})] - [0.56 \times A_{450}]$/FW

Estimation of Antioxidant Enzymes

Catalase Assay

Catalase activity was determined using catalase calorimetric activity kit (Invitrogen, EIACATC). Nitrogen stressed (N^-) and control (N^+) algal pellet (100 mg) were collected by centrifugation at 8000 rpm for 10 min. Further, the pellet was homogenized or sonicated in 1 ml of cold 1× assay buffer (as provided by the manufactures) per 100 mg of cells. Then the content was centrifuged at 10,000 rpm for 15 min at 4°C. Collect the supernatant and assay immediately, or store at ≤ -70°C.

As dilution of standards for catalase assay was prepared as described by the manufactures instructions. In brief, one unit of catalase decomposes 1 μmol of H_2O_2 per minute at pH 7.0 and 25°C. About 10 μl of catalase standards was added to one tube containing 190 μl 1× assay buffer and labeled as 5 U/ml catalase. 100 μl of 1× assay buffer was added to each of six tubes labeled as follows: 2.5, 1.25, 0.625, 0.313, 0.156, and 0 U/ml catalase. Serial dilutions of the standard were prepared as described in the kit manual.

Accurately 25 μl of standards or diluted samples were added to the appropriate wells. Then added 25 μl of H_2O_2 reagent into each well and incubated for 30 min at room temperature. After that 25 μM of the substrate was added into each well. Again added 25 μl of 1× Horse Radish Peroxidase (HRP) solution into each well and incubated for 15 min at room temperature, further, the absorbance was read at 560 nm. Curve fitting software with a four-parameter algorithm (Graph pad prism2) was used to generate the standard curve and catalase activity of samples. The mean values of three independent replicates were plotted as a graph with standard deviation as an error bars.

Peroxidase Assay

The peroxidase activity was quantified using Peroxidase activity assay kit (SIGMA-ALDRICH, MAK092). As dilution of standards for peroxidase assay was prepared as described by the manufactures instructions. In brief, about 10 μl of the 12.5 mM H_2O_2 solution was diluted with 1240 μl of assay buffer to prepare a 0.1 mM standard solution. Then 0, 10, 20, 30, 40, and 50 μl of the 0.1 mM standard solution was added into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 nmol/well standards. Further, the assay buffer was added to each well to make up the volume to 50 μl. To each standard curve well, 50 μl of the standard curve reaction mix was added. Each well was mixed well and incubated at room temperature for 5 min and absorbance was read at 570 nm.

About 10 mg of the algal pellet (N^+ and N^-) was sonicated with 150 μl of assay buffer and centrifuged at 15,000 rpm for 10 min. Then 50 μl of the master reaction mix was added to each sample and positive control well. The contents in the well were mixed well by pipetting and incubated the plate at 37°C for 3 min, then the initial measurement was read at 570 nm (T initial). The assay was performed in the dark. The measurements were taken until the value of the test exceeded that of the standard. The final measurement [(A_{570}) final] for calculating the enzyme activity would be the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T final.

The change in measurement from T initial to T final for samples was calculated.

$$\Delta A_{570} = (A_{570})\,\text{final} - (A_{570})\,\text{initial}$$

The Δmeasurement value (ΔA_{570}) of each sample was compared to the standard curve to determine the amount of H_2O_2 reduced during the assay between T initial and T final (B). The Peroxidase activity of a sample was determined by the following equation:

Peroxidase Activity = [B × Sample Dilution Factor]/(Reaction time) × V

B, Amount (nmol) of H_2O_2 reduced between T initial and T final; Reaction Time, T final–T initial (min); V, Sample volume (ml) added to well.

Peroxidase activity reported as nmol/min/ml = milliunit/ml, where one unit of peroxidase is defined as the amount of enzyme that reduces 1.0 μmole of H_2O_2 per minute at 37°C. The mean values of three independent replicates of peroxidize activity were plotted as a graph with standard deviation as an error bars.

Targeted Metabolite Analysis by LC-MS

The *S. quadricauda* cells were collected by centrifugation at 8000 rpm for 10 min at room temperature. Metabolites were extracted from control or different time point samples from two independent biological replicate by homogenizing with liquid nitrogen in a prechilled sterile mortar and pestle. The samples then suspended with a mixture of 1 ml of methanol: water (80:20). Subsequently, the supernatant was collected by centrifugation at 8000 rpm for 10 minutes at 4°C and the extracted metabolites were stored at −20°C for LC-MS analysis. The mobile phase used for LC-MS is a mixture of triethylamine (A, 60%) and methanol (B, 40%) containing 0.1% formic acid adjusted to pH 4.2 and separated through a 1.9 μM C18 Shimadzu shim pack GISS column (Dimension 2.1 mm × 150 mm). The column temperature was maintained at 4°C and the temperature of the drying gas in the ionization source was 300°C. The gas flow was 10 l/min and the capillary voltage was 4 kV and the detection was using electrospray ionization (ESI)-MS. The LC-MS 8045 (Shimadzu, Japan) chromatogram was analyzed and the results were plotted by a heat map. The mean values of two experimental results were calculated and the data were used for the heat map generation (**Supplementary Table 1**). The heat map was generated using heat mapper (an online tool to interpret the metabolomic analysis) (Babicki et al., 2016).

Statistical Analysis

All the experiments were carried out in triplicate unless otherwise specified. The results are represented as mean value ± standard deviation with error bars in the figure. The data were analyzed by one-way ANOVA and the *P* value was calculated using Tukey HSD test.

RESULTS AND DISCUSSION

Nitrogen Stress-Induced Morphological Variation in a Population of *S. quadricauda*

Nitrogen being an integral part of biomolecules such as proteins of an organism and thus it's deficiency in the medium affects the enzymes required for cell division and eventually the growth of microalgae. As a primary analysis, microscopic images were observed. The nitrogen stress-induction leads to morphological changes and cell death (**Supplementary Figure 1a,b**). Morphological changes studied in a few cells under a microscope will not represent the phenomena at population level. Thus the variation in size of the cell due to nitrogen stress induction was studied in a population of *S. quadricauda* by flow cytometry analysis. The forward scatter analysis, the control cells were gated in such a way that large cells were presumed to represent 10% of the population and it compares to nitrogen stressed cells. The gated region represents the "region of hypertrophy" (**Figure 1A**). The population statistics of the cell enlargement showed that there is a 2.6% cell size enlargement in nitrogen stress-induced *S. quadricauda* (**Figure 1B**). The values were obtained from three independent replicates and standard deviation as an error bars.

According to Anand and Arumugam (2015), the cell size of *S. quadricauda* was enlarged in nitrogen starved condition. The accumulation of lipid droplets in *S. quadricauda* leads to variation in cell size. Similarly, the cell length was doubled in *Acutodesmus dimorphus* under nitrogen starved conditions (Chokshi et al., 2017). *Symbiodinium*, when cultured under nitrogen stress, the average cell size was observed as 7.35 and 6.96 μm at day 5 and 7 when compared to control (6.54 μm). Moreover, significant changes in the size and lipid droplets induced the morphological changes in *Scenedesmus obtusiusculus* and *Symbiodinium* during nitrogen starvation (Jiang et al., 2014).

Biochemical Changes During Nitrogen Starvation

Changes in Mitochondrial Membrane Potential During Nitrogen Starvation

Nitrogen stress induces perturbations in mitochondrial membrane potential ($\Delta\psi_m$), which is one of the signals to the stress through mitochondria. The increased mitochondrial membrane potential is directly proportional to the increased fluorescence of Rhodamine 123 (Rh123). The higher the $\Delta\psi_m$, the more Rhodamine 123 is taken up into the matrix. Also, the increased mitochondrial membrane potential leads to an elevated ROS generation. Here in *S. quadricauda* the rate of fluorescence of Rh 123 is increased during nitrogen stress. Eight thousand cells were taken to analyze the population and from that the fluorescence retained by the cells were represented in percentage. Thus is a 25% elevation in fluorescence of Rh 123 retained in the mitochondria of 24 h nitrogen stress-induced *S. quadricauda* when compared to control cells (**Figure 2**). Also, the 48 and 72 h samples revealed an increased membrane potential during the onset of nitrogen stress. Thus it implies that there is a fluctuation in mitochondrial membrane potential during onset of nitrogen stress. As the stress progresses, increased ROS generation observed in mitochondria and which eventually leads to metabolic rearrangements.

Mitochondrion plays a major role in cellular adaptation to abiotic stresses and is known to induce oxidative stress (Pastore et al., 2007). Mitochondrial membrane potential ($\Delta\psi_m$) is the driving force for ATP synthesis in mitochondria and it is generated by the proton-pumping electron transport chain. It has been reported that a correlation between membrane potential and ROS, as it generates more ROS at high membrane potential (Suski et al., 2012). Similarly, mitochondrial membrane potential and ROS generation were elevated in *S. quadricauda* during nitrogen starvation. Rhodamine 123 is a cationic, lipophilic fluorescent probe used to assay mitochondrial membrane potential in populations of apoptotic cells and it was measured according to the rate of fluorescent decay which is proportional to the mitochondrial membrane potential (Baracca et al., 2003).

Reactive Oxygen Species Generation During Nitrogen Stress-Mediated Lipid Accumulation

The mitochondria are the primary producers of ROS and also it depends on the metabolic state of mitochondrion during

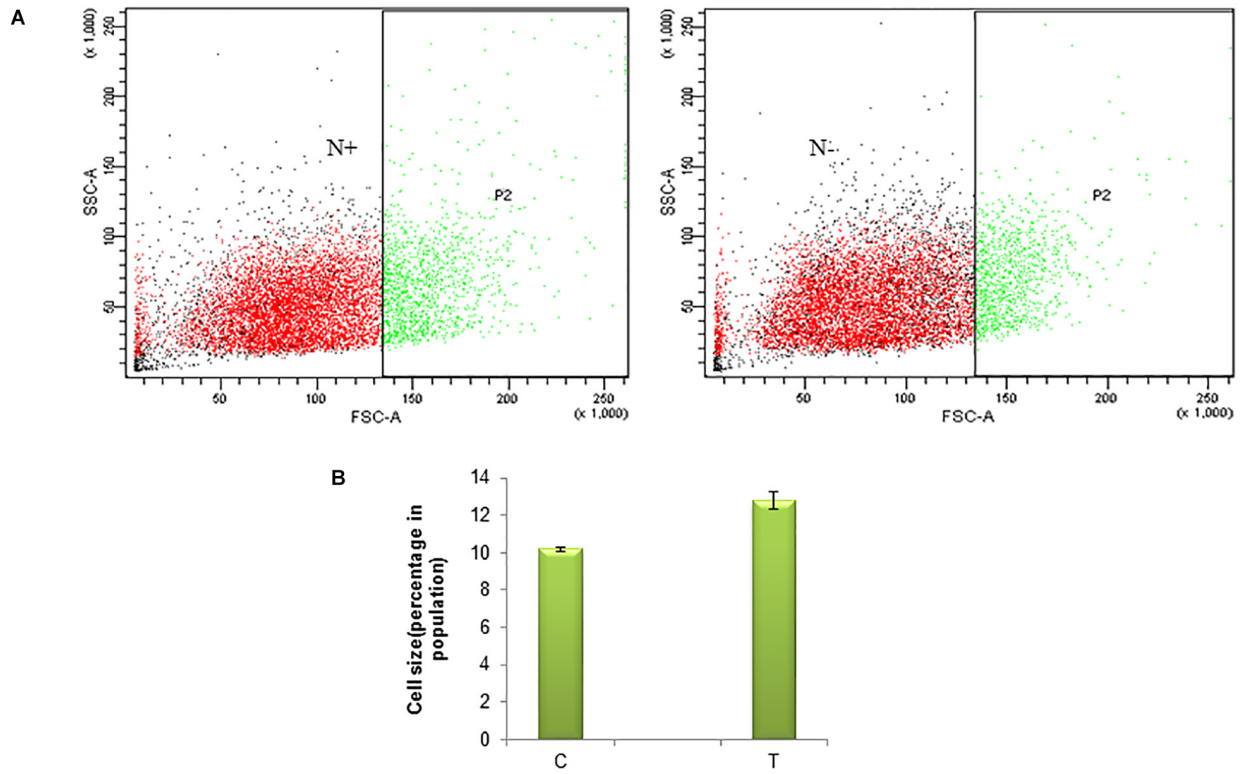


FIGURE 1 | Nitrogen stress induced morphological variation in a population of *S. quadricauda.* **(A)** Flow cytometry analysis of control (N^+) and nitrogen starved (N^-) *S. quadricauda* P2 represents the population and SSC-A, FSC-A represents the Side Scattered light-Area. Forward Scattered light-Area respectively. **(B)** Population statistics of enlarged cells during nitrogen starvation (T) and control *S. quadricauda.* The mean values of three independent triplicates ($n = 3$) were plotted with standard deviation as error bars.

nitrogen stress. The increased production of ROS is a sign of stress at a molecular level and the subsequent accumulation of oxidative damage. The H_2O_2 accumulation during nitrogen stress-induced *S. quadricauda* showed an elevated level at 24 and 48 h ($P < 0.0001$) of incubation around 7 and 11 μM respectively compared to control (0.17 μM) (**Figure 3A**). The $O_2\cdot^-$ radical in the nitrogen stressed *S. quadricauda* showed around 3.09 μM on 24 h of incubation and it was a lower concentration compared to nitrogen-rich *S. quadricauda* where the $O_2\cdot^-$ concentration was about 7.13 μM (**Figure 3B**). The $O_2\cdot^-$ concentration in the treated samples (N^-) found to be significant at 99% confidence level with respect to control. Also, the level of hydroxyl radical elevated during the initial hours of nitrogen stress induction (**Figure 3C**).

Lipid peroxidation is the oxidative degradation of lipids. The free radicals steal electrons from the membrane lipids and cause severe cell damage. Lipid peroxidation was determined in terms of MDA content in the cells. The MDA was elevated during the 72 h of incubation and it was around 1.13 μM. The MDA content was lower during the initial hours of stress induction (**Figure 4A**).

Even though ROS are highly reactive and potent toxic to the cells, they are having beneficial roles in abiotic stress. These include (i) diversion of electrons from the photosynthetic machinery in chloroplast to prevent the overload of the antenna and subsequent damage (Choudhury et al., 2017); (ii) regulation of metabolic fluxes during abiotic stress; and vital role as (iii) mediating signal transduction reactions which make the cells adapt to the stress by activating other pathways (Vaahtera et al., 2014; Considine et al., 2015; Mignolet-Spruyt et al., 2016; Mittler, 2017). Chokshi et al., 2017, reported that 3 days nitrogen starved *Acutodesmus dimorphus* showed 2-fold elevated levels of H_2O_2 than the control and simultaneously 4-fold reduction in $O_2\cdot^-$ in nitrogen starved cells. H_2O_2 and $O_2\cdot^-$ are showing the inverse relationship, as highly reactive $O_2\cdot^-$ is converted into H_2O_2 by the enzyme superoxide dismutase (SOD). According to them the OH- and MDA did not vary significantly in nitrogen stressed *A. dimorphus*. But in *Chlorella sorokiniana* C3 showed a significant increase in the MDA level during nitrogen starvation-induced oxidative stress (Zhang et al., 2013).

Antagonistic Antioxidant Enzymes During Nitrogen Stress

Catalase and Peroxidase Activity in *S.quadricauda* Under Nitrogen Stress

In order to clear-off the highly ROS, the free radical scavenging enzymes were also elevated during nitrogen stress induction. Catalase is the enzymes which speed up the

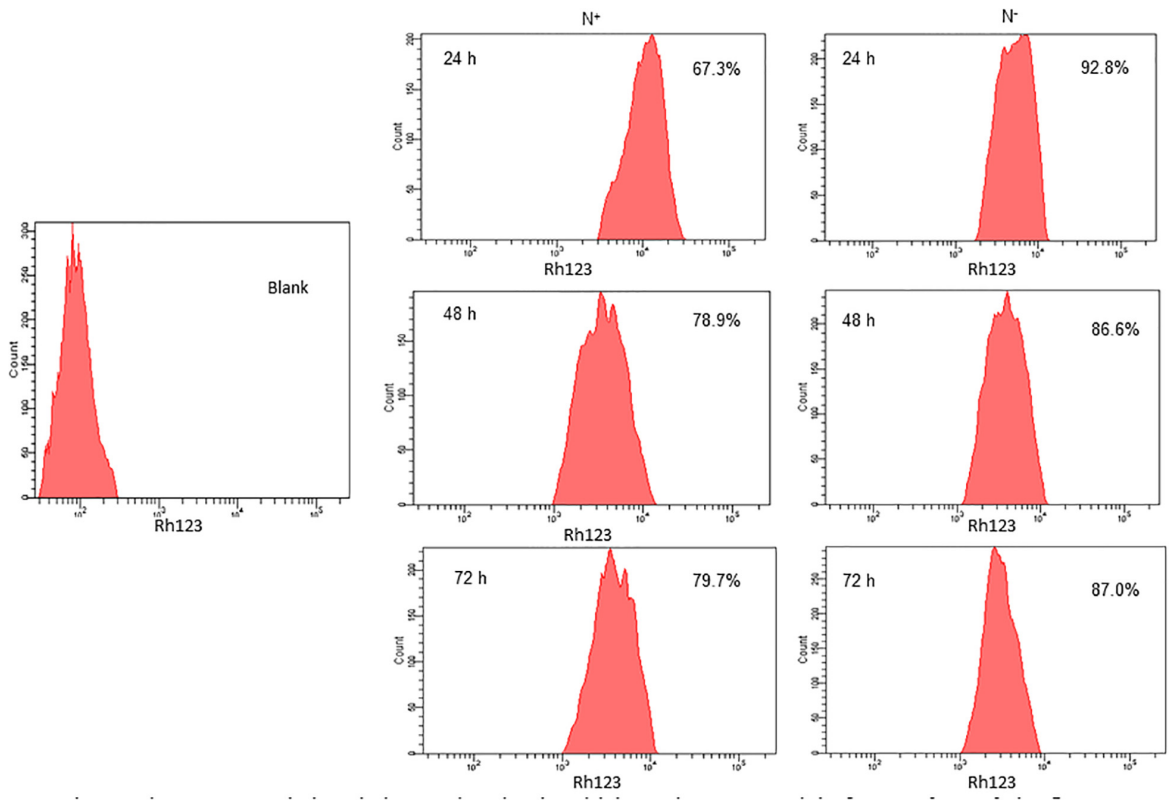


FIGURE 2 | Nitrogen stress induced changes in mitochondrial membrane potential of 5. *quadricauda* by flow cytometry. Mitochondria were stained by Rhodamine123. Blank represents the auto fluorescence of *S.quadricauda*; N^+-control. N^- – Nitrogen starved *S. quadricauda* at 24 h. 48 and 72 h. The experiments were carried out in duplicates ($n = 2$). The percentage values in each histogram represents the percentage cells retained the fluorescence of Rh 123 and the population of cells were fixed at 8000.

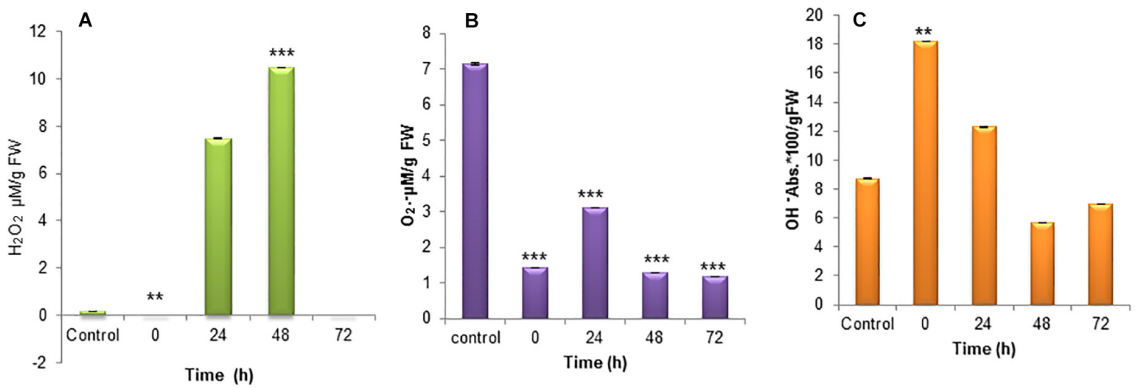


FIGURE 3 | Reactive Oxygen species generation in nitrogen stress induced (N^-) S. *quadricauda* and control (N^+). **(A)** H_2O_2, **(B)** $O_2\cdot^-$, and **(C)** OH^- generation during nitrogen stress induced at 0, 24, 48, and 72 h and the control *S. quadricauda.* The experiments were carried out in triplicate ($n = 3$) and the values were represented as a mean value with ± standard deviation as error bars. One-way ANOVA followed by Tukey HSD test for each treatment with respect to control. **lindicate significant differences compared to control ($P < 0.01$). ***Indicates highly significant differences compared to control ($P < 0.001$).

conversion of H_2O_2 to water and oxygen. During nitrogen stress, H_2O_2 generation was elevated at the same time the catalase activity was also found to be increased significantly ($P < 0.01$). The catalase activity was observed to be about 0.8 U/ml (**Figure 4B**).

The peroxidase is heme-containing proteins which catalyze the conversion of H_2O_2 into the water and an activated donor molecule. It utilizes H_2O_2 from various organic and inorganic substrates. Relatively, peroxidase enzyme in *S. quadricauda* was less active for H_2O_2 oxidoreduction. As it was evidenced

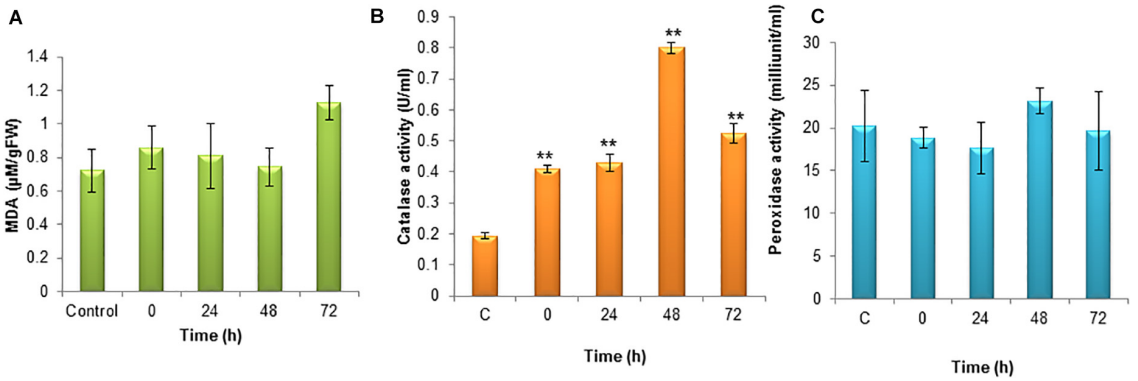


FIGURE 4 | (A) Level of lipid peroxidation (MDA) under nitrogen stress induction in *S. quadricauda*. (B) Catalase activity in nitrogen stress induced (N^-) *S. quadricauda* and control (N^+). (C) Peroxidase activity in nitrogen stress induced (N^-) *S. quadricauda* and control (N^+). The experiments were carried out in three independent triplicates ($n = 3$) and standard deviation were represented as error bars. One-way ANOVA followed by Tukey HSD test for each treatment with respect to control. **Indicate significant differences compared to control ($P < 0.01$).

from **Figure 4C**, there is a deviation in peroxidase activity at 48 h of nitrogen stress induction (23.16 mU/ml) compared to control (20.22 mU/ml).

The overproduction of toxic ROS was neutralized by the antioxidant scavenging enzymes such as SOD, catalase and ascorbate peroxidase during nutrient starvation (Ali et al., 2005; Bhaduri and Fulekar, 2012; Fan et al., 2014; Yilancioglu et al., 2014; Ruiz-Dominguez et al., 2015; Salbitani et al., 2015). Also, the Yilancioglu et al. (2014), in *D. salina* observed an elevated level of catalase and peroxidase activity under nitrogen-deficient conditions. Their experimental evidence suggested that the lipid accumulation might be partially induced by ROS mediated oxidative stress under nitrogen starvation. In order to prove that, they induced oxidative stress by H_2O_2 and the results showed that increased lipid accumulation during induced oxidative stress with full strength nitrogen source in *D. salina*. In addition to

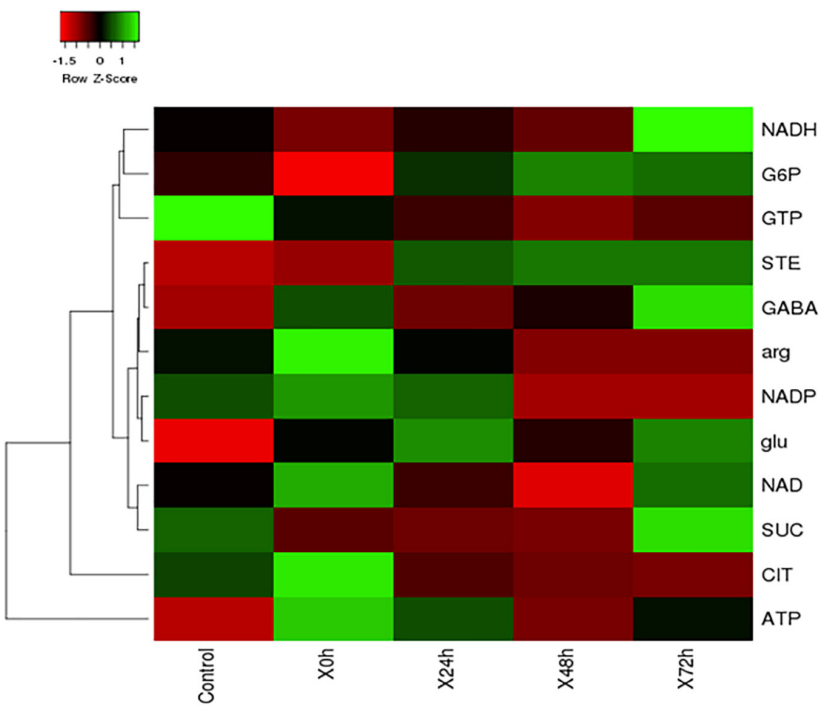


FIGURE 5 | The heat map of nitrogen stress induced *S. quadricauda* at 0, 24, 48, and 72 h and the control i.e., nitrogen sufficient *S. quadricauda* represented in X axis. The targeted metabolites were represented in Y axis and the map was generated by heat mapper. The mean values of experimental duplicates ($n = 2$) were used to generate metabolite expression heat map.

TABLE 1 | Role of different metabolites induced by nitrogen stress in eukaryotes.

Targeted metabolites	Role
GABA	Regulation of energy metabolism Bypasses two steps in TCA cycle
Glutamate	Precursor of chlorophyll
Arginine	Regulation of energy metabolism
Sucrose	Promotes cell expansion and storage
Citrate	Intermediate of TCA cycle
Succinate	Intermediate between the glyoxylate cycle and TCA cycle
GTP	Regulation of energy metabolism
ATP	Regulation of energy metabolism
Glucose-6-Phosphate	Intermediate of glycolysis
NAD	Regulation of energy metabolism
NADH	Regulation of energy metabolism
NADP	Regulation of energy metabolism
NADPH	Regulation of energy metabolism

that, they have claimed that oxidative stress itself can trigger lipid accumulation and suggested that the lipid accumulation was mediated by oxidative stress during nitrogen starvation.

Metabolic Changes During Nitrogen Starvation

Targeted Stress Metabolite Analysis by LC-MS

During nitrogen stress, several changes are happening in the cell and the cellular events triggered by the stress, finally leads to TAG accumulation as an energy reserve. Metabolomics is one of the omics studies which help to understand the metabolic rearrangement of the cell during nitrogen stress. In order to address the metabolic changes governed by nitrogen stress, several metabolites were listed and its role was discussed in **Table 1**. The metabolic changes are mainly associated with the liberation of low molecular weight biomolecules and their levels during abiotic stress condition (**Supplementary Table 1**). The integrated targeted metabolic analysis was characterized by LC-MS analysis. The heat map results showed stress-related non-proteinogenic amino acids and energy equivalents elevated during the initial hour of nitrogen starvation (**Figure 5**). The non-proteinogenic amino acids like Gamma Amino Butyric Acid (GABA), glutamate and arginine were observed in maximum peak area at 72, 24 and 0 h of nitrogen stress induction respectively. Also, the energy equivalents such as NADH and ATP are highly reactive during 72, 0 h of nitrogen stress induction (**Figure 5**).

The metabolic changes during nitrogen starvation showed low molecular weight secondary metabolite accumulation and metabolic rearrangement to cope up the stress (Salama et al., 2019). To adjust the metabolic changes, microalgal species modulates their metabolite synthesis (Paliwal et al., 2017). An elevated level of sugars (glucose, sucrose, and fructose) was observed in salinity and they have a role in osmotic homeostasis, carbon storage as well as scavenging of free radicals (Rosa et al., 2009). Several researchers proposed that the fatty acid synthesis was promoted by the hyperactivity of Tricarboxylic acid (TCA) cycle (Sweetlove et al., 2010; Hockin et al., 2012; Lee et al., 2012). According to Guerra et al., 2013, the hyperactivity of TCA cycle occurs because the lipid synthesis needs more ATP together with the reduction power of NADPH during nitrogen starvation. The lipid synthesis after nitrogen starvation creates a C/N imbalance and it can be adjusted by the protein degradation to take out the amino acids. The amino acids such as leucine, isoleucine, and valine take part in the synthesis of Acetyl CoA (Allen et al., 2011; Ge et al., 2014) which is the precursor of fatty acid synthesis. Also, the glutamate forms the precursor for chlorophyll synthesis. Gamma Amino Butyric Acid is a non- protein amino acid whose levels are found to be increased during the response to nitrogen stress (Xupeng et al., 2017). The present study also indicates that the energy equivalents and non-proteinogenic amino acid-like GABA was found elevated during nitrogen starvation in *S. quadricauda*. During abiotic stress metabolites of glycolysis and TCA cycle along with these amino acids showed an initial increase in levels followed by a decrease (Zhang et al., 2016).

CONCLUSION

The nitrogen stress leads to oxidative stress-induced ROS generation at high membrane potential ($\Delta\psi_m$). The predominant ROS generated were H_2O_2, OH^-, $O_2\cdot^-$ and in order to suppress the ROS, antioxidant scavenging enzymes like peroxidase and catalase were elevated. Also, it showed an inverse correlation between $O_2\cdot^-$ and H_2O_2, also the OH^- and lipid peroxidation in terms of Malondialdehyde. The Metabolic changes are mainly associated with the liberation of low molecular weight biomolecules and their levels during abiotic stress condition. The integrated metabolic analysis revealed that stress-related non-proteinogenic amino acids and energy equivalents are elevated during nitrogen starvation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MA conceptualized the study, contributed to funding acquisition, project administration, resources, supervision, writing – review and editing, conducted the experiments and analyzed the primary data, and corrected and communicated the manuscript. SS prepared the manuscript draft. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Evaluation of Microalgae as Immunostimulants and Recombinant Vaccines for Diseases Prevention and Control in Aquaculture

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Microalgae are often used as nutritional supplements for aquatic animals and are widely used in the aquaculture industry, providing direct or indirect nutrients for many aquatic animals. Microalgae are abundant in nature, of high nutritional value, and some of them are non-toxic and rich in antioxidants so that they can be explored as a medicinal carrier for human or animals. Natural wild-type microalgae can be adopted as an immunostimulant to enhance non-specific immune response and improve growth performance, among which *Haematococcus pluvialis*, *Arthrospira* (*Spirulina*) *platensis*, and *Chlorella* spp. are commonly used. At present, there have been some successful cases of using microalgae to develop oral vaccines in the aquaculture industry. Researchers usually develop recombinant vaccines based on *Chlamydomonas reinhardtii*, *Dunaliella salina*, and cyanobacteria. Among them, in the genetic modification of eukaryotic microalgae, many examples are expressing antigen genes in chloroplasts. They are all used for the prevention and control of single infectious diseases and most of them are resistant to shrimp virus infection. However, there is still no effective strategy targeting polymicrobial infections and few commercial vaccines are available. Although several species of microalgae are widely developed in the aquaculture industry, many of them have not yet established an effective and mature genetic manipulation system. This article systematically analyzes and discusses the above problems to provide ideas for the future development of highly effective microalgae recombinant oral vaccines.

Keywords: aquaculture industry, microalgae, immunostimulant, oral vaccine, polymicrobial infections

INTRODUCTION

The aquaculture industry has developed rapidly in recent years and provided more fish products for human food supply than that did capture fisheries for the first time in 2014 (Pauly and Zeller, 2017). According to the model proposed by Kobayashi et al. (2015), in face of the rapid expansion of global fish demand and the relatively stable capture fisheries, aquaculture is expected to fill the widening

gap between food supply and demand, especially in Asia. By 2030, aquaculture will provide about 62% of fish for human consumption. Moreover, after 2030, aquaculture is likely to continue to dominate the future global fish supply and grow sustainably.

However, the outbreak of diseases will bring severe losses to the aquaculture industry economically. Severe diseases in aquaculture are mostly caused by viruses and various protists, as well as bacteria (Lafferty et al., 2015). Although some bacterial diseases can be treated by several vaccines and antibiotics effectively, others cannot be solved by antibiotics. With the abuse of antibiotics, the amount of antibiotic-resistant bacteria and the risk of remaining antibiotics and antibiotic-resistant bacteria in aquatic products transferring to the human body have been increasing (Costa et al., 2015), arising great attention globally, which leads to the development of new antibiotic alternatives to control the diseases. Recently, the developed vaccines using microalgae as carriers have been increasingly applied to the aquaculture industry, which provides new ideas for diseases prevention and control in aquaculture.

As an essential part of aquatic ecosystems like oceans and lakes, microalgae is of great significance to the aquatic environment, the health of aquatic animals, and the balance of the ecosystem (**Figure 1**). The cells of microalgae are rich in active nutrients such as proteins, polyunsaturated fatty acids (PUFAs), polysaccharides, and essential amino acids, which can promote the growth of fish, shrimp, crab, and shellfish. Therefore, they can be used as a basic feed for fish and other economic aquatic animals directly or indirectly (Yaakob et al., 2014). In addition, microalgae also play an important role in regulating and judging the quality of aquaculture water. For example, algae cells can absorb nutrients such as nitrogen and phosphorus to improve the water quality and maintain a good dynamic balance, thereby enhancing the disease resistance of aquatic animals and improving the survival rate (Taelman et al., 2013). Due to the increasing importance of microalgae in aquaculture, this article reviews the aspects of microalgae in the prevention and control of diseases in aquaculture. Before conceiving and writing this review, we had searched the literature thoroughly, whatever in the past few years or decades. There are roughly hundreds of papers in the field of algae application in aquaculture. However, there is few of systematic summary of the existing research conclusions, especially few about evaluation of microalgae as immunostimulants and recombinant vaccines for disease prevention and control in aquaculture. Charoonnart et al. (2018) reviewed the genetic engineering of microalgae to produce therapeutic proteins and biomolecules against aquaculture diseases, in which some research cases were listed and summarized. Abidin et al. (2020) focused on the potential and application of genetically modified microalgae in aquaculture, and evaluated the feasibility of microalgae as a vaccine carrier from a technical perspective. This article systematically analyzes and discusses the above problems, including some examples of commercial application, in order to provide ideas for the future development of highly effective microalgae recombinant oral vaccines.

THE IMPORTANCE OF MICROALGAE AS FEED FOR AQUACULTURE INDUSTRY

For a long time, microalgae have provided direct or indirect nutrition to the early stages of growth of many aquaculture fish, shellfish, and invertebrates (Priyadarshani and Rath, 2012). In the larval stages of mollusks, echinoderms, crustaceans, and some fish, the feeding method is usually filter feeding, during which microalgae are the source of nutrients (Hemaiswarya et al., 2010; Kaparapu, 2018). Tredici et al. (2009) have reviewed the cultivation patterns of microalgae used for feeding. They introduced the development of microalgae biotechnology, especially the new culture techniques, and focused on the practical and potential applications of algae in the nutrition of aquatic animals, which can take a dominant position in this ecosystem. Shields and Lupatsch (2012) have summarized the current state of algae use in aquaculture and developments in algal biomass as an ingredient in formulated animal feeds. Microalgae provide an important direct or indirect feed source for the early developmental stages of many aquatic species, and traditionally, in hatcheries for aquatic animals, microalgae farming is large-scale, generally in outdoor ponds or large pools. However, in intensive aquaculture hatcheries, microalgae breeding is usually carried out in specialized bioreactors, managing different algae species regularly through artificial or automated means.

Microalgae are generally single cells of size from several to dozens of micrometers. The individual size and structural function of microalgae as well as the feeding structure and digestive function of aquatic animals exist differences. Thus different aquatic animals require nutrients from respective types of feeding microalgae at different growth stages. For example, microalgae rich in eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (*Chaetoceros calcitrans*, *Isochrysis galbana*, etc.) are usually provided for marine mollusks in the early development stage (Ran et al., 2020). Microalgae that are inseparable from the whole-life growth and development of shellfish, such as *Tetraselmis* spp., *Thalassiosira pseudodonana*, *and C. calcitrans*, are usually supplied for crustaceans. *Nannochloropsis* spp. are mainly used in the culture of rotifers. *Dunaliella salina* is often used for pigmentation of aquatic animals (Borowitzka, 1997). As the number and species of aquaculture animals increase, so does the demand for suitable microalgae in the aquaculture industry. At the growth and metamorphic development stage of fish, shrimp, shellfish, and crab larvae, the mixed feeding of microalgae and animal-based natural bait would lead to better results (Shields and Lupatsch, 2012).

At present, there are dozens of kinds of bait microalgae that can be popularly applied in large-scale seedling production all over the world. Microalgae are the major source of food for zooplankton and small-size fish, and subsequently a valuable

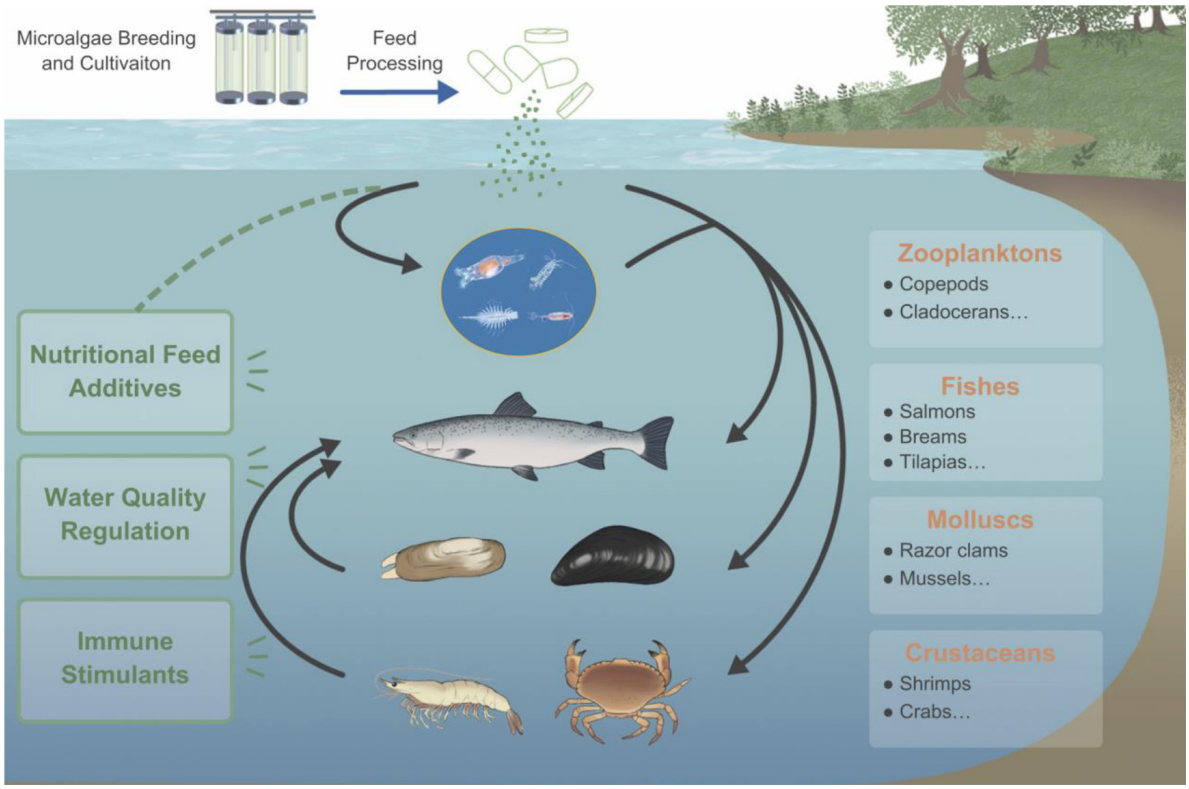

FIGURE 1 | The importance of microalgae in the aquaculture industry. Microalgae can provide direct or indirect nutrition for aquatic animals and can be immune stimulants for diseases control, also play an important role in regulation of water quality.

source of vital nutrients as fodder for fish in the upper echelons of the food chain, microalgae-based feeds offer promising food sources for sustainable aquaculture industry (Yarnold et al., 2019). Shah et al. (2017) reviewed the latest progress of microalgae as a supplement or feed additive to replace fishmeal and fish oil in aquaculture. According to the nutrient requirements of fish, good selection of microalgae species can improve its conversion rate in fish body, thus studying the nutrient composition of different microalgae is necessary to support aquaculture. In addition, the safety and regulatory issues of microalgae feed applications also need to be considered. Also, the cost of high-quality algal biomass is considerably higher than fishmeal or grain-based feed components, possibly limiting its large-scale application. It is worth noting that the algal biomass applied to the aquaculture feed industry is mainly in the form of pasting, thus the cost could be greatly reduced in this form (Raja et al., 2018). Of course, the algae species that are being developed and commonly used are usually non-toxic, harmless, nutritious, and easy to grow on high density at large scale.

Although the value of bait microalgae is increasingly recognized, in order to meet the development needs of aquatic animal larvae, further screening and directional cultivation of microalgae is indispensable. Moreover, it is urgent to establish the nutritional value evaluation system of microalgae bait and study new breeding methods and technologies to improve the nutritional value of bait microalgae. In conclusion, microalgae are absolute highly-demanded products in the field of aquaculture, with unique advantages and broad application prospects.

MICROALGAE CAN BE EXCELLENT IMMUNOSTIMULANTS OR ANTIOXIDANTS

Natural microalgae are rich in natural products, pigments, proteins, vitamins, PUFAs, and polysaccharide derivatives, and are the natural feed of aquatic animals, so it has inherent advantages to use them to develop microalgae additives. Bioactive substances from microalgae have natural antibacterial activity, which could eventually kill or inhibit the growth of pathogenic bacteria. Some polysaccharides were reported to increase the phagocytic capacity of macrophages and the gene expression level of pro-inflammatory cytokines, thereby activating natural immune response (Mohan et al., 2019). In addition, some PUFAs have unique regulatory effects on growth performance, membrane permeability, enzyme activity, immune function, etc. Yaakob et al. (2014) reviewed the contribution of microalgae in the nutritional requirements of aquatic feed. The nutrients contained in different microalgae are also different, and these main biomass ingredients in aquatic animals can play a role in enhancing the immune system and improving the function of

anti-infection. From the perspective of the application status of microalgae feed in fish nutrition, microalgae also play a positive role in improving the growth performance, disease resistance, and skin color of edible fish (Roy and Pal, 2014; Molino et al., 2018). Because the nutrient characteristics of different algae are different, they can be used differently in aquaculture, so it is necessary to study the economic benefits of microalgae as feed additives to different aquaculture animals.

Haematococcus pluvialis

There are lots of studies that used pigment-rich algae species as feeding additives. Sheikhzadeh et al. (2012) found that astaxanthin-rich *Haematococcus pluvialis* was added to fish feed in different proportions. Especially when the ratio is 0.3%, it promotes the physiological and metabolic functions of rainbow trout (*Oncorhynchus mykiss*), and improves the antioxidant activity of fish tissue effectively. Li et al. (2014) compared the effects of adding astaxanthin and *H. pluvialis* in feed on growth performance, antioxidant activity, and immune response of large yellow croakers (*Pseudosciaena crocea*). They found that dietary supplementation of both astaxanthin and *H. pluvialis* can improve the growth performance of large yellow croakers, whereas the latter is more effective. Furthermore, when the additive proportion of *H. pluvialis* is 0.28–0.56%, it can significantly improve the blood indices of large yellow croakers, and improve antioxidant and immune capacity. Astaxanthin can enhance the salinity stress tolerance, and salinity stress test is a widely used criterion to predict the health status of shrimp. Xie et al. (2018) found that dietary supplementation of 0.33–0.67% *H. pluvialis* improved the survival rate of white shrimp *Litopenaeus vannamei* under the salinity stress. One of the lipid peroxidation products malondialdehyde (MDA) and mRNA expression of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) decreased in white shrimp larval livers after salinity stress, whereas total antioxidant capacity (T-AOC) increased. In addition, they demonstrated that the addition of *H. pluvialis* is mainly enhanced by the regulation of the NF-κB pathway, which indicated that the astaxanthin might improve the anti-inflammation and immune property. Thus, the intake of these substances or algae powder can improve the antioxidant performance as well as has a beneficial effect on innate immunity. Notably, there is a significant economic barrier in natural algal powder application, as that synthetic astaxanthin produced from petrochemicals is considerably cheaper than natural sources from *H. pluvialis*, and therefore the synthetic form dominates in the aquaculture industry (Shah et al., 2016).

Arthrospira platensis (*Spirulina*)

Arthrospira platensis (also known as *Spirulina*) is a kind of cyanobacteria that has become increasingly popular worldwide as a dietary supplement, one of the most nutritious foods known to human, and is often used as a feed additive in aquaculture. Mahmoud et al. (2018) studied the effect of dietary adding *A. platensis* on nile tilapia (*Oreochromis niloticus*) growth performance, immune response, and the oxidation resistance. They found that dietary supplementation of *A. platensis* has no effect on the growth performance of tilapia, but significantly improves the antioxidant capacity and ability to resist the infection of *Pseudomonas fluorescens*, especially when adding proportion is 1%. Radhakrishnan et al. (2014) used *A. platensis* and Chlorella *vulgaris* instead of fishmeal to feed *Macrobrachium rosenbergii*. In all feeding groups, 50% of the fishmeal replacement group showed better non-enzymatic antioxidant activity, while no significant increases were shown in enzymatic antioxidant performance. Macias-Sancho et al. (2014) added different proportions of *A. platensis* to the fishmeal of the white shrimp *L. vannamei*. With the increasing proportion, the number of granular hemocytes increased significantly and the reduction of hemolymph cellular apoptosis prevented the early stage of virus infection. Yeganeh et al. (2015) evaluated the effect of dietary addition of *A. platensis* on the immune function of rainbow trout (*O. mykiss*). They found the immune response increases when supplementation ratio at 7.5 and 10%, and hematology and serum biochemistry related indicators of rainbow trout improved. Adel et al. (2016) have explored the effects of adding *A. platensis* diet on humoral immunity and mucosal immune response, and disease resistance of great sturgeon (*Huso huso*). They found that adding 5 or 10% of *A. platensis* significantly increased serum IgM, and the activity of lysozyme and the total protein content on the mucosa increased, thereby improving the ability of the great sturgeon to resist various pathogens. Chen et al. (2016) investigated the effects of *A. platensis* dried powder on the immune response of white shrimp *L. vannamei*. *A. platensis* can induce degranulation of shrimp hemocytes and increase oxidative stress response *in vitro* experiments. The 3 and 6% *A. platensis* diet feeds significantly stimulated shrimp innate immunity by increasing the expression of pattern recognition proteins (PRPs) like LGBP, as well as by increasing lysozyme activity, phagocytic activity, and resistance against *Vibrio alginolyticus*. Yu et al. (2018) found that feeding coral trout (*Plectropomus leopardus*) diets containing *A. platensis* (especially 10%) significantly improved their growth performance and the antioxidant status of livers, and enhanced the immune ability and resistance against *Vibrio harveyi*. Promya and Chitmanat (2011) found that adding 5% *A. platensis* or *Cladophora* spp. to the diet increased the number of red and white blood cells of African sharptooth catfish, and improved lysozyme activity in sera. Raji et al. (2018) explored fishmeal replacement with *A. platensis* and *C. vulgaris* in African catfish *Clarias gariepinus*, which have significantly improved the activity of CAT and the number of white blood cells. This indicates that the supplementation of these microalgae can stimulate immune response and improve the ability to fight infection.

Chlorella spp.

From the view of nutrition, *C. vulgaris* is rich in nutrients, containing 61.6% proteins, 12.5% fat, 13.7% carbohydrates, trace elements, various vitamins, and minerals, and is generally used as growth promoter and immunopotentiator (Ahmad et al., 2018). Khani et al. (2017) have studied the effect of *C. vulgaris* on immunological parameters of koi carp *Cyprinus carpio*. They found that the addition of 5% dry *C. vulgaris* powder to koi carp diet can make fish with the highest level of IgM and lysozyme activity, resulting in resistance to both unsuitable

environmental conditions and outbreaks of infectious diseases. Xu et al. (2014) indicated that when feeding gibel carp *Carassius auratus gibelio* with *C. vulgaris* as the dietary additive, with the increase of dietary proportion (0.4–2.0%), the relevant immunological parameters (SOD, POD, LZM, etc.) of gibel carp also showed an increasing trend. Zhang et al. (2014) also studied the effect of feeding *C. vulgaris* on the immune status of gibel carp (*C. a. gibelio*) and analyzed the relevant immunological parameters, reporting that *C. vulgaris* could increase the level of IgM and IgD, interleukin-22, and chemokine(C-C motif) ligand 5 in some tissues. Zahran and Risha (2014) and Zahran et al. (2018) found that dietary supplementing *C. vulgaris* at 10% could protect nile tilapia *O. niloticus* against arsenic-induced immunotoxicity and oxidative stress. Maliwat et al. (2017) added *C. vulgaris* to the giant freshwater prawn *M. rosenbergii*, which improved the prophenol oxidase activity and the total amount of hematocytes of *M. rosenbergii* postlarvae and could enhance the larval survival to *Aeromonas hydrophila* infection, especially at the supplementation ratio of 6%. Galal et al. (2018) found that *C. vulgaris* dietary supplementation could protect nile tilapia *O. niloticus* from being exposed to sub-lethal concentrations of penoxsulam herbicide and improve its anti-infective capacity against *Aeromonus sobria*.

Other Algae Species

Research by Das et al. (2013) has shown that long-term dietary supplementation of dry *Microcystis aeruginosa* can significantly improve the immunity and the survival rate of Indian major carp *Labeo rohita*. However, *M. aeruginosa* can secrete some toxins that threaten health. They found that when feeding *L. rohita* in a ratio of 0.1%, it could significantly stimulate the immune system, improving the defense against *A. hydrophila*. Lyons et al. (2017) found that controlling the intestinal microbial community by dietary supplementation of 5% *Schizochytrium limacinum* might be a promising method to improve the intestinal health and nutrient utilization of rainbow trout *O. mykiss*. Nevertheless, it should be highlighted that the heterotrophic protist *S. limacinum* is technically not an algae, although it is often (incorrectly) named as such in publications and marketing (Leyland et al., 2017). What is noteworthy is that *S. limacinum* is rich in PUFAs and can be applied to the aquaculture industry. Salvesen et al. (1999) fed juvenile turbot *Scophthalmus maximus* L. with microalgae *I. galbana* matured water and found that such green water could improve the survival rate of juvenile turbot as well as accelerate its growth performance and ability to inhibit the proliferation of bacteria. Molina-Cárdenas and Sánchez-Saavedra (2017) found that six benthic diatom species had inhibitory effects on the growth of three common pathogens (*V. alginolyticus*, *Vibrio campbellii*, and *V. harveyi*), which could infect mollusks, shellfish, and fish. Moreover, many reports about the antibacterial properties of microalgae have pointed out that some metabolites secreted by microalgae can prevent pathogenic microorganisms from infecting their host. For example, marine algal polysaccharides play an antiviral role by inhibiting the adhesion of viruses (Ahmadi et al., 2015). And dietary supplementation of marine-derived polysaccharides can improve the growth, immune response, and disease resistance of aquatic animals (Mohan et al., 2019).

The researches mentioned above have shown that these microalgae can successfully enhance the innate immune function of the host, improve the antioxidant capacity, or reduce the infection of the pathogens to a certain extent. Therefore, some wild-type microalgae can act as effective immunostimulants. In addition, some studies have shown that microalgae can be used as a platform for protein production and drug delivery through genetic engineering. Although microalgae and its extracts have significant ability to prevent and control aquatic animal bacterial diseases, the antibacterial mechanism is still unclear, which should be strengthened in the future.

GENETIC MODIFIED MICROALGAE HAS GREAT POTENTIAL IN DISEASE CONTROL

The Potential for Microalgae as Bioreactors to Produce Vaccines or Other Products

The development of microalgae cells as vaccine has long attracted the attention of scientists. In human diseases, the first reported antigen expressed using microalgae as a carrier was the capsid protein VP1 of foot-and-mouth disease virus (FMDV). Researchers assembled the gene of the cholera toxin B subunit (CTB) and the gene encoding VP1 into a chloroplast expression vector of *Chlamydomonas reinhardtii*, and finally integrated the chloroplast genome for expression. The expression level of the fusion protein can reach 3% of the total soluble protein (TSP) after detection (Sun et al., 2003). So far, researchers have used microalgae as a host to express relevant antigens of human diseases into preclinical trials (Rosales-Mendoza, 2016b). For example, in the field of parasitic and infectious diseases, multiple antigens such as Pfs25/28, D2-CTB, and Pfs25/28, D2-CTB, have been expressed in microalgal cells, respectively, against *Plasmodium falciparum*, *Staphylococcus aureus*, human papilloma virus, hepatitis B virus, and HIV (Specht and Mayfield, 2014; Rasala and Mayfield, 2015; Yan et al., 2016). In the field of non-infective diseases, genetically engineered microalgae have been developed for diseases such as type I diabetes, atherosclerosis, hypertension, allergies, and tumors (Rasala and Mayfield, 2015; Rosales-Mendoza, 2016b). There are also many successful reports on the use of microalgal cells to develop animal pathogenic vaccines. A number of microalgal recombinant subunit vaccines have been developed against classical swine fever virus, FMDV, etc. (Specht and Mayfield, 2014; Rasala and Mayfield, 2015; Rosales-Mendoza, 2016a; Yan et al., 2016). Throughout these studies, *C. reinhardtii* was used as a mature host system to express antigenic proteins, and the expression of foreign proteins is mainly between 0.1 and 5% TSP (Specht and Mayfield, 2014). Although *C. reinhardtii* is a model organism, due to random integration event, nuclear transformation is often accompanied by transgenic silencing, while transgenes inserted into the

TABLE 1 | Research on developing genetically modified microalgae vaccine in aquaculture.

Number	Pathogen	Antibacterial peptides (AP) Antigen (Ag)	Expression host	Vector	Promoter	Position	Expression (yield)	Protection Object	Effect	References
1	*R. salmoninarum*	Ag:p57	*C. reinhardtii* [CC744]	/	/	Chloroplast and plasma membrane	/	*Oncorhynchus mykiss*	Produced p57-specific immunoglobulins (IgM) in different tissues	Siripornadulsil et al., 2007
2	*A. salmonicida*	Ag:AcrV, VapA	*C. reinhardtii* [WT, FUD50, FUD7]	pGA4	psaA, atpA psbD, psbA	Chloroplast	AcrV: 0.8% TP VapA: 0.3% TP	/	/	Michelet et al., 2011
3	WSSV	Ag:VP28	*C. reinhardtii* [CC741 mt+, Fud7 mt−]	pBA155 pSR229	psbA, atpA, psbD	Chloroplast	0.1–10.5% TSP	Shrimp	/	Surzycki et al., 2009
4	WSSV	Ag:VP28	*D. salina* [UTEX-1644]	pUX-GUS	Ubil	Chloroplast	78 mg/100 mL culture	Shrimp	59% Protection rate	Feng et al., 2014
5	WSSV	Ag:VP28	*Anabaena* sp. [PCC 7120]	pRL-489	psbA	Cytoplasm	34.5 mg/L culture Expression efficiency: 1.03% (dry weight)	Shrimp	68% Protection rate	Jia et al., 2016
6	WSSV	Ag:VP28	*Synechocystis* sp. [PCC 6803]	pRL-489	psbA	Cytoplasm	/	Shrimp	88.42% Relative survival	Zhai et al., 2019
7	WSSV	Ag:VP28	*C. reinhardtii* [TN72]	pASapI	atpA	Chloroplast	detectable	Shrimp	87% Relative survival	Kiataramgul et al., 2020
8	WSSV	Ag:VP19, VP28	*Synechococcus* sp. [PCC 7942]	pRL-489	psbA	Cytoplasm	vp19, vp28, vp (19 + 28) 5.0, 4.7, and 4.2%, (dry weight)	Shrimp	Activity of PO, SOD, CAT, and LYZ changed	Zhu et al., 2020
9	YHV	dsRNA-YHV(RNA)	*C. reinhardtii* [CC503 cw92mt+]	pSL18	psbD	Nucleus	41 ng/100 mL(1 × 10^8 cells)	Shrimp	Increasing 22% protection	Somchai et al., 2016
10	YHV	dsRNA-YHV(RNA)	*C. reinhardtii* [*CC-5168*]	pSRSapI	psaA	Chloroplast	16.0 ± 0.9 ng dsRNA/L late-log phase culture	*Penaeus vannamei* Shrimp	50% survival at 8 day-post infection	Charoonnart et al., 2019

chloroplast genome by homologous recombination are not silenced and offer a platform for the production of recombinant proteins. Hence, the chloroplast homologous recombination is the main expression method, and nuclear transformation is randomly integrated as the auxiliary. Researchers also explored *Chlorella ellipsoidea* as an expression host to heterologously express and purify the rabbit defensin (NP-1). They obtained a small molecule defensin peptide NP-1 with high bacteriostatic activity, which also made it possible to produce such antibacterial peptides on a large scale with algae (Bai et al., 2013). At present, the application of microalgae and microalgae products in the pharmaceutical industry is attracting more and more attention. As the biotechnology for algae active substance research gradually matures, the use of microalgae for research and development of new drugs or vaccines has great potential.

Prevention of Aquaculture Diseases by Microalgae Vaccine

It is worth noting that the microalgae oral vaccines have been designed and developed for specific pathogens in the field of aquaculture, and their immunoprotection has been explored. In order to prove the feasibility of the microalgae can be utilized as a oral delivery vector, Kwon et al. (2019) fed zebrafish (Danio rerio) on a diet of green fluorescent protein (GFP)-expressed *C. reinhardtii*, clear fluorescent signals in the intestinal tract can be detected by laser confocal image and immunostain, and GPF can also be detected in zebrafish serum, which indicates that the orally delivered proteins were protected until they were released in the gut. The research demonstrated the ability of *C. reinhardtii* as an oral delivery platform for recombinant bioactive proteins. The researchers Siripornadulsil et al. (2007) expressed the antigen protein p57 of *Renibacterium salmoninarum* in *C. reinhardtii*, and studied the effects of *in vivo* algae cell soaking and algae meal addition on the immune response induced by iris fish larvae. Their results showed that antibody production can be detected by soaking for 2 h or by adding 4% of the microalgae feed. Moreover, it was confirmed that the transgenic microalgae oral administration of this antigen presentation method could completely induce antibody production in blood, skin, epithelial tissue, and mucosa. Michelet et al. (2011) successfully genetically modified *C. reinhardtii* and efficiently expressed the two antigenic proteins AcrV and VapA of *Aeromonas salmonicida* in chloroplasts with different promoters and different expression methods. Feng et al. have successfully achieved the heterologous expression of VP28 protein with green alga *D. salina* and cyanobacteria *Anabaena* sp., and have explored the ability of the transgenic microalgae vaccine against white spot syndrome virus (WSSV). The results showed that genetically modified microalgae can effectively improve disease resistance and delay the death of shrimp (Feng et al., 2014; Jia et al., 2016). Subsequently, Zhai et al. (2019) highly expressed the VP28 protein in *Synechococcus* sp., and the expression efficiency was three times higher than that in *Anabaena*. In their recent research, *Synechocystis* PCC6803 was successfully carried out heterologous expression of VP28 protein. Oral transgenic *Synechocystis* PCC6803 can increase the enzyme activities in immune system and enhance the defense ability of

TABLE 2 | Recent patents related to recombinant microalgae vaccine in aquaculture.

Number	Patent Number	Pathogen	Antibacterial peptides (AP) Antigen (Ag)	Expression host	Vector	Promoter	Position	Effect	References
1	US20160346373-A1	*Beta-nodavirus*	Nervous Necrosis Virus (NNV) Capsid protein or fragment thereof	*P. tricornutum*	pPhaT1	fcpA, fcp B	Vacuole	Improving the effectiveness of oral vaccines	Chen, 2016
2	WO2008027235-A1	WSSV	/	*D. salina*	pRrMDWK	Constitutive Inducible	Cytoplasm	Improving survival after virus attack	Durvasula and Durvasula, 2008
3	US20110147708-A1	/	LFB VP28	*N. oculata*	pCB740 pGEM-T	RBCS2, HSP70A	Nucleus Cytoplasm	Improving antibacterial and antiviral activity	Tsai and Li, 2011
4	US20172029400-A1	*R. salmoninarum*	P57 protein	*C. reinhardtii*	pUC18 pSSRC7	psbA β2-tubulin	Nucleus Chloroplast	Detecting antibodies *in vivo*	Sayre et al., 2017
5	US20141701810-A1	WSSV	VP28	*Chlorella* sp.	pGA4	/	Cytoplasm	100% protection rate	Moi et al., 2014

WSSV infection in juvenile prawn. And these VP28 transgenic algae cells can be directly fed as juvenile shrimp bait without extraction and purification, which are expected to be applied to the aquaculture industry on a large scale. Somchai et al. (2016) constructed a shrimp yellow head virus (YHV) RNAi vector in the *C. reinhardtii* system, which improved survival by 22% after oral administration to prawn larvae. In the same way, Charoonnart et al. (2019) engineered the chloroplast genome of *C. reinhardtii* to express double-stranded RNA (dsRNA) designed to knock down key viral genes, Shrimps fed with dsRNA-expressed algal cells prior to YHV infection had 50% survival at 8 day-post infection, whereas only 15.9% survival rate was observed in control groups, and RT-PCR results revealed a lower infection rate in dsRNA-expressing algae treated shrimp compared to control groups. In all cases, aquatic animals were direct protected by feeding genetic modified microalgae. The above research and explorations give the direction for the development and large-scale application of genetically modified microalgae recombinant vaccines in the aquaculture field (**Table 1**).

In addition, many international patents about recombinant microalgae vaccines for the prevention and control of aquatic diseases have been published or authorized (**Table 2**). This indicates that the theoretical research of microalgae vaccines is also extending to practical commercial applications. *Betanodavirusare* causes severe diseases, such as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER), which is detrimental to the growth and reproduction of marine fish, especially their larvae. In a patent filed by TransAlgae lnc. in Israel (Chen, 2016), the researchers selected Nervous Necrosis Viruses (NNV) capsid protein or fragment as an antigen to be expressed in microalgae subcellular compartment. The recombinant protein was delivered into the mucosal immune system of white grouper *Eyineyhelus aeneus* or the European sea bass *Dicentrarchus labrax* juvenile fish. The experimental results showed that the exogenous antigens could be presented successfully and stimulate the specific immune response in the fish body, so that transgenic microalgae could improve the survival rate of juvenile fish. At present, the company has been developing pipeline products for a fish disease and two major shrimp's diseases in the aquaculture industry. In a patent filed by Durvasula and Durvasula (2008), the researchers introduced a DNA fragment encoding the antigen into a variety of microalgae, and the exogenous DNA fragment encoding product containing one or more key epitopes of the pathogen, such as WSSV, *V. harveyi*, etc. In one embodiment, the transgenic *D. salina* was initially fed to Artemia, and then the latter was used to feed shrimp larvae. In this method, the antigen molecule was presented indirectly to shrimp and successfully induced antibody within the gut tissue. Therefore, the oral administration of recombinant microalgae step by step is a measure conducive to the safety of commercial aquaculture. Similarly, in the patent applied by Tsai and Li (2011), one of the preferred examples used *Nannochloropsis* as an expression vector to express the foreign gene-encoded products, including rYGH of *Acanthopagrus latus*, Bovine lactoferricin (LFB), and capsid protein of WSSV (VP28). They, respectively, demonstrated that recombinant microalgae had the effects of the promotion of growth, the resistance to pathogens, and the resistance to viruses for the aquatic animals. The patent filed by the Ohio State University showed that the antigen can be delivered to the host through oral vaccination with transgenic microalgae and successfully induced immune response (Sayre et al., 2017). In one preferred embodiment, the successful expression of the foreign protein p57 can be detected from the fish mucus.

Recently, a number of researches on the application of genetically modified microalgae in aquaculture and the protection of intellectual property rights have been emphasized (**Tables 1**, **2**). It is worth noting that many emerging algae companies (TransAlgae, Microsynbiotix, and Triton Algae) embarked on this trend and developed genetically modified organism (GMO) methods. In the current cases, oral administration leads to significant protective immunity and survival rate of orally vaccine animals. Oral delivery of antigens to aquatic organisms with microalgae can protect the antigen molecule from the degradation of metabolic digestive system. However, the detailed mechanism of antigen molecular presentation has not been dissected. The research on the direct or indirect oral feeding of recombinant microalgae for aquatic organisms is expected to better prevent, ameliorate, or treat diseases or disorders of aquatic animals in the aquaculture industry.

Resistance of Microalgae Oral Agent to Polymicrobial Diseases

Various microorganisms such as bacteria, fungi, viruses, and even parasites infect the same biological host in different combinations. This process that causes acute or chronic diseases is called polymicrobial infection (Brogden et al., 2005). In the field of aquaculture, polymicrobial infection is a long-term and common phenomenon. Currently, the strategy for developing oral agents against polymicrobial infections using microalgae as a platform is mainly to heterologously express antimicrobial peptides. LFB is an antimicrobial peptide that can kill or

TABLE 3 | Comparison of several systems used in oral vaccine development.

Organism	Diversity of genetic tools	Growth rate	Modification capacity	Cultivation cost	Biosecurity
Bacteria	++++	++++	+	+	++
Yeast	++++	+++	+++	+	+++
Mammalian cell	+++	+++	++++	++++	++++
Higher plant	+++	+	+++	++	++++
Microalgae	++	+++	+++	+	++++

inactivate many pathogens (Tsai and Li, 2011). Li and Tsai (2009) used the *Nannochloropsis oculata* as a host, expressed a broad-spectrum antibacterial peptide bovine lactoferricin (LFB), which has explored the antibacterial ability *in vitro* and *in vivo*. The experimental result showed that medaka fish fed with LFB-containing transgenic microalgae would have bactericidal defense against Vibrio *parahaemolyticus* infection in its digestive tract. After being infected by *V. parahaemolyticus*, the survival rate of the experimental group fed with transgenic microalgae increased to 85%. He et al. (2018) introduced the heterozygous antimicrobial peptide gene (Scy-hepc) into *Chlorella* sp., and evaluated the antibacterial effect of transgenic microalgae *in vitro* and *in vivo*. It was found that the extract of transgenic microalgae had antibacterial ability to the experimental bacteria. *In vivo* experiments, the relative survival rates of the *Sparus macrocephalus* and hybrid grouper fed with transgenic *Chlorella* after infected by *A. hydrophila* were 80 and 55%, respectively. Overall, the above examples provide some ideas for the development of a polymicrobial targeted recombinant microalgae vaccine in the aquaculture industry.

PROSPECT OF ALGAL-BASED ORAL RECOMBINANT VACCINES

In fact, algae cells are known as the natural green factories. They have the advantages of high photosynthesis efficiency, direct use of solar energy, and CO_2 fixation ability. Through genetic manipulation, a series of high value-added products such as pharmaceutical proteins, functional enzyme, and food additives can be efficiently expressed, which has unparalleled advantages (Georgianna and Mayfield, 2012; Dyo and Purton, 2018). The specific attributes and limitations of each system should be evaluated before selecting the most suitable oral vaccine development platform (**Table 3**). Compared with bacterial expression systems, eukaryotic microalgae can complete complex protein folding and modification to form active proteins that meet people's specific needs for antigens and antibodies. Compared with yeast expression systems, microalgae can photosynthesize, sequestrate carbon, and reduce greenhouse gas emissions. Compared with genetically modified higher plants, it has a shorter culture cycle and is less restricted by seasonal weather conditions. Compared with the mammalian cell culture, it has lower production costs and can be easily scaled up. In addition, the microalgae-derived protein is biocompatible and can be directly consumed by animals without isolation and purification, thereby avoiding the cost of purification and extraction (Leon-Banares et al., 2004). It is important to stress that microalgae and cyanobacteria are extremely diverse with 100,000s of different species spread across the tree of life. Nowadays, a handful of these are recognized as edible (e.g., have GRAS status) and are used as food or feed components, which can serve as the cell factory to synthesize valuable products and oral delivery vehicle for subunit vaccines at the same time (Rosales-Mendoza et al., 2016). Therefore, it is completely feasible to apply algae cell metabolic engineering and synthetic biology research to the development of aquatic vaccines.

At present, a variety of aquatic vaccines have been developed (Plant and Lapatra, 2011; Dadar et al., 2016). However, most of the vaccines usually need to be purified or need to be packaged with adjuvants. High cost and complicated processes (e.g., most of them require immunization by injection) have limited their application in large-scale aquaculture. Microalgae can be used as bait feed additive for aquatic animals in the form of living cells or powder, or made into granular bait as an additive, which generally has the effects of promoting growth, enhancing resistance, improving larval survival and body color for a variety of aquatic animals. At the same time, aquatic animals can obtain considerable immunity by oral administration, avoiding the physical damage caused to animals by injection or immersion immunization, reducing the operating cost and the burden on animals themselves. Therefore, if the antigen is highly expressed in the microalgae cells and the transgenic microalgae can be consumed as oral vaccine, it is expected to play an important role in the prevention and control of aquatic diseases (Mutoloki et al., 2015). As a feed for aquatic animals, algae cells can be used as a carrier to present heterologous expressed antigens which not only supplement nutrition but also play the role of vaccine.

Microalgae meet the sustainable development needs of multiple industries due to its unique advantages. However, there are still some problems with the microalgae expression system, such as low expression efficiency and poor stability, low recombinant protein content, immature genetic platform, etc. In addition, the situation of polymicrobial co-infection in aquaculture is becoming increasingly severe, whereas at present, there are no effective prevention and treatment measures for multi-pathogen infections, and related research is relatively scarce. In the future, with the development of oral vaccines using microalgae as a carrier, broad-spectrum antibacterial activity and multivalent recombinant microalgae vaccines are expected to flourish.

AUTHOR CONTRIBUTIONS

JF contributed to conceptualization and supervision. HW contributed to conceptualization, project administration, and supervision. KM and QB contributed to investigation, writing—original draft, review, and editing. YW, SC, and SZ contributed to writing—original draft. All the authors listed have approved the manuscript and agreed to authorship and submission of the manuscript for peer review.

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Environmental Regulation of PndbA600, an Auto-Inducible Promoter for Two-Stage Industrial Biotechnology in Cyanobacteria

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Cyanobacteria are photosynthetic prokaryotes being developed as sustainable platforms that use renewable resources (light, water, and air) for diverse applications in energy, food, environment, and medicine. Despite the attractive promise that cyanobacteria offer to industrial biotechnology, slow growth rates pose a major challenge in processes which typically require large amounts of biomass and are often toxic to the cells. Two-stage cultivation strategies are an attractive solution to prevent any undesired growth inhibition by de-coupling biomass accumulation (stage I) and the industrial process (stage II). In cyanobacteria, two-stage strategies involve costly transfer methods between stages I and II, and little work has been focussed on using the distinct growth and stationary phases of batch cultures to autoregulate stage transition. In the present study, we identified and characterised a growth phase-specific promoter, which can serve as an auto-inducible switch to regulate two-stage bioprocesses in cyanobacteria. First, growth phase-specific genes were identified from a new RNAseq dataset comparing two growth phases and six nutrient conditions in *Synechocystis* sp. PCC 6803, including two new transcriptomes for low Mg and low K. A type II NADH dehydrogenase (*ndbA*) showed robust induction when the cultures transitioned from exponential to stationary phase growth. Behaviour of a 600-bp promoter sequence (PndbA600) was then characterised in detail following the expression of PndbA600:GFP in *Synechococcus* sp. PCC 7002. Culture density and growth media analyses showed that PndbA600 activation was not dependent on increases in culture density *per se* but on N availability and on another activating factor present in the spent media of stationary phase cultures (Factor X). PndbA600 deactivation was dependent on the changes in culture density and in either N availability or Factor X. Electron transport inhibition studies revealed a photosynthesis-specific enhancement of active PndbA600 levels. Our findings are summarised in a model describing the environmental regulation of PndbA600, which can now inform the rational design of two-stage industrial processes in cyanobacteria.

Keywords: cyanobacteria, biotechnology, two-stage cultivation strategy, stationary phase, promoter, transcriptomics, nutrient limitation

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INTRODUCTION

Cyanobacteria are being developed as sustainable platforms that use renewable resources (light, water, and air) for diverse industrial applications, including the manufacturing of commodity and high-value products and remediation of heavy metals or salt (Amezaga et al., 2014; Al-Haj et al., 2016; Singh et al., 2016, 2017; Miao et al., 2020). This phylum of oxygenic photosynthetic bacteria inhabits virtually every niche across the planet and, coupled with its metabolic plasticity, lends itself to a vast variety of industrial settings and processes (Thajuddin and Subramanian, 2005; Xiong et al., 2017). Cyanobacteria produce a diverse palette of natural products with applications in energy, food, environment, and medicine, and are easily engineered for recombinant production (Camsund and Lindblad, 2014; Dittmann et al., 2015; Singh et al., 2017).

Despite the attractive promise that cyanobacteria offer to industrial biotechnology, they present unique challenges which have hampered its adoption by an industry currently dominated by well-established heterotrophic systems, such as *Escherichia coli* and *Saccharomyces cerevisiae*. While similarly amenable to high-throughput screening and engineering, many cyanobacterial strains still pose key technical difficulties such as cultivation and transformation—issues that have long been optimised for their heterotrophic competitors. With the collection of data and progress in technology, these barriers are gradually coming down. For instance, energetic and economic costs of cultivation and product purification are alleviated by rapid developments in photobioreactor and downstream processing technologies (Pierobon et al., 2018). Substantial effort has also been directed towards the development of molecular tools to genetically engineer these photosynthetic prokaryotes in which both standard prokaryote and photosynthetic eukaryote toolboxes are ineffective (Berla et al., 2013; Camsund and Lindblad, 2014; Santos-Merino et al., 2019). Despite the improvements in cultivation systems and the identification of relatively fast-growing cyanobacterial strains, however, slow growth rates continue to pose a major challenge to cyanobacterial biotechnology (Gale et al., 2019).

Industrial applications typically require a large biomass to obtain sufficient productivity. It is, therefore, important to avoid any growth inhibition during biomass accumulation in order to generate the optimal biomass as quickly as possible. In addition, computational analyses indicate trade-offs between biomass production and product synthesis in cyanobacteria (Knoop and Steuer, 2015). Two-stage cultivation strategies are, therefore, an attractive approach to decouple the industrial process (stage II) from biomass accumulation (stage I). Growth inhibition is thus minimised by alleviating issues arising from product/process toxicity, and stage I growth rates/stage II productivity is maximised by preferentially allocating resources (carbon precursors, ATP energy, and NAD(P)H reducing power) to growth or productivity, respectively (Burg et al., 2016).

In cyanobacteria, two-stage cultivation strategies typically require extra steps between stages I and II that add monetary and energetic costs to the process. The most common approach involves physical transfer of cultures from stage I to stage II, promoting conditions using centrifugation, filtration, flocculation, or sedimentation (Monshupanee et al., 2016; Kushwaha et al., 2018; Testa et al., 2019; Aziz et al., 2020). Alternatively, stage II can be induced by the application of physical and/or chemical stimuli such as changing light conditions for pigment production or temperature and salt stress for polysaccharide production in *Spirulina* (Lee et al., 2012, 2016). Strategies which eliminate these extra steps between stages I and II can greatly improve the economic feasibility of these systems.

Batch grown systems are well-suited for two-stage approaches. Bacterial growth is characterised by three successive phases: lag phase, exponential growth phase, and stationary phase. Cyanobacterial batch cultures show a similar growth pattern with the exception of an extended growth phase comprised of a shorter early growth phase, from which exponential growth rates are commonly reported, and a longer late growth phase, often termed the linear growth phase, as the cultures transition to the stationary phase (Schuurmans et al., 2017). Inherent differences between the growth and stationary phases of batch systems can be used to regulate two-stage processes and initiate stage II once maximum culture density has been achieved in the late growth/early stationary phase neither with any manipulation of culture nor with any added cost to the process.

Promoters are regulatory elements in the DNA that function as biological switches. Ideally, promoters controlling two-stage processes should be inactive during stage I and become active at the onset of stage II (**Supplementary Figure S1**). Auto-inducible promoters, which respond to endogenous signals, have the distinct advantage of not requiring any additional supplements, thus simplifying and improving the sustainability of the process. In the case of two-stage processes, promoters that specifically respond to changes in growth phase, particularly the transition to stationary phase, are ideal candidates. Libraries of stationary phase promoters have been developed for *E. coli* (Miksch et al., 2005). While orthogonal promoters derived from other organisms are generally preferred in order to avoid interference of engineered systems by host machinery, well-established prokaryotic tools perform poorly in cyanobacteria (Huang et al., 2010). In cyanobacteria, several growth phase-responsive genes and promoters have been reported for some model strains (Ludwig and Bryant, 2011; Berla and Pakrasi, 2012; Kopf et al., 2014; Ruffing et al., 2016). However, we still lack a detailed understanding of activation/deactivation behaviours and of performance across strains.

In this study, we aimed to identify and characterise an auto-inducible promoter for two-stage batch cultivation strategies in cyanobacteria using the approach presented in **Figure 1**. Two different species of cyanobacteria were used to avoid potential issues with genetic instability or cross-talk between native expression machinery and engineered expression systems (Camsund and Lindblad, 2014; Gordon and Pfleger, 2018). First, RNA sequencing analyses comparing transcriptional profiles across growth phases and nutrient conditions led to the identification of robust growth phase-specific genes and thus candidate promoters in the freshwater cyanobacterium *Synechocystis* sp. PCC 6803. Next, the behaviour of a promoter, PndbA600, was characterised in response to changing culture

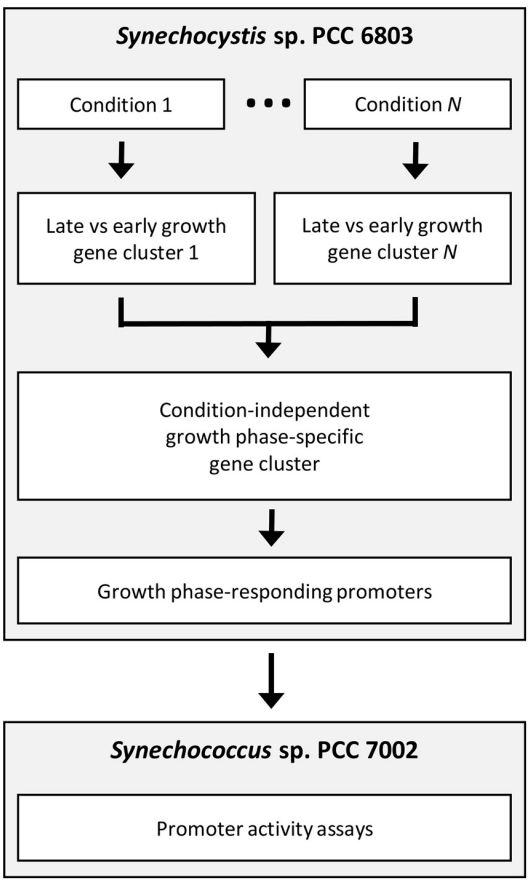

FIGURE 1 | Workflow for the identification and characterisation of growth phase-specific promoters. *Synechocystis* sp. PCC 6803 was cultivated in numerous different conditions. RNA sequencing of samples harvested during early and late growth phases led to the identification of condition-independent, growth phase-specific genes and candidate promoters. Heterologous promoter behaviour was characterised using a green fluorescent protein (GFP)-based assay in *Synechococcus* sp. PCC 7002.

density, growth media, and cellular redox status using a green fluorescent protein (GFP) assay in *Synechococcus* sp. PCC 7002. Our findings are summarised in a model describing the environmental regulation of PndbA600, which can inform the rational design of sustainable, two-stage industrial processes in cyanobacteria.

MATERIALS AND METHODS

Strains and Culture Conditions

Cyanobacterial Strains

Synechocystis sp. PCC 6803 was grown photoautotrophically in Dreschel flasks in water baths equilibrated to 30°C with photoperiod 12 h/12 h light/dark and light intensity 80 μmol photons m^{-2} s^{-1}. *Synechococcus* sp. PCC 7002 was grown photoautotrophically in Bijou bottles maintained at 30°C in a walk-in environmental growth chamber (Conviron model MTPS72) with photoperiod 16 h/8 h light/dark and light intensity 150 μmol photons m^{-2} s^{-1}. Cultures had a working volume of 60–75% relative to the culture vessel capacity, which were illuminated with fluorescent cool white lights and sparged with humidified ambient air.

Growth Media

For control conditions, *Synechocystis* cultures were grown in BG11 medium (Stanier et al., 1971) and *Synechococcus* cultures were grown in A + medium (Stevens and Porter, 1980). For low nutrient conditions, individual nutrients (N, P, K, S, and Mg) were reduced to the indicated concentrations relative to the concentration in control BG11 or A + medium, and counter ions were replaced with control concentrations of KCl, MgCl, Na_2SO_4 or NaH_2PO_4 (see **Supplementary Tables S1** and **S2** respectively). To obtain "spent" media, the supernatant of stationary phase *Synechococcus* cultures (cultivated for ≥ 5 weeks) was harvested after centrifugation at 4,000 *g* for 20 min at room temperature, and filter-sterilised.

Culture Setup

Twenty millilitre control medium was inoculated with strains maintained as DMSO stocks at −80°C or on solid media maintained at 23°C. For control and low nutrient conditions, 20 ml cultures were grown to optical density (OD) 1–5, diluted to OD 1 in the relevant growth medium and 0.5 ml was used to inoculate 150 ml in the relevant growth medium. For promoter activation and deactivation experiments, 750 and 150 ml cultures were grown in control conditions to low density (OD < 5, GFP < 300) and high density (OD ≥ 12, GFP > 850), respectively. Cells were harvested by centrifugation at 4,000 *g* for 20 min at room temperature, the supernatant was removed, and the pellets were washed and resuspended at OD 1 or 12 (for low and high density, respectively) in the relevant growth medium.

Electron Transport Inhibitors

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, aka Diuron; Sigma) or malonic acid (Sigma) were added at the indicated concentrations to either young (1-week old cultures, OD < 5, GFP < 300) or mature (4-week old cultures, OD ≥ 12, GFP > 850) 150 ml cultures grown in control conditions.

Growth Monitoring

Growth was monitored by measuring OD at 730 nm (OD_{730}) within the linear range (OD 0.05–1.00) of a Lambda 45 UV/VIS Spectrophotometer (PerkinElmer).

RNA Analyses

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Frozen cell pellets were resuspended in 700 μl Buffer RLT and cells were disrupted using 0.5 g of 0.5 mm diameter glass beads for 5 min at 30 Hz in a TissueLyser (Qiagen, Venlo, Netherlands). Following centrifugation at 10,000 *g* for 1 min, the supernatant was applied to the RNeasy spin column and RNA purified as recommended by the supplier.

For RNA sequencing, messenger RNA (mRNA) was enriched using the MICROBExpress Kit (Ambion, Austin,

TX, United States). RNA quality was assessed before and after mRNA enrichment using an Agilent® 2100 Bioanalyzer™. Complementary DNA (cDNA) libraries were generated using TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced using the Illumina MiSeq System at Glasgow Polyomics. Reads were processed and mapped to the *Synechocystis* genome (GenBank assembly accession GCA_000009725.1) using TopHat and Cuffdiff software (Trapnell et al., 2012). A total of 640,131,273 reads were obtained with average reads of 16.4 million reads per sample and an average length of 75 base pairs. A total of 546,270,878 reads (85.3%) were mapped to the genome with a tolerance of a 2 base pair mismatch. The number of reads mapped to each coding sequence was calculated and normalised for gene length (number of fragments mapped per kilobase of gene) and library depth (total number of aligned reads in the experiment). Data are thus presented as fragments per kilobase of gene per million reads mapped (FPKM).

For each condition and time point, replicate samples from three independently grown cultures were sequenced, resulting in a total of 39 RNAseq datasets (1 control condition × 3 time points × 3 replicates + 5 low nutrient conditions × 2 time points × 3 replicates). The raw RNA sequencing data are available from the European Nucleotide Archive, accession number PRJEB40560. Significant differences between conditions and time points were determined using Cuffdiff software (Trapnell et al., 2012). Multi-dimensional scaling of the RNA sequencing data was performed using the CummeRbund visualisation package (Trapnell et al., 2012).

For quantitative real-time PCR (qPCR), total RNA was isolated from different cultures to those used for RNA sequencing experiments, resulting in a total of 36 qPCR samples (6 nutrient conditions × 2 time points × 3 replicates). cDNA libraries were generated using random primers with the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, Netherlands). qPCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies). Primers were designed to amplify 110–155 bp products with a 60°C annealing temperature (**Table 1**) using Primer 3 software (Untergasser et al., 2012) and interrogated using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent). Primer pair efficiencies were determined using serial dilutions of gel purified RT-PCR product. Standard curves were included in all qPCR runs to transform threshold cycles into RNA concentrations, which were ratioed against the internal control *slr0211*. For replication, assays were performed using cDNA from three independently grown cultures. Significant differences between conditions and time points were determined by two-way ANOVA with Tukey (HSD) *post-hoc* analysis using SigmaPlot software (Systat). Negative (no template) controls were included and a melting curve analysis was performed in all assays.

TABLE 1 | Primer sequences for quantitative real-time PCR.

Target gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
slr0211	CCTGCTCCGGGCCTTGG	CTGGTATTGAATGGGGCCAC
slr0451	GAACAACAGGCCAGGGTAG	CGTAGTTCTTGCCGTTGGTG
sll0401	GAGAGTAGAAGCCGTTACCC	GCTGACGGAGAAGGAGCC
slr1697	CCCGATTTAACACCAATGTCC	GACTCAATATTGCTGGTAGCC
slr0073	GGAATATTGCACTCGTCTGGG	GCCAAGGTACGGTAGGAATG
slr2144	CAACAGTGACGGTCTGACC	CACCACTGCTTGCCCATCC
psbA1	CCTGTGGTCACGGTTCTGTT	TGCCATCAATATCCACCGGG
rnpB	GTGAGGACAGTGCCACAGAA	GATACTGCTGGTGCGCTCTT
ndbA	GACAAAAACGGTGCTCTGGG	CTCAAATCCGGGTTGACCAC
slr1747	GTTGCCCTCCCCTTGGTG	GAATATGGCTCGAATCCAACAC

Promoter Analyses

A 600 bp sequence occurring directly upstream of the *ndbA* gene, PndbA600, was amplified from *Synechocystis* genomic DNA using primers containing BioBrick prefix and suffix overhangs for cloning into the pAQ1BB transformation vector: forward primer 5′- gaattcgcggccgcttctagagTTAATGGATCGTTACCATTCCCAC -3′ and reverse primer 5′- ctgcagcggccgctactagtaAGCAACGGCG AAAATATTACGATTTG-3′. Following sequence confirmation in the pGEM-T Easy vector, PndbA600 was cloned upstream of a synthetic RBS-reporter construct comprised of RBS3 and GFP BioBrick part BBa_E0040 in the pAQ1BB vector (Madsen et al., 2018) to generate pAQ1BB:PndbA600:RBS3:GFP (**Supplementary Figure S2**). The sequence of the promoter-reporter construct is presented in **Supplementary Figure S3**. The promoter-GFP construct was integrated into a neutral site in the *Synechococcus* genome by natural transformation and verified by PCR amplification and sequencing to generate the transgenic *Synechococcus* PndbA600:GFP strain. GFP was measured at regular intervals during culture growth by adjusting culture density to OD_{730} 0.25–0.30 and measuring fluorescence using 480 nm excitation and 514 nm emission wavelengths using a LS 55 Luminescence Spectrophotometer (PerkinElmer, Waltham, MA, United States).

RESULTS

Environmental Conditions to Control Growth Phase Transition in *Synechocystis* Batch Cultures

Stationary phase can be induced by many different factors including nutrient limitation, toxic by-product accumulation, or a variety of stress factors such as pH or temperature (Nyström, 2004). For robust separation of general responses to changes in the growth phase from specific responses to individual factors, several different conditions inducing transition to stationary phase were required. Nutrient deficiencies limit the growth of cyanobacteria (Hirani et al., 2001; Richaud et al., 2001) and were used here to induce the stationary phase. *Synechocystis* sp. PCC 6803 was cultivated under control conditions (BG11) and low levels of individual nutrients as reported before, with 12.5% N, P, or S in BG11 background. In addition, two new low nutrient conditions were tested, 12.5% Mg and 12.5% K in BG11 background. Nutrients are co-supplied with a counter ion as an electrically neutral salt, so counter ions were replaced up to the control concentration (**Supplementary Table S1**). **Figure 2A** shows that *Synechocystis* sp. PCC 6803 batch cultures cultivated

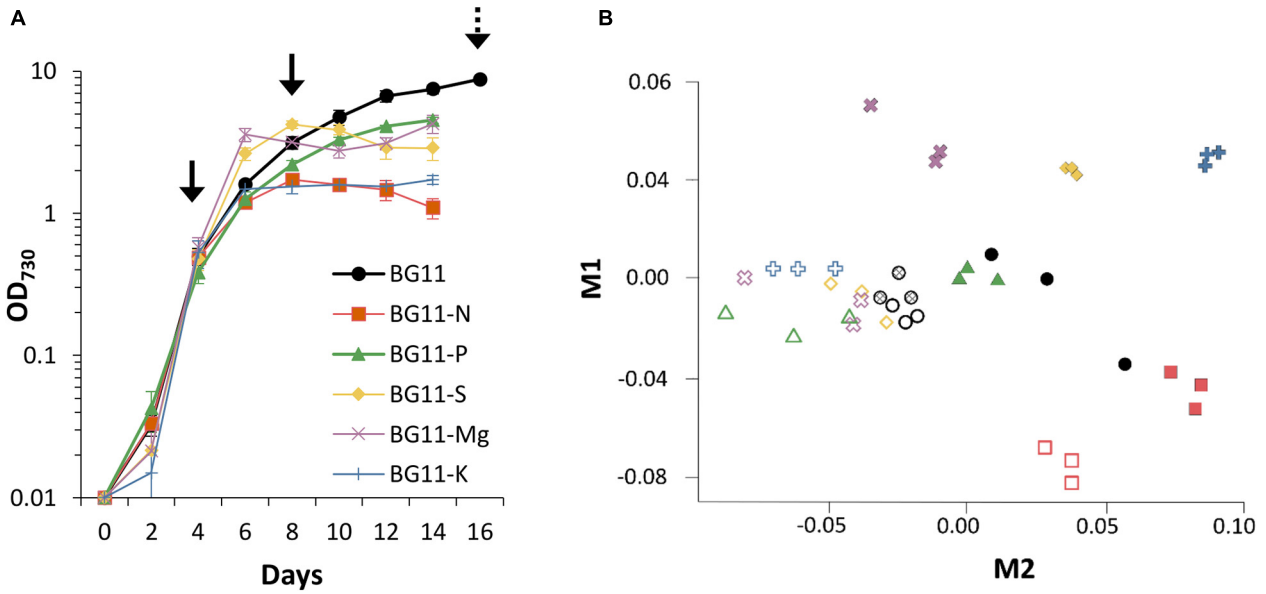

FIGURE 2 | Growth and transcriptomics of *Synechocystis* sp. PCC 6803. **(A)** Culture density (OD_{730}) of *Synechocystis* batch cultures cultivated under control (BG11) and low nutrient conditions (12.5% N, P, S, Mg, or K in BG11 background, **Supplementary Table S1**). Solid arrows show when samples were collected for RNA sequencing from all conditions. Dashed arrow indicates additional samples collected from control conditions. Data are means ± S.E.M. of three independent cultures. **(B)** Multi-dimensional scaling plot of 39 RNAseq expression profiles. Distances between points reflect relative similarities between samples based on genome-wide transcript levels. Symbols and colours are same as in **(A)**. Open symbols represent early growth phase samples (day 4). Closed symbols represent late growth phase samples (day 8 in low nutrient and day 16 in control conditions). Hatched circles represent control samples harvested on day 8.

in all five low nutrient conditions transition to the stationary phase at an earlier time (day 8) and lower density (OD_{730} 1.5–4.2) compared to the control condition (day 16, OD_{730} 8.7). Thus, low nutrient conditions were used to induce early transition to the stationary phase in response to specific environmental stimuli.

RNA Sequencing of Early and Late Growth Phases in *Synechocystis*

To compare the transcriptomes of early and late growth phases, time points were selected for RNA sequencing based on the growth curves (black arrows in **Figure 2A**). Early samples were harvested during the exponential growth phase (day 4), and late samples were harvested as the cultures transitioned into the stationary phase (day 8). To account for later transition to the stationary phase, control cultures were also harvested on day 16. For replication, three cultures were independently grown in each condition. RNA purification and sequencing is described in Materials and Methods (Section "RNA Analyses"), and the normalised transcript levels for all genes in all 39 samples, together with statistical parameters, are available in **Supplementary Table S3**. Transcript counts are presented as FPKM.

Figure 2B shows a multi-dimensional scaling plot based on normalised FPKM values in the 39 RNAseq samples. Early samples generally group together with the exception of BG11-N, suggesting a distinct early response to low N. Additionally, samples harvested on day 8 under control conditions cluster with early samples, reflecting that nutrients were not yet limiting in this condition and control cultures were still in the early growth phase. Late samples separate according to condition with close grouping of replicates, demonstrating nutrient-specific transcriptional responses. Greater variation is observed in late control samples, possibly due to simultaneous limitation of multiple nutrients in the optimised BG11 medium in which nutrients deplete at equal rates.

Robust, Late Growth Phase-Specific Genes of *Synechocystis*

To identify condition-independent, growth phase-responsive genes, we looked for genes that were upregulated in the late growth phase compared to the early growth phase in all of the conditions tested. Genes were, therefore, selected from the RNA sequencing dataset based on a significance value of $p < 0.05$ between early and late samples within each condition and $\log_2$(late/early) > 1 for all six conditions. **Table 2** presents 24 late growth phase-specific genes from the *Synechocystis* RNA sequencing dataset. The majority of genes are annotated as hypothetical proteins (16/24, 67%); however, there are also genes with annotated functions in cell killing, energy metabolism, photosynthesis and respiration, regulatory functions, and transport.

RNAseq expression profiles were verified using real-time qPCR. Three new *Synechocystis* cultures were grown for each of the six nutrient conditions, and samples were harvested during the early and late growth phases (36 samples total). To control for potential biases introduced during mRNA enrichment for the

TABLE 2 | Late growth phase-specific genes of *Synechocystis* sp. PCC 6803.

Gene ID	Gene symbol	Gene product	Functional category
slr1747		Cell death suppressor protein Lls1 homologue	Cellular processes
slr2132		Phosphotransacetylase	Energy metabolism
sll0549		Hypothetical protein	Hypothetical
sll0528		Hypothetical protein	Hypothetical
slr1119		Hypothetical protein	Hypothetical
sll1675		Hypothetical protein	Hypothetical
sll1355		Hypothetical protein	Hypothetical
sll1274		Hypothetical protein	Hypothetical
sll1769		Hypothetical protein	Hypothetical
sll1158		Hypothetical protein	Hypothetical
sll1884		Hypothetical protein	Hypothetical
slr1674		Hypothetical protein	Hypothetical
slr0959		Hypothetical protein	Hypothetical
slr0292		Hypothetical protein	Hypothetical
sll6052		Hypothetical protein	Hypothetical
sll6053		Hypothetical protein	Hypothetical
sll6054		Hypothetical protein	Hypothetical
sll6055		Hypothetical protein	Hypothetical
slr1498	*hypD*	Putative hydrogenase protein HypD	Other categories
slr1675	*hypA1*	Putative hydrogenase protein HypA1	Other categories
slr0851	*ndbA*	Type 2 NADH dehydrogenase	Photosynthesis and respiration
slr0741		Transcriptional regulator	Regulatory functions
slr0096		Low affinity sulphate transporter	Transport and binding proteins
slr0529	*ggtB*	Glucosylglycerol transport system substrate-binding protein	Transport and binding proteins

RNA sequencing analyses, cDNA for qPCR analyses was generated from total RNA. *slr0211* (encoding a hypothetical protein) was selected as a reference gene for qPCR normalisation based on low standard error of FPKM across the 39 RNAseq samples and low variation of Ct values across the 36 qPCR samples (**Figure 3A**). While there were small differences in the level of upregulation within individual conditions, there was an overall excellent agreement between RNAseq and qPCR expression profiles for *ndbA* (*slr0851*, encoding a type II NADH dehydrogenase, **Figures 3B,C**) and *slr1747* (encoding a homologue of the cell death suppressor protein Lls1, **Supplementary Figure S4**). While these genes are adjacent to one another on the *Synechocystis* chromosome, they are independent transcriptional units (Kopf et al., 2014), and both showed significantly higher transcript levels in the late growth phase compared to the early growth phase in all six nutrient conditions. The level of *ndbA* upregulation was relatively consistent across the conditions tested; therefore, this gene was selected for promoter analyses.

Heterologous Activity of the *ndbA* Promoter, PndbA600, in *Synechococcus*

Heterologous systems have several advantages for the characterisation of molecular tools for metabolic engineering. For example, the interference from native regulatory machinery is minimised, such as small RNAs important for adaptation to changing environments in cyanobacteria (Hu and Wang, 2018). Furthermore, introduction of high copy numbers of an endogenous, or even homologous, promoter could outcompete the native promoter (or vice versa) and potentially cause genetic instability in cyanobacteria (Gordon and Pfleger, 2018). Although there is a potential for common regulatory machinery, such as transcription factors across cyanobacteria, *Synechocystis* promoters are routinely used to drive the heterologous expression in *Synechococcus* sp. (Huang et al., 2010; Wang et al., 2012). We therefore used an established GFP-based promoter assay in *Synechococcus* sp. PCC 7002 (Madsen et al., 2018), to assess whether the *ndbA* promoter controls growth phase-specific transcription. For this, we analysed the 600 bp sequence directly upstream of the *ndbA* start codon, which, in addition to the core promoter, includes the 5′UTR and other potential genetic features. This sequence was designated PndbA600 and cloned upstream of a GFP reporter gene and integrated into a neutral site in the *Synechococcus* genome. Following confirmation by PCR and sequencing, the transgenic strain *Synechococcus* PndbA600:GFP was grown in A + (control) medium, and GFP fluorescence per cell (GFP normalised to OD_{730} of the GFP sample) was measured throughout culture growth. **Figure 4A** shows that under control conditions the OD_{730}-normalised GFP fluorescence was low during the early stages of growth followed by a sharp increase in week 3, concomitant with transition to the late growth phase. High levels were then maintained over several weeks of the stationary phase. Notably, PndbA600 showed two distinct levels of the promoter activity with low activity at low density (OD < 10.7, GFP < 450) and high activity at high density (OD > 10.75, GFP 636-3590; **Figure 4B**). The GFP signal reflects

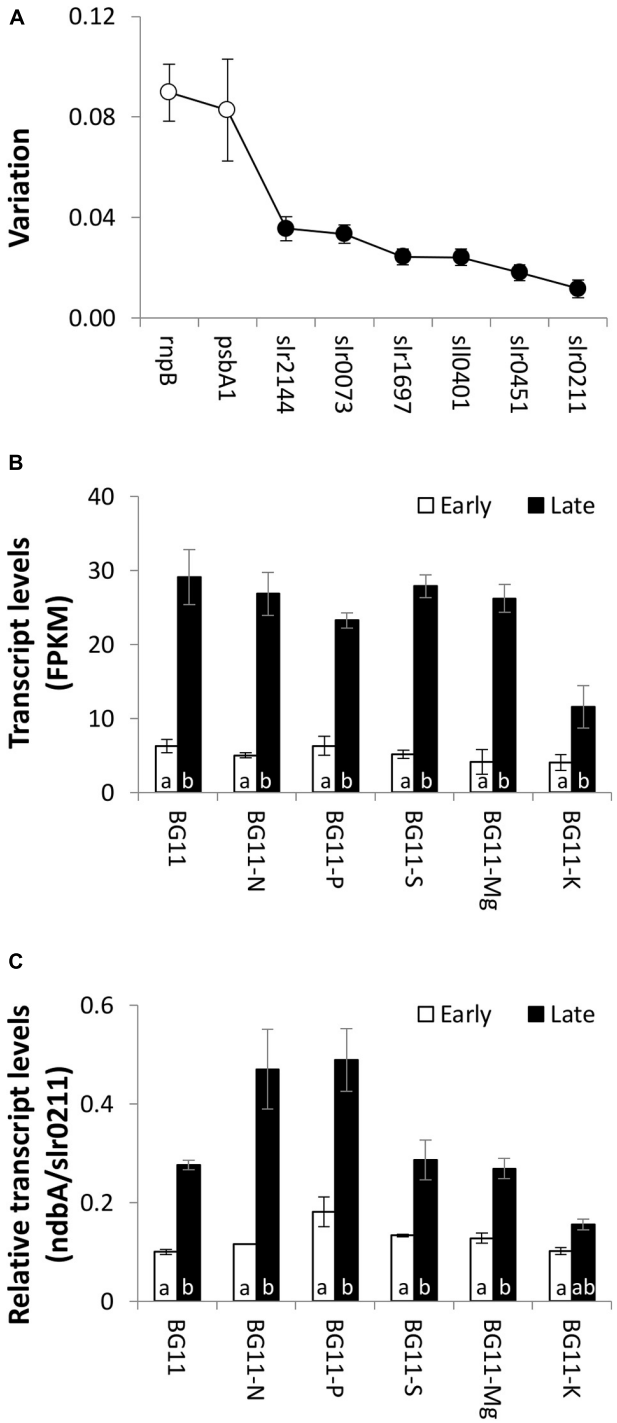

FIGURE 3 | Native *ndbA* transcript levels. **(A)** Variation of Ct values determined using NormFinder software (Andersen et al., 2004). qPCR was performed with 36 samples representing the early and late growth phases of three independent cultures of *Synechocystis* cultivated under control (BG11) and low nutrient conditions (12.5% N, P, S, Mg, or K in BG11 background). White circles represent reference genes used in other studies (Foster et al., 2007; Pinto et al., 2012). Black circles represent genes selected from the RNA sequencing dataset based on low standard error. Data are means ± S.E.M. of three independent cultures. *slr0211* was selected as reference gene for further *(Continued)*

FIGURE 3 | Continued
experiments. **(B,C)** Transcript levels of *ndbA* determined by **(B)** RNA sequencing (normalised to gene length and read counts as FPKM) and **(C)** qPCR (normalised to *slr0211*) in the early (white bars) and late (black bars) growth phases of *Synechocystis* cultivated under control (BG11) and low nutrient conditions (12.5% N, P, S, Mg, or K in BG11 background). Data are means ± S.E.M. of three independent cultures. Different letters indicate significant difference across all conditions ($p < 0.05$; two-way ANOVA using Tukey (HSD) *post-hoc* analysis).

PndbA600 promoter activity: background fluorescence levels in the wild-type and no promoter controls were lower than those observed in *Synechococcus* PndbA600:GFP (GFP < 120). These combined results show that the 600 bp upstream sequence of *ndbA*, PndbA600, shows both growth phase- and culture density-specific activity in *Synechococcus* sp. PCC 7002 grown in control conditions.

Effect of Culture Density on PndbA600 Activity

Culture density, possibly due to direct cell-to-cell interactions, has been suggested as a factor controlling cell division and the transition into stationary phase in cyanobacteria (Esteves-Ferreira et al., 2017). Additional factors could include self-shading and thus light limitation in higher density cultures or extracellular metabolites e.g., signalling molecules secreted to the growth medium (Abisado et al., 2018; Clark et al., 2018). To investigate the relationship between culture density and promoter activity in more detail, we attempted to separate the effect of culture density from the effect of the growth medium. For this experiment, *Synechococcus* PndbA600:GFP cultures were grown to either low density to investigate PndbA600 activation or high density to investigate PndbA600 deactivation. Culture density was then modulated by harvesting the cells and resuspending them at either low or high density. Growth media were modulated by resuspending the cells in either a fresh (control) medium or a spent medium harvested from stationary phase cultures.

We first investigated PndbA600 activation using young, low density cultures with low promoter activity. **Figure 5A** shows that increasing culture density from low to high OD is not sufficient to activate PndbA600. GFP fluorescence only increased after a period of growth in the fresh medium for both low to low and low to high OD cultures (see also **Supplementary Figure S5A**). By contrast, resuspending low density cultures in the spent medium led to rapid PndbA600 activation with faster activation in cultures resuspended at low density compared to high density (**Figure 5B** and **Supplementary Figure S5B**). These results suggest that PndbA600 activation is not dependent on high culture density *per se* but requires one or more components of the spent stationary phase medium, designated as Factor X.

PndbA600 deactivation was then investigated using mature, high density cultures with high promoter activity. **Figure 5C** shows that decreasing culture density from high to low OD in

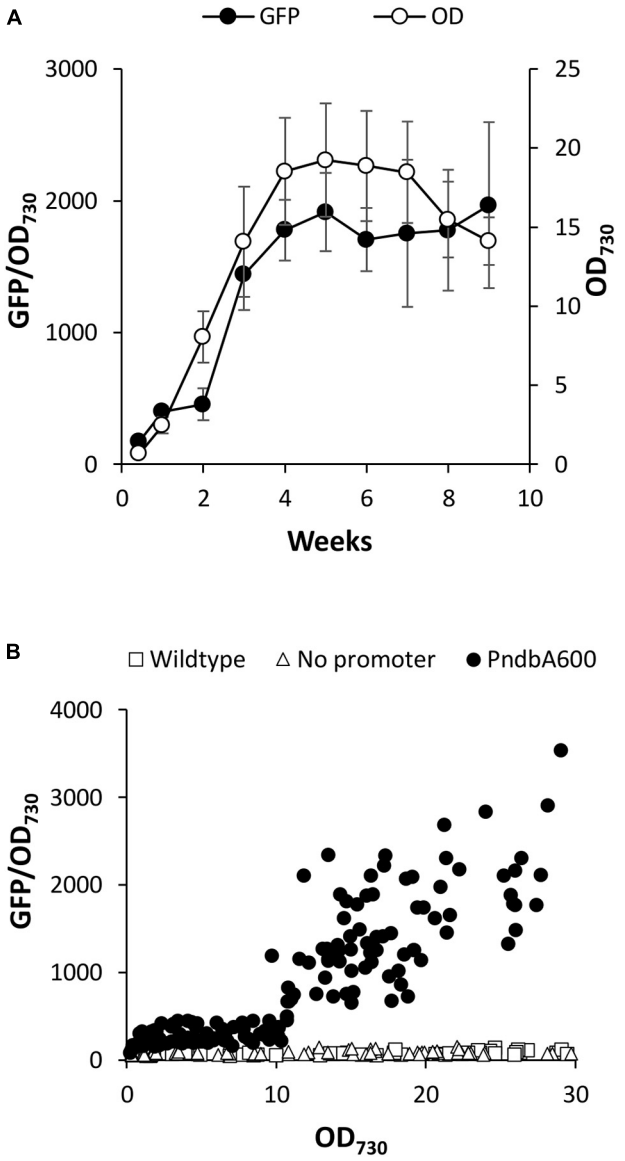

FIGURE 4 | Heterologous PndbA600 activity under control conditions. **(A)** Promoter activity (black circles, GFP fluorescence normalised to OD_{730}, left axis) in *Synechococcus* PndbA600:GFP. Culture density (OD_{730}, right axis) is shown with white circles. Data are means ± S.E.M. of three independent cultures grown in control A + media. **(B)** Promoter activity (GFP fluorescence normalised to OD_{730}) against culture density (OD_{730}) of *Synechococcus* cultures grown in control conditions. Data are shown for the following lines: wild-type (no GFP, squares, $n = 7$), no promoter (*Synechococcus* transformed with GFP, triangles, $n = 6$), *Synechococcus* expressing GFP under the control of PndbA600 (*Synechococcus* PndbA600:GFP, circles). Measurements taken after maximum culture density was achieved are not shown.

a fresh medium resulted in a rapid loss of GFP fluorescence (within 1 week), indicating rapid deactivation of PndbA600. The promoter then regained activity as the culture moved again into the late growth phase (see also **Supplementary Figure S5C**). PndbA600 deactivation requires the decrease in cell density: mature, high density cultures resuspended at high density in fresh media maintained high promoter activity. PndbA600 deactivation also requires the fresh growth medium: mature, high density cultures resuspended at low density in spent media maintained high promoter activity (**Figure 5D** and **Supplementary Figure S5D**). This suggests that the alleviation of Factor X from the spent stationary phase medium is a co-requirement for PndbA600 deactivation. In summary, PndbA600 deactivation requires both low cell density and fresh growth medium.

The Effect of Nutrient Availability on PndbA600 Activity

We have shown that the spent medium of stationary phase cultures is able to modulate PndbA600 activity by both inducing promoter activation and inhibiting promoter deactivation. A possible explanation for these responses may be the low nutrient levels in the spent stationary phase medium. The native expression profile in *Synechocystis* suggested that PndbA600 activation in the late growth phase occurs independent of nutrient depletion. However, this might not be the case in *Synechococcus*. We, therefore, investigated the nutrient dependence of PndbA600 activity in *Synechococcus* PndbA600:GFP using A + with low levels of individual nutrients. Counter ions co-supplied with nutrients were replaced up to the control concentration (**Supplementary Table S2**). Unlike the optimised BG11 medium for *Synechocystis*, we found that nutrient ratios in the standard A + medium used for *Synechococcus* are not optimally adjusted, and different nutrients become limiting at different concentrations (**Figure 6**). Timing of PndbA600 activation and, thus, correlations between the promoter activity, culture density, and the growth phase differed across nutrient conditions.

When comparing the promoter activity relative to the growth phase transition, promoter activation occurred during the transition to the stationary phase at > 75% of maximum culture density under control conditions (**Figure 4A**). Lowering N in the growth medium accelerated PndbA600 activation, whereby promoter activation occurred during the active growth phase at < 50% of maximum culture density (**Figure 6A**). By contrast, PndbA600 activation was delayed in media with low K or low Mg with promoter activation first occurring > 1 week after reaching maximum culture density (**Figures 6B,C**) and was completely abolished in low P (**Figure 6D**). In summary, PndbA600 activation, relative to the growth phase transition, is accelerated by lowering N and delayed by lowering other essential nutrients (and thus increasing the relative N level), suggesting that PndbA600 may be activated by the depletion of N relative to other nutrients in the media.

Plotting promoter activity directly against culture OD demonstrated promoter activation at high density (OD > 10.75) under control conditions (**Figure 4B**). **Figure 6E** shows that PndbA600 activation occurred at lower culture densities in media with 20% N (OD > 1.84) and 2% K (OD > 6.15). By contrast, PndbA activation required a much higher culture density (OD > 17) in media with 0.5% Mg. No PndbA activation was seen in 10% P at any OD. The varying culture OD

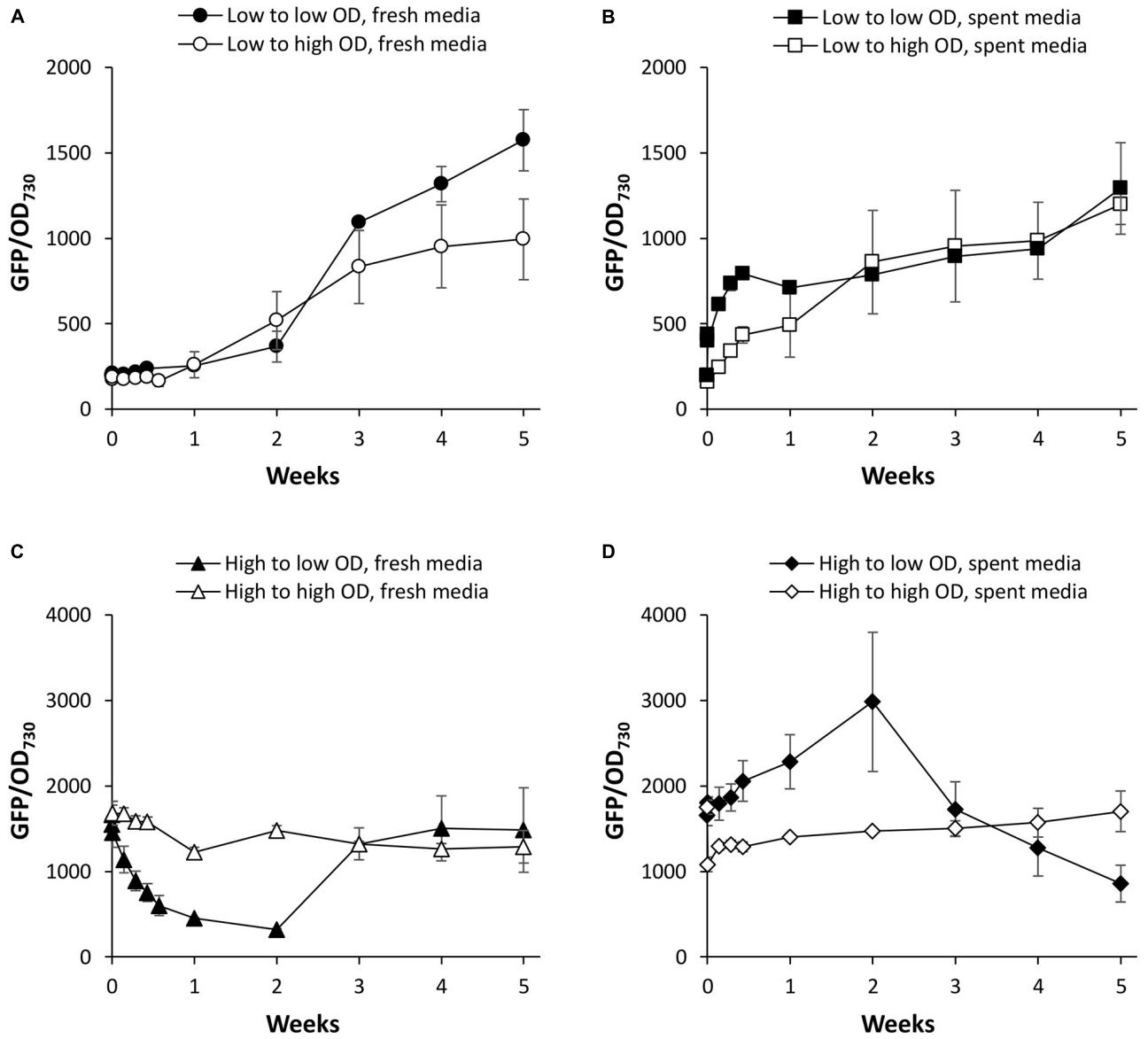

FIGURE 5 | The effect of culture density on PndbA600 activity. PndbA600 activity (GFP fluorescence normalised to OD_{730}) in *Synechococcus* PndbA600:GFP cultures pre-grown to certain density and resuspended to another density at time point 0. Cultures shown in **(A,B)** were started from young, low density cultures with low promoter activity (OD < 5, GFP < 300). Cultures shown in **(C,D)** were started from mature, high density cultures with high promoter activity (OD > 12, GFP > 850). Cultures were resuspended to low (black symbols) or high (white symbols) density in fresh control media **(A,C)** or spent media of stationary phase cultures **(B,D)**. Data are means ± S.E.M. of three independent cultures. Accompanying growth curves are presented in **Supplementary Figure S5**.

requirements for PndbA activation under different low nutrient conditions reflect a combination of the maximum culture OD achieved and the timing of PndbA activation relative to the transition to stationary phase.

To investigate the effects of N supply in more detail, *Synechococcus* PndbA600:GFP cultures were grown in control conditions and resuspended at OD 1 in growth media containing either 0 or 100% N in a background of the control A + medium. Again, young *Synechococcus* PndbA600:GFP cultures grown to low density were used to investigate PndbA600 activation, and mature cultures grown to high density were used to investigate PndbA600 deactivation. **Figure 7A** confirms that PndbA600 activation requires the lack of N: early promoter activation was observed in N-deficient media (see also **Supplementary Figure S6A**). Similarly, PndbA600 deactivation requires the presence of N: mature, high density cultures resuspended at low density in N-deficient media maintained active levels of GFP fluorescence, albeit at a slightly lower level (**Figure 7B** and **Supplementary Figure S6B**). Combined, these results confirm that PndbA600 specifically responds to N levels, with promoter activation upon N depletion and promoter deactivation upon N replenishment.

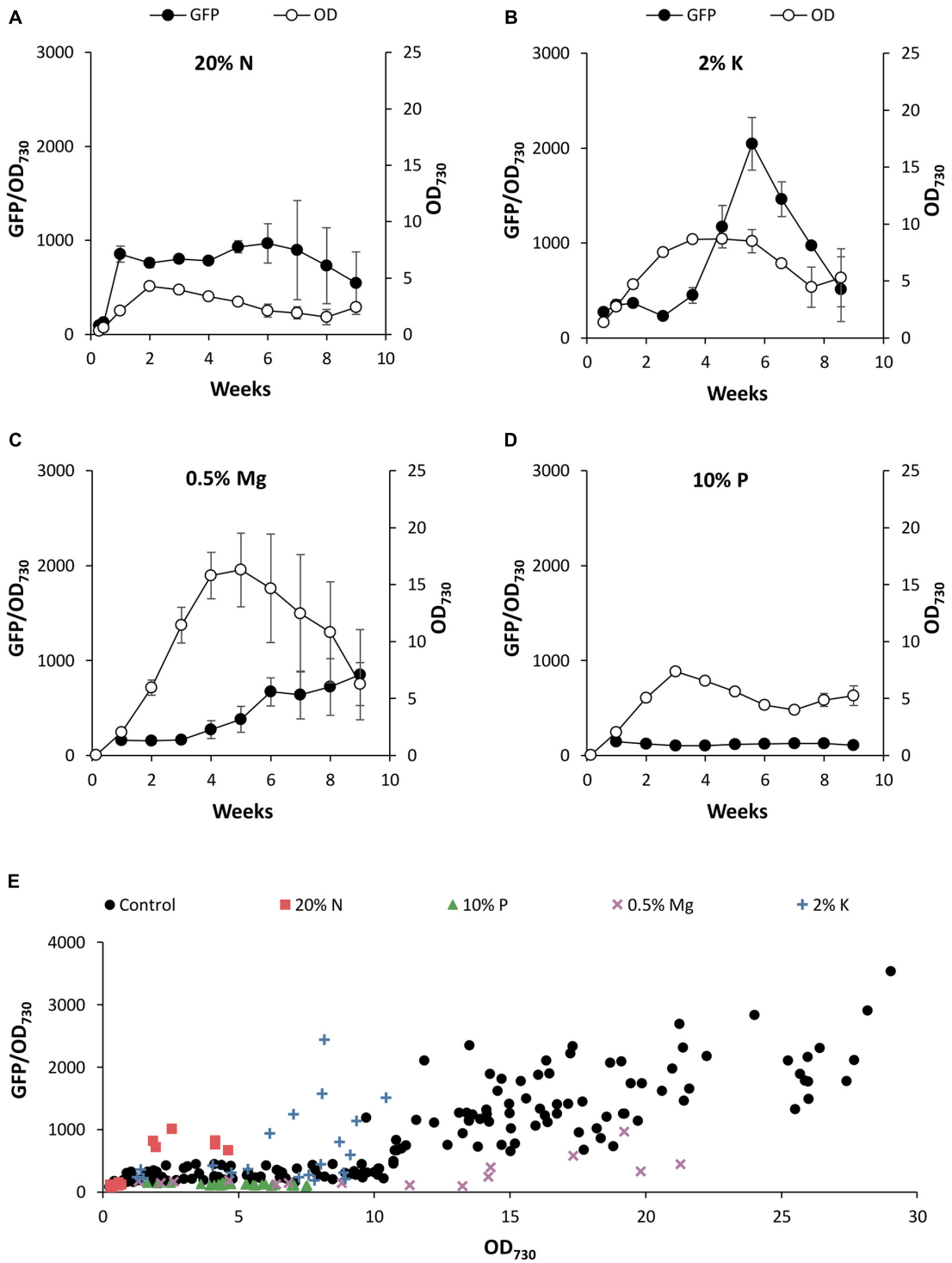

FIGURE 6 | The effect of nutrient deficiency on PndbA600 activity. PndbA600 activity (black circles, GFP fluorescence normalised to OD_{730}, left axis) in *Synechococcus* PndbA600:GFP cultures grown under low nutrient conditions (**Supplementary Table S2**). **(A)** 20% N, **(B)** 2% K, **(C)** 0.5% Mg, and **(D)** 10% P in A + background. Culture density (OD_{730}, right axis) is shown with white circles. Data are means ± S.E.M. of three independent cultures. **(E)** PndbA600 activity (GFP fluorescence normalised to OD_{730}) against culture density (OD_{730}) in *Synechococcus* PndbA600:GFP grown in control (A+, black circles, $n = 20$) and low nutrient conditions (A + background; 20% N, red squares, $n = 3$; 10% P, green triangles, $n = 3$; 0.5% Mg, purple crosses, $n = 3$; 2% K, blue plusses, $n = 3$). Data represent measurements taken as culture density increased. Measurements taken after maximum culture density was achieved are not shown. For promoter activity under control conditions see **Figure 4**.

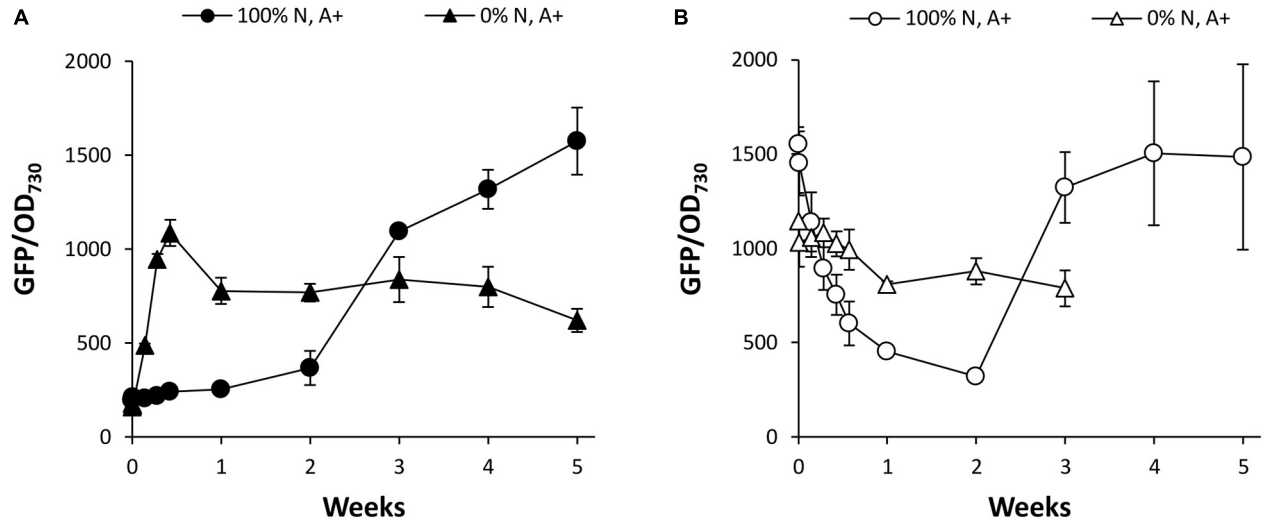

FIGURE 7 | The effect of N supply on PndbA600 activity. PndbA600 activity (GFP fluorescence normalised to OD_{730}) in *Synechococcus* PndbA600:GFP grown in media containing 0 or 100% N in A + background. Cultures shown in **(A)** were started from young, low density cultures with low promoter activity (OD < 5, GFP < 300, black symbols), and cultures shown in **(B)** were started from mature, high density cultures with high promoter activity (OD > 12, GFP > 850, white symbols). All cultures were resuspended to OD 1 in the indicated media at time point 0. Data are means ± S.E.M. of three independent cultures. Accompanying growth curves are presented in **Supplementary Figure S6**.

Early Kinetics of PndbA600 Activation

We have identified two types of media that accelerate the activation of PndbA600: the spent medium of stationary phase cultures and N-deficient medium. The spent medium is a complex solution comprised of a combination of different levels of multiple nutrient deficiency, as well as extracellular metabolites that have been secreted by the cells throughout culture growth. To check whether N depletion is the cause of PndbA600 activation in the spent medium, we compared PndbA600 activation in response to the spent medium with N-deficient medium (**Figure 8**). In the spent medium, PndbA600 activation is > 10-fold faster (within 30 min in low to low OD, spent media) compared to N-deficient medium (with 24 h in low to low OD, 0% N, A+). This suggests that nutrient deficiency, specifically N depletion, is not sufficient to explain the rapid activation in the spent medium and that other factor(s) contribute to PndbA600 activation.

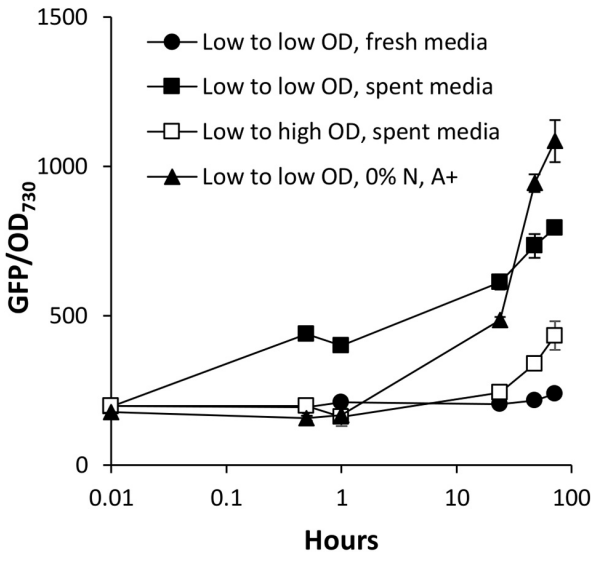

FIGURE 8 | Early kinetics of PndbA600 activation. Early responses of PndbA600 in *Synechococcus* PndbA600:GFP to different treatments at time point 0: low OD < 5 to low OD 1 in fresh control medium (black circles), low OD < 5 to low OD 1 in spent medium (black squares), low OD < 5 to high OD 12 in spent medium (white squares), or low OD < 5 to low OD 1 in N-deficient medium (0% N in A + background, black triangles). Data are means ± S.E.M. of three independent cultures.

The Effect of Electron Transport Inhibition on PndbA600 Activity

Type II NADH dehydrogenases play a central role in the respiratory metabolism of bacteria; however, this is not the case in cyanobacteria where NdbA function remains unclear (Howitt et al., 1999; Lea-Smith et al., 2016; Huokko et al., 2019). A role in redox sensing has been proposed (Howitt et al., 1999), so we tested the effect of changing cellular redox status on promoter activity by disturbing electron transport. For this experiment, electron transport inhibitors specific to photosynthesis [DCMU, which blocks the plastiquinone binding site of photosystem II (Duysens and Sweers, 1963)] or respiration [malonic acid (MA), which competitively inhibits succinate dehydrogenase complex II (Pardee and Potter, 1949)] were applied to low or high density cultures of *Synechococcus* PndbA600:GFP grown under control conditions. Inhibitors were applied at two concentrations: a lower concentration, which allowed for growth of low density cultures (0.1 μM DCMU and 4 mM MA), and a high concentration, which inhibited the growth of low density cultures (1 μM

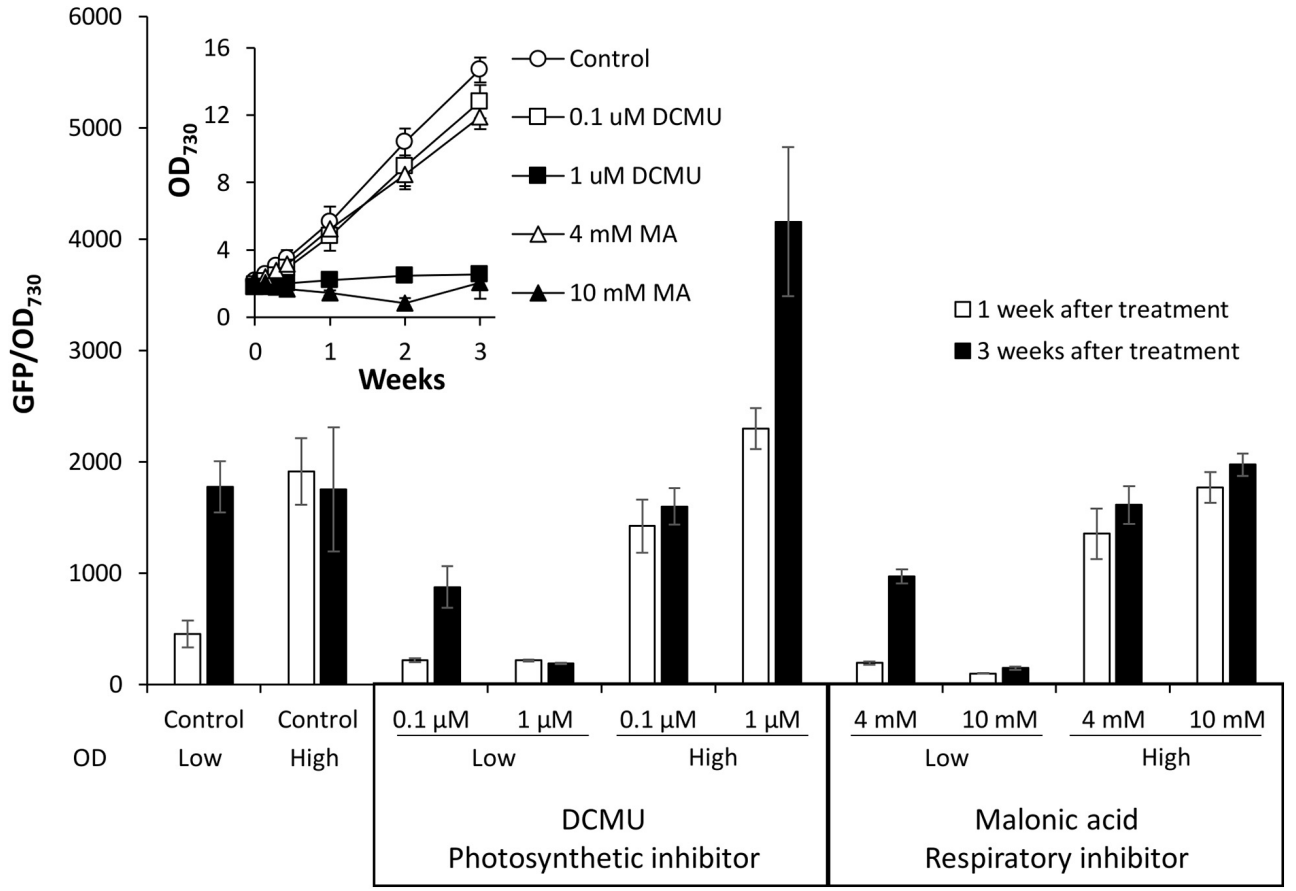

FIGURE 9 | The effect of electron transport inhibition on PndbA600 activity. PndbA600 activity (GFP fluorescence normalised to OD_{730}) in *Synechococcus* PndbA600:GFP 1 week (white bars) and 3 weeks (black bars) after treatment with DCMU or malonic acid at the given concentration. OD indicates low (OD < 5) or high (OD < 12) culture density at the start of treatment. Control are untreated cultures. Data are means ± S.E.M. of at least three independent cultures. Inset shows culture growth starting from low OD in the different conditions.

DCMU and 10 mM MA; **Figure 9**). When the inhibitors were applied at low concentration in young, low density cultures, PndbA600 activation in the late growth phase still occurred, albeit at a lower level than without inhibitor. The slight decrease in activity may reflect the slower growth and lower density of the inhibitor-treated cultures. By contrast, high inhibitor concentrations resulted in a lack of culture growth and of PndbA600 activation. In mature, high density cultures, low concentrations of inhibitors did not alter PndbA600 activity. At high concentrations, however, DCMU increased PndbA600 activity 1.79-fold in high density cultures whereas MA did not. These results show that the inhibition of photosynthesis, but not respiration, enhances PndbA600 activity in high density cultures.

DISCUSSION

Two-stage cultivation strategies are an attractive solution to growth/productivity trade-offs in cyanobacteria; however, the costly addition of extra steps between growth (stage I) and production (stage II) is often required to initiate stage II (Lee et al., 2012, 2016; Monshupanee et al., 2016; Kushwaha et al., 2018; Aziz et al., 2020). Less effort has been made towards utilising inherent features of cyanobacterial cultures to distinguish between stages and thus auto-induce transgene expression in stage II, cutting costs and improving economic feasibility. Auto-inducible production systems have been engineered using nutrient-deficiency responsive regulators (Liu et al., 2011; Asada et al., 2019); however, their applications may be limited by the regulatory nutrient and the timing of deficiency. Here, we instead opted to develop regulatory systems based on growth phase transitions in batch cultures and endogenous regulation by stationary phase promoters. A few studies led to the identification of cyanobacterial growth phase-responsive genes and promoters (Foster et al., 2007; Berla and Pakrasi, 2012; Ruffing et al., 2016); however, the knowledge is still very limited for the stationary phase and its regulation in cyanobacteria. In this study, we identified a small subset of genes that specifically respond to growth phase transition in *Synechocystis* sp. PCC 6803. Furthermore, we report the first detailed description of the complex environmental responses of a growth phase-responsive promoter of cyanobacteria.

General Responses to Changes in Growth Phase

Common responses of nutrient limitation studies form the main foundation of knowledge surrounding stationary phase in cyanobacteria and generally involve increased catabolism and decreased anabolism (Schwarz and Forchhammer, 2005). Direct comparisons of cyanobacterial growth phases have focussed on transcriptional responses in at most two conditions (Foster et al., 2007; Ludwig and Bryant, 2011; Berla and Pakrasi, 2012; Kopf et al., 2014). These studies have led to the identification of differentially expressed genes; however, it is difficult to differentiate between genes involved in general responses to changes in the growth phase and specific responses to the conditions in which the cultures were grown. Furthermore, it is often difficult to confirm that these datasets do in fact reflect stationary phase gene expression as low stationary phase ODs are commonly reported without any accompanying growth curves. Here, we present a comprehensive RNA sequencing dataset that enables the robust separation of growth phase-specific responses from condition-specific responses in *Synechocystis* sp. PCC 6803 (**Supplementary Table S3**). Our dataset agrees with general, growth phase-related downregulation of genes involved in photosynthesis, energy metabolism, and translation reported by previous nutrient limitation studies (Hirani et al., 2001; Richaud et al., 2001). Of the genes upregulated in the late growth phase/early stationary phase, 67% are annotated as hypothetical proteins reflecting a large gap in knowledge that persists on growth phase-specific responses in the extensively studied model cyanobacterium *Synechocystis* sp. PCC 6803. Besides their purpose for this study, the datasets provided in **Supplementary Table S3** represent a new resource for understanding transcriptional responses of *Synechocystis* sp. PCC 6803 to individual nutrient deficiencies (including new transcriptomes in low Mg and low K).

Orthogonal Promoter Behaviour in Cyanobacteria

Orthogonal molecular parts are preferred for metabolic engineering in order to avoid the interference of engineered systems by host machinery and genetic instability arising from endogenous DNA sequences (Camsund and Lindblad, 2014). Well-established prokaryotic tools such as IPTG- and tetracycline-inducible systems from *E. coli* perform poorly in cyanobacteria (Huang et al., 2010). Promoters derived from other species of cyanobacteria appear to have greater success despite regulatory differences between cyanobacterial species (Huang et al., 2010; Wang et al., 2012; Gordon and Pfleger, 2018). For example, the phycocyanin promoters (Pcpc) of *Synechocystis* sp. PCC 6803 and PCC 6714 have been used to drive heterologous expression in *Synechococcus* sp. PCC 7002 and PCC 7942 (Markley et al., 2015; Gao et al., 2016). Furthermore, a previous report showing higher activity of the heterologous phycocyanin promoter in the early growth phase of *Synechococcus* sp. PCC 7002 agrees with the expression profiles of the native phycocyanin operon in our *Synechocystis* sp. PCC 6803 RNAseq dataset (Madsen et al., 2018). Nevertheless, our detailed analysis of heterologous PndbA600 activity in *Synechococcus* sp. PCC 7002 revealed clear differences to the endogenous *ndbA* expression profiles in *Synechocystis* sp. PCC 6803. While expression patterns were similar under control conditions (**Figures 3B,C**, **4A,B**), closer examination under nutrient limitation revealed differences in correlations between the gene expression, culture density, and the growth phase in the two species (**Figure 6**). The promoter activation during the late growth phase observed under control conditions in this study is not an artefact of the assay, as evidenced by previous characterisation of an early growth phase-specific promoter using the same approach (Madsen et al., 2018). While this suggests that the condition-dependent activation profiles of PndbA600 should reflect promoter response to growth phase status, further studies using a truncated version of PndbA600, which has lost the ability to respond to changes in the growth phase, will strengthen this finding. This study, therefore, highlights the importance of thorough characterisation of molecular components to enable rational design and accurate prediction of the behaviour of more complex assemblies in non-standard conditions.

Environmental Regulation of PndbA600

This study also presents the first detailed analysis of a growth phase-responsive promoter of cyanobacteria. Responses to changing environmental stimuli, including culture density, growth media, nutrient availability, and cellular redox status, showed differing requirements for PndbA600 activation and deactivation (**Figure 10**). PndbA600 activation could be induced either by lowering N supply or presenting Factor X from the spent medium of stationary phase cultures. Interestingly, culture density *per se* had no effect on PndbA600 activation, but low culture density was required for PndbA600 deactivation in addition to sufficient N supply and the absence of Factor X. Furthermore, PndbA600 may respond in a dose-dependent manner to Factor X. Increasing the relative amount of Factor X per cell may have a proportionate effect on PndbA600 activity. For example, PndbA600 activation in spent media was > 100-fold faster in young cultures resuspended at low density compared to high density (**Figures 5B**, **8**). PndbA600 activity in spent media was also higher in mature cultures resuspended at low density compared to high density (**Figure 5D**). Similarly, mature cultures transferred from spent to N-deficient media showed a visible reduction in GFP fluorescence, which could be due to the removal of Factor X (**Figure 7B**). Additional analyses are required to identify Factor X, which could be either a downstream response to N limitation or an unrelated extracellular metabolite e.g., signalling molecule secreted by stationary phase cells. Compositional analyses of spent media fractions paired with gene expression and promoter analyses could yield further insights into stationary phase and its regulation in cyanobacteria.

While heterologous promoter activity may not accurately reflect endogenous transcriptional regulation, this study could provide initial insights into growth phase-specific regulation and NdbA function in cyanobacteria. The *ndbA* gene encodes a type II NADH dehydrogenase of unknown function. The ability of *ndbA* knockout strains of *Synechocystis* sp. PCC 6803 to grow under otherwise lethal high-light conditions in

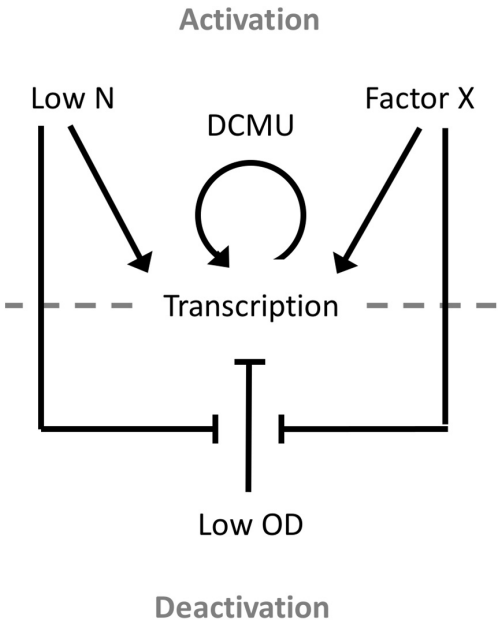

FIGURE 10 | Model of the environmental regulation of PndbA600. Promoter activation and, thus, transcription occur in response to either low N or Factor X of the spent stationary phase medium. Once activated, promoter activity can be further increased using the photosynthesis-specific inhibitor DCMU. Promoter deactivation requires low culture density, sufficient N supply, and the absence of Factor X.

a PSI-less background led to the suggestion of a regulatory role for NdbA and monitoring of, e.g., cellular redox status (Howitt et al., 1999). In support of these functions, native NdbA localises to the thylakoid membrane (Huokko et al., 2019), and the PndbA600 promoter responds to disturbances in electron transport in the heterologous expression host (**Figure 9**). Specifically, heterologous PndbA600 responds to photosynthetic, but not respiratory, inhibition, whereby the key difference is the reduction of NADP + in photosynthesis and oxidation of NADH in respiration. These findings, paired with the presence of NADH binding motifs in the *ndbA* coding sequence (Howitt et al., 1999), suggest that NAD(P) + /NAD(P)H balance may be an important factor regulating PndbA600/NdbA activity levels.

Industrial Applications

This study describes first steps towards developing regulatory systems to drive stage II of a two-stage cultivation system in cyanobacteria. Libraries of stage II promoters can now be constructed based on PndbA600 or other late growth phase-responsive promoters and, subsequently, used to optimise heterologous metabolic pathways for industrial production or fine-tune endogenous metabolic pathways supplying precursors necessary for the engineered process. Similarly, libraries of stage I promoters can be constructed to optimise growth, potentially improving growth rates and thus decreasing time scales until the initiation of stage II. Furthermore, many additional analyses can be performed on the RNAseq dataset to identify genes/promoters with any combination of growth phase- and/or nutrient-specific activity for diverse applications in industry, e.g., biosensors.

Stationary phase may not be suitable for all industrial applications or commercial products, and therefore, a careful selection of products and processes is important. While general decreases in anabolism occurs during the stationary phase, select processes continue at appreciable levels even after prolonged starvation (Schwarz and Forchhammer, 2005). Notable examples are secondary metabolites important for human health, particularly as anti-infective drugs such as antibiotics (Ruiz et al., 2010). Here, we used a new approach for the robust separation of growth phase- vs. condition-specific processes. This approach can also complement bioprospecting for new secondary metabolites in cyanobacteria and other microorganisms by comparing transcriptomic and metabolomic data across a variety of conditions to identify genes and unravel biosynthetic pathways underpinning the production of interesting metabolites. The large proportion (67%) of late growth phase-specific genes encoding hypothetical proteins identified in *Synechocystis* sp. PCC 6803 highlights the strength of this approach, as well as the great potential for the identification of new cyanobacterial products and pathways.

As a chassis, *Synechococcus* sp. PCC 7002 has numerous advantages for industrial production, including relatively fast growth rates and high tolerance to various parameters such as light, temperature, and salinity (Nomura et al., 2006). Another desirable feature we have observed in this strain is sedimentation in the stationary phase, which allows for easy biomass harvest at the end of stage II without the need for energy-demanding techniques such as centrifugation (data not shown). Perhaps, the most sustainable application of PndbA600-driven two-stage cultivation strategies involves seeding the engineered *Synechococcus* cultures in the fresh growth medium, biomass accumulation during stage I until nutrient depletion results in the auto-induction of stage II, and finally application-specific downstream processing of the biomass and supernatant. Recycling stage II cells to seed new cultures is not ideal as we observed a decrease in the amount of biomass attained, no increase in promoter activity, and no decrease in time to stage II (high to low OD in fresh media, **Supplementary Figure S5C**). By contrast, maximum biomass can be further increased by concentrating low density cultures in fresh nutrients to generate higher culture densities compared to control conditions (low to high OD in fresh media, **Supplementary Figure S5A**). Alternatively, if the expense of time is greater than the benefit of high biomass, stage II can be induced at lower culture densities by using nutrient limitation to significantly reduce timescales. Early induction could prove particularly profitable if nutrient-specific responses increase the productivity of stage II cells. Finally, stage II productivity can be further improved by increasing the activity of the auto-inducible promoter, either by engineering PndbA600 or adding supplements such as DCMU.

This study provides the first insights into the regulation of stationary phase in cyanobacteria. Additional studies to identify DNA motifs present within growth phase-responsive

promoters, transcription factors that bind to these motifs, and other regulatory molecules will provide further important insights to this still elusive phase of cyanobacteria. Unravelling these mysteries and expanding the foundation of knowledge surrounding these organisms will be of great value to both academia and industry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB40560.

AUTHOR CONTRIBUTIONS

MAM and AA designed the study and wrote the manuscript. MAM performed the experiments. MAM, GH, PH, and AA analysed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.619055/full#supplementary-material

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REVIEW
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Advantages of Heterotrophic Microalgae as a Host for Phytochemicals Production

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Currently, most commercial recombinant technologies rely on host systems. However, each host has their own benefits and drawbacks, depending on the target products. Prokaryote host is lack of post-transcriptional and post-translational mechanisms, making them unsuitable for eukaryotic productions like phytochemicals. Even there are other eukaryote hosts (e.g., transgenic animals, mammalian cell, and transgenic plants), but those hosts have some limitations, such as low yield, high cost, time consuming, virus contamination, and so on. Thus, flexible platforms and efficient methods that can produced phytochemicals are required. The use of heterotrophic microalgae as a host system is interesting because it possibly overcome those obstacles. This paper presents a comprehensive review of heterotrophic microalgal expression host including advantages of heterotrophic microalgae as a host, genetic engineering of microalgae, genetic transformation of microalgae, microalgal engineering for phytochemicals production, challenges of microalgal hosts, key market trends, and future view. Finally, this review might be a directions of the alternative microalgae host for high-value phytochemicals production in the next few years.

Keywords: microalgae, heterotroph, phytochemical, transformation, host system

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INTRODUCTION

Plant chemicals or phytochemicals are chemicals that may have biological activities produced by plants. Phytochemical sources come from fruits, vegetables, whole grains, nuts, seeds, leaves, bark, flowers, and other part of plants. Bioactive phytochemicals have been extensively studied *in vitro* and *in vivo* models due to their great potential for human consumption. Generally, phytochemicals were classified into six major categories based on their chemical structures and characteristics (**Figure 1**) including lipids, carbohydrates, terpenoids, phenolics, alkaloids, and other nitrogen-containing compounds (Xiao et al., 2016). Similarly, microalgae are promising natural sources of various bioactive compounds, such as polysaccharide paramylon, polyunsaturated fatty acids, and pigments (e.g., phycocyanin, phycoerythrin, astaxanthin, and etc.) (Chakdar et al., 2020).

Currently, most commercially obtainable recombinant technologies rely on host systems, which are organisms that can produce valuable proteins and bioactive compounds via genetic engineering, such as bacteria, yeast, transgenic animals, and transgenic plants. However, each host has their own benefits and drawbacks, depending on the target products. When eukaryotic plant compounds are the set goal, bacteria and yeast are not suitable because they lack post-transcriptional and

post-translational mechanisms (e.g., glycosylation, splicing, and protein assembly) (Koo et al., 2013). Even though bacteria are frequently used for recombinant proteins, bacterial endotoxin and protease contaminants are concerned in biopharmaceutical products. Yeast is an excellent eukaryotic host because of its low cost and up-scalability, however, hypermannosylation, which commonly occurs in yeast, leads misfolded proteins and activity malfunction (Yusibov and Mamedov, 2010). Most biopharmaceutical products are manufactured in animal cells, but animal hosts still have some limitations, such as low yield, high cost, expensive medium, and virus contamination, making them unsustainable as a host in medical applications. Plant-based expression systems can solve the following problems, such as having a eukaryotic mechanisms, no hypermannosylation, and etc. However, plant hosts have to deal with some limitations and environmental issues, including the spread of genetically modified plants (GMO), allergic reactions to plant components, contamination of proteins, regulation of medical protein permission, and a long production period (Koo et al., 2013).

Eukaryotic algae, especially green microalgae, share evolutionary ancestry with land plants (Novoveska et al., 2019; Saini et al., 2019). They hold incredible metabolic potential and possess most criteria for being a good host of eukaryotic phytocompound expression. These criteria include: (i) microalgae are a various group of microscopic plants that share a common ancestor, thus it might have less complexity to modify their genetic pathway for producing plant chemicals, (ii) many microalgal species have ability to grow in extreme conditions, so the cost will be minimized related to no steady environmental conditions, (iii) post-translational modification pathways of microalgae are numerous to enable proper maturation for a variety of protein, especially for plant compounds (Scaife et al., 2015; Weiner et al., 2018).

Normally, microalgae are considered photoautotrophic organisms, whereas heterotrophic cultivation, which can use external carbon sources under dark conditions, has also been used to obtain high value products. Heterotrophs have many advantages compared to autotrophs, such as growing on a larger scale, having more FDA-approved standards and protocols for industrial fermenters, and ability to grow in higher cell density, among others (Rasala and Mayfield, 2015). Green microalgal hosts have been continually developed for expression. In this paper, several green microalgal hosts and their genetic toolboxes, including transformation methods, vectors, promoters, and selectable markers are presented, with a major focus on heterotrophic microalgae for phytochemical biosynthesis in an attempt to address the above concerns.

ADVANTAGES OF HETEROTROPHIC MICROALGAE AS A HOST

Microalgae are also known as single-cell algae that have a vital role in the food chain. Interestingly, microalgae can produce other nutrients that are also found in higher plants, including synthesizing lipids, fatty acids, proteins, nucleic acids, carbohydrates, fibers, starches, vitamins, and antioxidants (Klamczynska and Mooney, 2017). Unicellular microalgae present in a wide range of habitats and can be cultured in three cultivation conditions: autotrophic, heterotrophic, or mixotrophic mode (**Figure 2**). Autotrophic microalgae use energy from photosynthesis to grow, while some microalgae can grow in the dark using organic compounds as carbon and energy sources, which is called heterotrophic microalgae. Mixotrophic microalgae can use both supplied organic carbons and light energy in cultivation. Nowadays, many researchers have studied the production of pharmaceutical proteins, antibodies, and valuable compounds in microalgae (Koo et al., 2013; Dreesen et al., 2010).

Recently, attention has been drawn to microalgae as simple models for a sustainable source of high-value compounds, ranging from therapeutic proteins to biofuels (Rosenberg et al., 2008; Huang et al., 2010; Gong et al., 2011; Yang et al., 2016). Apparently, autotrophs and mixotrophs have drawbacks, which are described in detail below. Hence, the focus moves to heterotrophic microalgae that can grow well in the dark, like yeast and bacteria, by using simple carbon sources, such as glucose. Other advantages of heterotrophic microalgae for expression of phytochemicals include the following:

(1) Compared with traditional used host, prokaryotic hosts are the most commonly used platforms. Due to post-translational modification and protein localization are important for the production of phytocompounds or eukaryotic substances, whereas, prokaryotic *Escherichia coli* is not always the easiest hosts for this process (Yang et al., 2016). However another eukaryotic hosts including insect, mammalian cells, and transgenic animals may overcome these obstacles, but these systems might suffer from other limitations, such as virus contamination, proteolysis, expensive cost, incorrect glycosylation, high nutrient requirement, and long generation time (Gomes et al., 2016). Hence, alternative hosts are still needed. For example, eukaryotic microalgae, this is because they give the advantages of fast growing, low cost, ease manipulation, and etc. (Yang et al., 2016). Moreover, they allow glycosylated proteins to be secreted into the cell from post-translational modification pathways (Lauersen et al., 2013). The comparison of advantages and disadvantages to produce plant compounds among host systems and other methods is summarized in **Table 1**.

(2) Compared to plant cultivation and synthesized phytochemicals, microalgae are easily scalable in fermenters or bioreactors compared to plant cultivation because they can be constructed on any land type or industrial site (Melis, 2012). This shows that microalgae are non-seasonal, not dependent on climatic conditions, and do not need arable land (Lopes et al., 2019). Even if plant compounds can be synthesized by using chemicals instead of cultivation, in some cases, the complexity of their structure, which requires difficult multistep reactions, leads to high costs, very low yield, and unwanted effects for pharmaceutical product. Synthesized compounds are designed and utilized synthetic DNA parts, whereas metabolic engineering involves protein and pathway optimization for improving the yield of products (Stephanopoulos, 2012).

(3) Compared to transgenic plant, microalgae share evolutionary ancestry with land plants. That means genetic

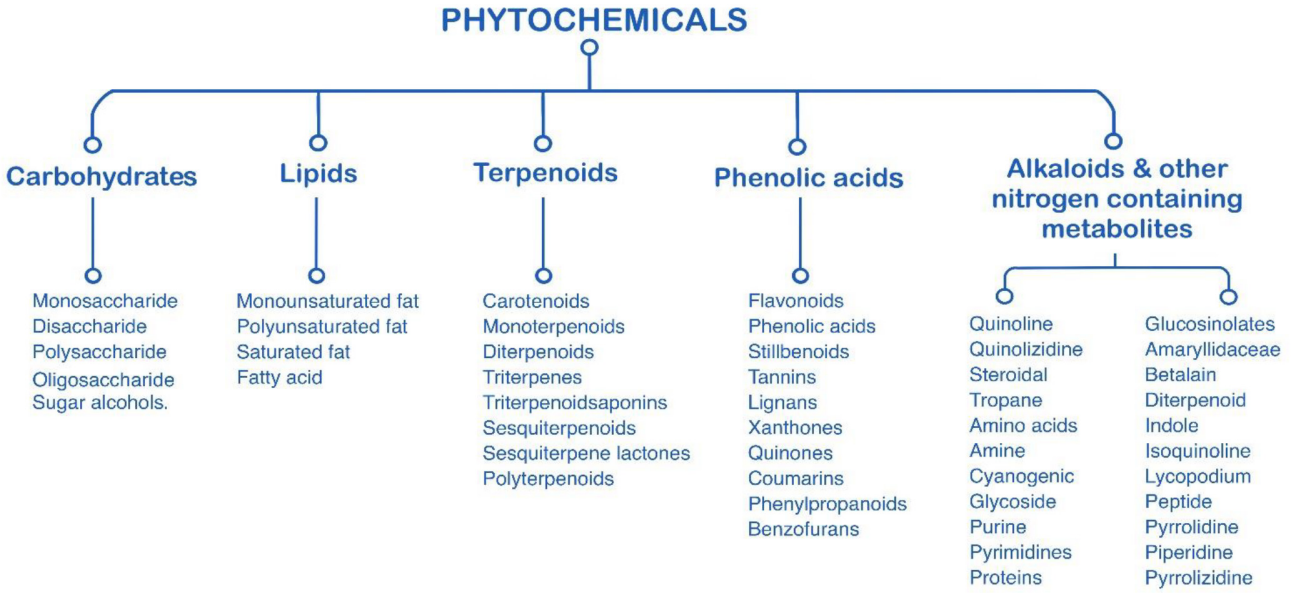

FIGURE 1 | Characterization of phytochemicals (adapted from Xiao et al., 2016).

manipulation techniques might be easily adapted to microalgae, such as codon optimization, intron addition, expression methods, and vectors (Scaife et al., 2015). For transgenic plants to express any gene, there are limitations. First, plant cell suspension culture or plant tissues can grow in fermenters, but they are limited to a few plant species compared to a wide range of microalgae. Microalgae cells might be more favorable for plant compounds production than yeast, bacteria, or others hosts because microalgal cellular environments are suitable for those exogenous plant enzymes. Additionally, microalgae metabolism contains production of precursors which are more associated with phytocompounds production more than prokaryotic host (Lauersen, 2018). For transgenic plants, there are only a few examples that have been commercially developed and there are still bottlenecks for commercial production, compared to a microalgal host. Second, the procedures to transform genes take longer periods of time than in a microalgal host; for example, expression in tomato requires more than a year, while green microalgae need a few days (Canto, 2016). Moreover, microalgae require only a few months to scale up compared to transgenic plants; for instance, tobacco plants take 6 months to grow after regeneration. However, apart from Faè et al. (2017) research, it is assumed that the specific activity of the enzyme produced by *Chlamydomonas* and tobacco are alike, as both proteins synthesis machinery in chloroplast is highly conserved. Faè et al. (2017) suggested that algal molecule farming is still desirable for high value pharmaceutical production. Third, there are concerns about transgenic plants transferring genes to the environment via pollen, which might not occur in microalgae, especially in heterotrophic microalgal hosts because there is no in and out for contaminated sources in the fermenter. Forth, product expression from plants might be contaminated with agrochemicals and fertilizers, so downstream cultivation after expression should be considered (Gomes et al., 2016). Finally, the main differences between the application of higher plant systems and microalgae for biotechnology is the scalability of cultivation in fermenters (Yu et al., 2013).

(4) Compared among microalgae cultivation, heterotrophic microalgae have more benefits, such as cheaper nutrients, low cost of instruments, and easy to operate and maintain. They can be adapted to a large scale with no cell density and less-stress concerns in only a few weeks (Yang et al., 2016). Autotrophs use CO_2 and light as inorganic carbon and energy sources, whereas heterotrophs use organic carbon as a source of carbon and energy (Lopes et al., 2019). Several species including *Chlamydomonas reinhardtii*, *Auxenochlorella protothecoides*, *Chlorella pyrenoidosa*, *C. vulgaris*, and *C. zofingiensis* can be grown in low-cost industrial waste products (Abreu et al., 2012). Although autotrophic microalgae can be cultured in large scale production, there are some disadvantages: only a few centimeters of light/sunlight penetrate the surface, which reduces cell growth; high cell density is related to low yield; high cost of transparent material for gaining light; difficult to design narrow photo-bioreactors; significant financial investment for energy use and maintenance; difficult to maintain in mono-culture; need continuous and clean water; and not compatible with pharmaceutical or food production (Wolf et al., 2016; Barros et al., 2019). For biomass yields, heterotrophs make 50–100 g/L of cell dry weight. This number is higher than that of autotrophs, which reach a maximum 30 g/L of cell dry weight (Perez-Garcia et al., 2011). Moreover, under heterotrophic conditions, *Chlorella* growth is approximately 5.5 times higher than cultures under light conditions (Yu et al., 2013). In particular, the period for scale-up of heterotrophic microalgae is shorter than autotrophic microalgae (**Figure 3**). In addition,

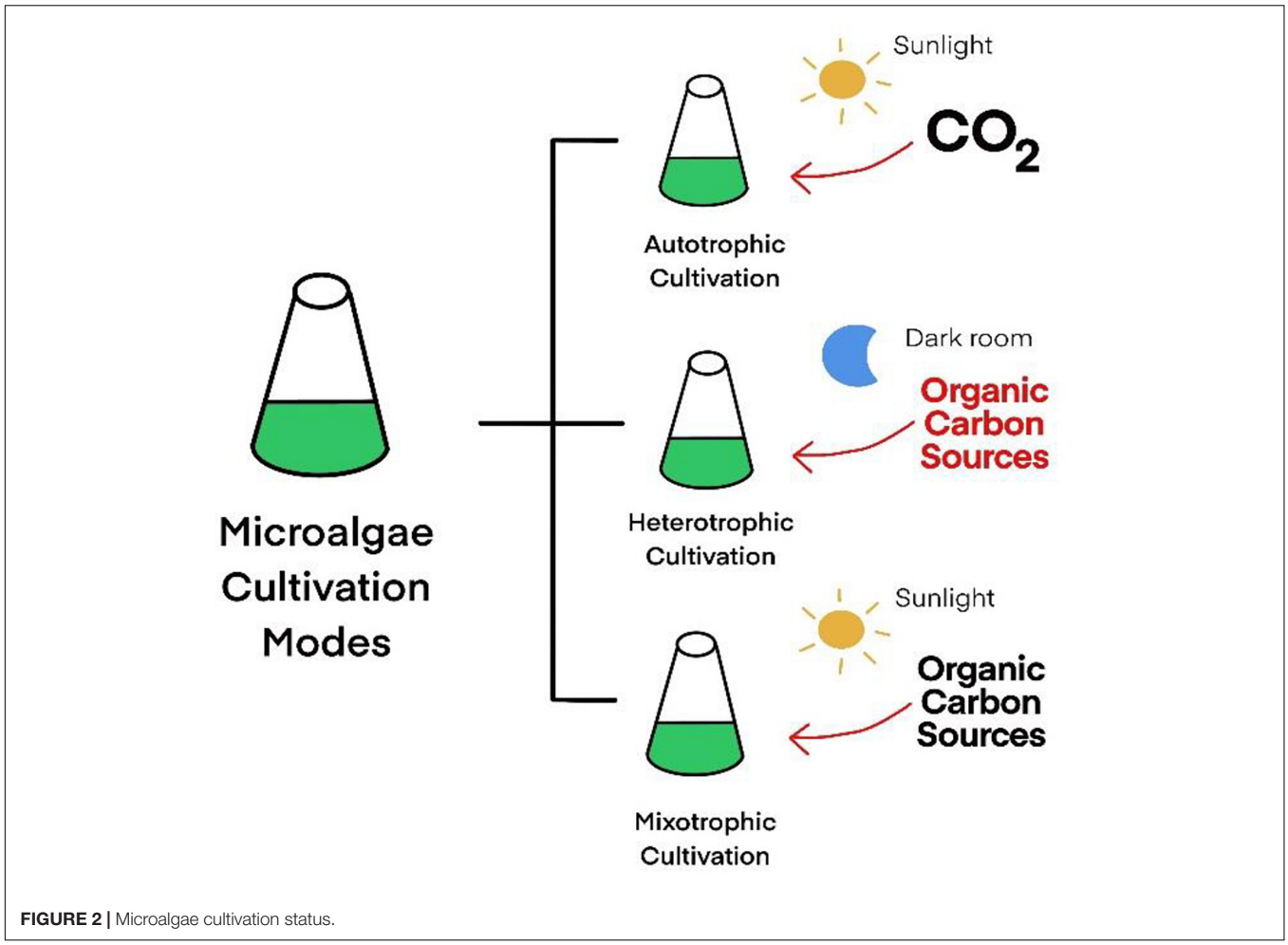

FIGURE 2 | Microalgae cultivation status.

the overall area cultivation for heterotrophs is 12 times less than that of autotrophs (Barros et al., 2019). From one study, it was shown that there is high impact of heat and energy use for autotrophs, but for heterotrophic microalgae, these are controlled by glucose feedings (Smetana et al., 2017). The carbon intermediates of heterotrophs are transformed into main metabolic pathways, replacing photosynthetically produced molecules (Morales-Sánchez et al., 2015). While, some autotrophs are able to grow in the dark, the central carbon metabolism of autotrophic growth involves incomplete pathways or the absence of an enzymatic reaction, which is a primary cause of obligation to consume vital substrates, particularly sugars, and other carbon sources (Morales-Sánchez et al., 2015). Thus, culturing heterotrophs in a fermenter might be a better option.

(5) In medicine, where production for humans is regulated under strict safety aspects (Gellissen, 2005), there are a variety of suitable microalgae that can be selected from their Generally Recognized as Safe (GRAS) status, depending on the purpose. For example, *Chlorella vulgaris* (a green alga) is normally used as a food additive, feed for animals, and diet supplements. Moreover, *Arthrospira platensis* (*Spirulina platensis*; a cyanobacterium), which has high protein and nutrient contents, is consumed as food and feed (Yaakob et al., 2014). Therefore, this is a great opportunity to develop these microalgae as a host.

(6) When considering the environmental impact of host systems, there are three main indicators, namely less greenhouse gas emissions, low water supply, and efficiency of land use. Heterotrophic microalgae offer these three main criteria. A study found that whole algae protein has a lower water footprint than beef and whey but more protein per hectare than other sources (Klamczynska and Mooney, 2017). Moreover, using simple media for the cultivation of algae is as low as $0.002 per liter compared to mainly using mammalian hosts, which cost $150 per gram (Taunt et al., 2018).

A suitable heterotrophic microalgae should have the following essential criteria: ability to grow without light, can be cultured on inexpensive and easily sterilized media, rapidly adapt to new surroundings, and the ability to endure hydrodynamic stress in fermenters and other equipments (Chen and Johns, 1996; Wen and Chen, 2003). Many factors have to be considered for culturing heterotrophic microalgae, including temperature, medium salinity (NaCl), pH, and dissolved O_2. In the heterotrophic status of *Chlorella sorokiniana*, high aeration increased cell growth, fatty acid yield, and unsaturated dienoic and trienoic fatty acids; conversely, this decreased

TABLE 1 | Brief comparison of merits and demerits among different host systems and plant cultivation.

Prokaryote host		**Eukaryote host**							**Plant cultivation (without engineering technology)**
Bacteria	**Yeast**	**Mammalian cells**	**Transgenic animals**	**Transgenic plants**	**Plant cell suspension**	**Microalgal host** • **Share evolutionary ancestry with plants** • **More favorable for plant compounds**			
						Autotroph	**Mixotroph**	**Heterotroph**	
• Common use • Rapid growth • Low cost • High yield	• Widely used • Rapid growth • Low cost • Biosafety	• Proper protein folding • Effective transcriptional and translational modification	• Proper protein folding • Appropriate post-translational modification • Proper glycosylation	• Effective transcriptional and translational modification • Low cost (1–5$/mg for production) • Localized to different organs	• Rapid growth (compared to transgenic plant) • Can secrete products into culture or maintain them in the cell • Grow in Fermenter (less environment concerns)	• Stainable source • Have more genetic toolboxes	• Higher growth rate and biomass	• Grow well in the dark (same as yeast and bacteria) • Use simple carbon sources and wastewater • Easy scaling up • Cheaper nutrients • Low industry cost • No cell density concerns • Requires less area than	• Extract the product from the
• No chaperones • No post-translational modifications • Have insoluble inclusion bodies • Endotoxin • Not suitable for plant products	• High cost (compared to bacteria) • Over-expression (e.g., mannose glycosylation or disulfide bond misfolding) • Not suitable for plant compounds	• Higher cost (150$/g) • Complicated technology • Protein contamination with animal viruses • Incorrect glycosylation • Long generation time	• Higher cost (500,000$/animal) • Low yield • Animal virus contamination • Long production period	• Long cycle time • Imprecise growth conditions • Gene flow contamination • Toxic alkaloids from tobacco	• Unclear permeability of plant cell wall • Limited to a few plant species • Less success • Lower yield	• a few depth of light penetration into surface • Low yield due to cell density • Higher cost • Requires clean water • Less yield than heterotrophs	• Effect of temperature is unknown	• Less genetic engineering research compared to autotrophs	• Cannot grow on every land type or industrial site • Depend on seasonality and climatic conditions • Less productive per unit land area compared to microalgae

, *advantages;* , *disadvantages.*

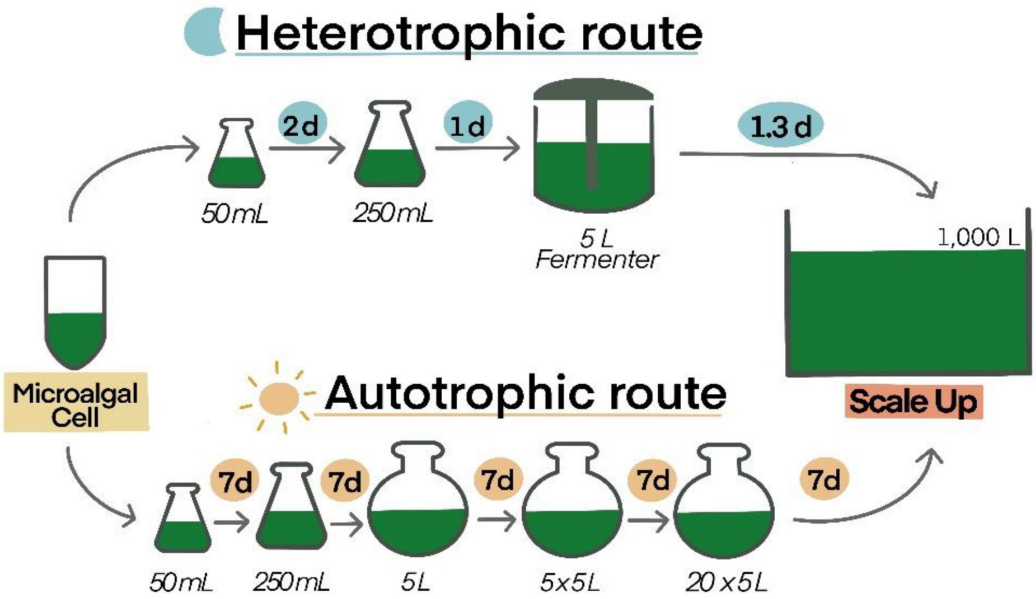

FIGURE 3 | Comparison of time consumption between autotrophic and heterotrophic routes of *Chlorella vulgaris*. Culture volumes (liters) and duration (d: days) of each scale up step are demonstrated (Barros et al., 2019).

cell lipid content (Chen and Johns, 1991). In heterotrophic metabolism, carbon is broken down in the same way used by bacteria. Complex molecules, like starch, are metabolized via the Embden-Mayerhoff-Parnas Pathway (EMP pathway or glycolysis) or the Pentose Phosphate pathway (PPP). However, heterotrophic culturing has some limitations, including high cost by adding more organic substrates, contamination or competition with other microorganisms, and unproduced light-induced metabolites (Perez-Garcia et al., 2011).

GENETIC ENGINEERING OF MICROALGAE

Recently, the development of microalgae biotechnological platforms has been continually progressed, especially from a genetic engineering perspective. Microalgae have potential to act like a cell factory to produce other compounds and proteins at economical levels. To date, over 40 different microalgae species, such as *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Chlorella vulgaris*, and *Haematococcus pluvialis*, have been successfully genetically manipulated. The available genetic tools are for both nuclear and chloroplast transformation for *C. reinhardtii* and *Phaeodactylum tricornutum*, however, there is still a lack of genetic toolboxes and applications compared to others host systems. In the green microalgal host area, algal genome data and transformation protocols are available (Gangl et al., 2015). However, research is rarely found for heterotrophic microalgal hosts, even if they are normally used on an industrial scale. These hosts have less limitation in the recombinant technological field compared to autotrophic microalgae and some other hosts.

Microalgae generally consist of nuclear, mitochondrial, and plastid genomes (Radakovits et al., 2012). Compared between nuclear and chloroplast transformation, which are crucially different (**Table 2**), chloroplast transformation allows higher accumulation of the desired protein (Faè et al., 2017). Several complete genomic resources are available in some species, such as the model of green microalga *C. reinhardtii*. Recently, many reports described genetic engineering of the chloroplast, which has small genome (205 kb) and less-complexity with only 99 genes. Although chloroplast transformation is also feasible for plenty of plant species, such as tobacco, tomato, and petunia, there are still fundamental challenges and less achievement reports than those for nuclear transformation (Gong et al., 2011). Nowadays, low cost sequencing technologies make more fully sequenced genomes of algae strains available (Jaeger et al., 2017). However, engineering strategies across all microalgae are difficult because their genetic contexts are highly specific, variable, and often poorly understood. Even if *C. reinhardtii* was a model organism, there is lack of a viable commercial production process and food safety (Taunt et al., 2018).

The chloroplast of green algae consists of gene machinery, including the ribosomes and translation factors, however, it is not similar to bacteria because the chloroplast contains a wide range of chaperones, protein disulfide isomerase, and peptidylprolyl isomerases. These chaperones aid in complex protein folding, and as a consequence, this unique biochemical environment allows for the expression of high-valuable biopharmaceuticals (Rasala and Mayfield, 2015). In fact, heterotrophic processes might limit the development of chlorophyll because it is no longer needed for metabolism (Klamczynska and Mooney, 2017).

TABLE 2 | Differences between nucleus and chloroplast transformation; adapted from Rasala and Mayfield (2015).

Genome engineering	Nucleus	Chloroplast
Gene expression mechanism	Eukaryotic	Prokaryotic
Silencing	More	Less
Protein localization	Cytoplasm, nucleus, chloroplast, ER*, mitochondria, secretion	Chloroplast
Modifications	Phosphorylation, glycosylation, disulfide bond	Phosphorylation, disulfide bond
Accumulation levels	Low (as high as 0.25% TSP reported)	High (1-21% TSP*)
Transformation methods	Electroporation, particle bombardment, glass beads, PEG*, Agrobacterium	Particle bombardment, glass beads, Agrobacterium
Integration mode	Non-homologous end joining	Homologous recombination
Inducible gene expression	Nutrient, chemical, physiological	Light inducible

**ER, endoplasmic reticulum; TSP, total soluble protein; PEG, polyethylene glycol mediated transformation.*

Currently, several new expression systems are commercially available, but some of them are private and need licensing. Many researchers are looking for other microalgal hosts because of the advantages of rapid growth, low cost, cheap medium, ease of culture, and board industrial applications. For example, *Chlorella* which has been chosen because for its fast growth with high cell density under various culture modes and adaptability to different conditions is interesting as a potential newcomer host for heterologous protein expression (Yang et al., 2016; Klamczynska and Mooney, 2017). They can be cultured in both autotrophic and heterotrophic culture.

Moreover, reducing culture time and high biomass might be better options for choosing microalgae that can double their biomass in less than 24 h, such as *Chlorella sorokiniana,* which has a doubling time of less than 3 h (Sorokin, 1967) and a new transgenic time of around 2 months on an industrial scale (Mayfield et al., 2007). One of the fastest growing species is *Chlorella vulgaris*, thus this specie is another promising algae model for genetic engineering. *Chlorella* species are future hosts for protein and glycoproteins, while diatom *Phareodactylum tricornumtum* has been shown to produce a fully functional anti-hepatitis antibody with high-mannose glycan (Mathieu-Rivet et al., 2014; Yang et al., 2016; Vanier et al., 2017).

For human consumption, *Spirulina* and *Chlorella* are best-known for nutritional properties. They are consumed in many forms, such as tablets, capsules, and liquids (Aron et al., 2020; Khoo et al., 2020), so this familiarization might be the answer for producing recombinant biopharmaceuticals in these microalgal hosts. Although there are many reports of successful recombinant technology in algae, there is only one report of transferring recombinant production to a large scale (Gangl et al., 2015). This shows that there are still gaps in the knowledge transfer from a lab scale to industrially relevant growth conditions for recombinant production. However, the cheap cost of culturing, potential for large-scale in fermenter growth, and many GRAS status species are advantages of heterotrophic microalgae. In the future, gaps might be filled in as the industry is continually growing.

GENETIC TRANSFORMATION OF MICROALGAE

There are many transformation methods for the delivery of genes into algal cells, including agitation by glass beads or silicon carbide whiskers, electroporation, polyethylene glycol (PEG) mediated transformation, particle bombardment, and Agrobacterium-mediated transformation (Kim et al., 2014). The cell wall of algae is a physical barrier for foreign DNA because of the cell membrane. Hence, many transformation methods depend on cell excluding the cell wall, which is called protoplasts. For instance, *Chlamydomonas* cell walls, which consist of glycoproteins and cellulose or chitin, can be degraded by autolysins, while *Chlorella* cell walls are composed of sugar polymers that can be degraded by sugar digesting enzymes (Kim et al., 2014). The most frequently used methods are particle bombardment and electroporation, however, agitation methods that have a lower transformation rate are often used because of the minimal equipment required. In contrast, Agrobacterium-mediated transformation has not been extensively used, and less information is known about its use in microalgae (Barrera and Mayfield, 2013). This transformation method is normally used in plant systems, thus, some researchers adapted this method for microalgae. From one report, some microalgae were electro-transformed, but the transformants were just a few. While Agrobacterium-mediated transformation had much more transformation rate when compared between ten microalgae (Suttangkakul et al., 2019). Thus, choosing the transformation method is determined by the cell size, nature of the cell wall, species, target organelles, cost, and especially the aim of the interested product. A comparison and some limitations of transformation methods are shown in **Table 3**.

Vector Construction

Common strategies have been considered, including increasing transcription levels by choosing strong promoters with appropriate enhancers and leader sequences, the improvement of translation via codon usage optimization, control of transgene copy number, gene product targeting by using signal peptide, and host genome position (**Table 4**).

To generate a plasmid vector, which is the critical step for genetic transformation, the vector might include the genetic elements (e.g., promoters, enhancers, reporters, marker genes, and codon usage). Promoters are a crucial factor for gene expression and have a significant transcriptional regulation effect. There are different types of optional promoters. In general,

TABLE 3 | Comparison between transformation methods.

Methods	Techniques	Cost	*Trans*-formant*	Limitations	References
Glass bead	DNA delivery is based on agitating protoplasts or cell wall-deficient using glass beads or silicon carbide whiskers with foreign DNA.	Low	1,000	- Effect of shear stress - Requires cell wall-deficient strain	Kim et al., 2014
Particle bombardment	-DNA-coated gold or tungsten micro-particle is delivered by using a specialized tool. -Does not require removal of the cell wall.	Very high	Very good	- Expensive tools - Size of the particle is an important factor for nuclear or plastid transformation (smaller size increases penetration) - Low repeatability - Complex operation process	Potvin and Zhang, 2010; Kim et al., 2014
Agro bacterium	Using Agrobacterium, DNA is transformed into host cells.	Low	20x glass bead	- Related to biological compatibility - Less known in microalgal host	Barrera and Mayfield, 2013
Electroporation	Using an electric pulse to push DNA into cells	High	2,500–7,137	- Uses specialized equipment - Requires strains without or a reduced cell wall - Random integration of genes - Optimal conditions depend on species (osmolality, temperature, concentration of DNA, voltage, electroporation buffer, pulse length, field strength, and capacitance) - If extreme conditions are used, it may cause a low cell viability due to the presence of cell walls.	Barrera and Mayfield, 2013
PEG-mediated	DNA delivery is based on agitating protoplasts or cell wall-deficient with PEG and foreign DNA.	Medium	356–2,250	- Requires cell wall-deficient strain - Factors affect the transformation (starting material, Agrobacterium density, co-cultivation conditions, acetosyringone concentration, etc.)	Cha et al., 2012

**transformant unit: cfu per μg DNA.*

high gene expression is positively correlated with a strong promoter. Some native promoters, including heat shock protein 70A (HSP70A), Rubisco small submit (RBCS2), or photosystem I protein D (psaD), are used in *C. reinhardtii* (Kim et al., 2018). Moreover, an inducible promoter is the one feasible choice for solving the effect of some proteins that might work on the growth of transgenic cells. Interestingly, some heterologous promoters that are widely used in plant transformation have been utilized in microalgae, such as the cauliflower mosaic virus (CaMV) 35S promoter and p1'2' *Agrobacterium* promoter, which drives the expression of GUS reporter genes (Jaeger et al., 2017). Thus, this can be a good sign for using microalgae as a plant compound host. Additionally, other commonly used promoters for microalgae are RBCS2, psaD, fcp, Pδ, GAPDH, CABII-1, NIT1, Ubi1-Ω, LIP, B12-responsive element, Actin1, NR gene, and CYC6 promoters. Currently, some researchers suggested that synthetic algal promoters (saps) can be used based on the characteristics of strong promoter motifs (Scranton et al., 2016). According to the research on *Chlorella* sp., expression promoters are in the early stages of development; only heterologous promoters from plant systems were used, such as 35S, ubiquitin, and NOs promoters (Run et al., 2016). Thus, further studies on expression and gene regulation in these microorganisms are necessary. From some studies, it was suggested that even when using the same construct, there are still variable expression patterns among different transformants, related to the number and location of recombination events. With supporting enhancers, transgene expression can be activated, no matter where the location of a target promoter is (Smallwood and Ren, 2013).

Reporter Genes

Reporter genes that encode easily recognizable proteins are useful for studying transformation efficiency, protein localization, and stability of transgenes. While selectable markers are proteins for helping the selection of positive transformants by

TABLE 4 | Some microalgal expression methods, vectors, and selectable markers.

Strains	Plasmids	Promoters	Expression methods	Selectable markers/ Reporter genes	References
Scenedesmus acutus	pCXSN-GEP	psaD, RBCS2	Agrobacterium	Hygromycin B	Suttangkakul et al., 2019
Chlamydomonas reinhardtii	pET-vp28	atpA	Glass bead	Spectinomycin	Kiataramgul et al., 2020
	pER123	–	Glass bead	Paromonycin	Mooi et al., 2018
	pSL18_HR	HSP70A	Electroporation	Paromomycin	Perozeni et al., 2018
	Atp B-int	psaA	Helium gun bombardment	Spectinomycin	Faè et al., 2017
	pChlamy3	LIP	Glass beads	Hygromycin	Baek et al., 2016
	pMS4-3	B12-responsive element	Electroporation	*METE* reporter gene	Helliwell et al., 2014
	pCRD1-5	CYC6	Electroporation	Luciferase	Quinn et al., 2003
	cabII-1 chimeric	CABII-1	Electroporation	GUS	Blankenship and Kindle, 1992
Phaeodactylum tricornutum	pHY21	Pt211	Electroporation	GUS, DGAT2	Zou et al., 2018
	pHY11	FCP	Electroporation	Chloramphenicol acetyltransferase (CAT)	Xue et al., 2015
Chromochloris zofingiensis	pCZT1	RBCS	Gold bombardment,electroporation	PDS gene for herbicides	Mooi et al., 2018
Chlorella pyrenoidosa	pGreeII 0029	Ubiquitin	Electroporation	NptII, eGFP	Run et al., 2016
Chlorella vulgaris	pCAMBIA1304	CaMV 35S	Electroporation	Hygromycin	Koo et al., 2013
	pPt-ApCAT	NR gene	Electroporation	Chloramphenicol	Niu et al., 2011
Chlorella ellipsoidea	pSoup	NIT1	Electroporation	NptII	Bai et al., 2013
Claculinopsis fusiformis	*p*ble	Pδ	Bombardment	Zeocin	Fischer et al., 1999
Dunaliella salina	pUCG-Bar	GAPDH	Electroporation	Herbicide PPT	Jia et al., 2012

being resistant to antibiotics (e.g., spectinomycin, kanamycin, erythromycin, chloramphenicol), herbicides (e.g., sulfometuron methyl, glufosinate, norflurazon), or having a function as a metabolic mutant (e.g., photoautotrophic growth, arginine free media, nitrate salt presented media) (Morales-Sánchez et al., 2015). Although antibiotic resistance genes are usually used for selecting the transformant, metabolic selection is considered to be environmentally friendly (Doron et al., 2016). Particularly, stable transformation depends on the use of a suitable selection marker.

Condon Optimization

Codon optimization is also important to consider because it significantly affects translation efficiency and protein expression levels. Codon bias from tRNA abundance can be quite different not only for various species genomes but for various organelles. The length of vector construction can lead to false positive transformants in microalgal hosts. The efficiency of positive transformants can range from 2–50% depending on the construct (Baier et al., 2018). Microalgae are still being used more than *P. tricoronutum* (diatom) because diatom is sensitivity and slow growth, even though they have less-complex genetic data.

When DNA synthesis is more reliable and cheap, it may soon be possible to design and construct complex metabolic pathways in microalgae (Lauersen et al., 2018). In recent years, many vectors, toolboxes, and strategies have been developed for the model microalgae *Chlamydomonas*, but these cannot be applied for all microalgae. Until now, non-model microalgae were still a challenge because of the lack of development in tools and strategies (Suttangkakul et al., 2019). In some cases, they can produce recombinant proteins in the same way as *Chladmydomonas reinhardtii*.

Protein Degradation

Proteases can degrade foreign proteins, so knockdown technologies, such as RNAi, are used to limit proteolysis. Methods to control this limitation are still required for further improvement in microalgae. Furthermore, foreign protein toxicity should also be considered; for example, the cholera toxin-B subunit is toxic to tobacco cells only when expressed in the cytosol (Daniell et al., 2001). Thus, similar aspects should be considered when using microalgae as a host.

Secretion Product

In eukaryotes, secretion can ensure proper glycosylation of proteins, which plays an important role in determining yield, biological function, stability, and half-life of production. Nevertheless, these mechanisms of protein glycosylation in higher plants remain unknown (Mathieu-Rivet et al., 2014). Therefore, secretion of expressed protein into the medium is widely used in heterotrophic microalgal hosts (Demain and Vaishnav, 2009). In general, secretion yields more than 10 mg/L are a minimum for commercial processes (Hellwig et al., 2014), while heterotrophic microalgae could have a yield more than 1 g/L. In 2017, reports supported potential of transgenic

microalgae as a host for the secretion of recombinant production (Ramos-Martinez et al., 2017).

MICROALGAL ENGINEERING FOR PHYTOCHEMICALS PRODUCTION

Microalgae have great potential to produce novel metabolites and other high-value compounds. Plant secondary products or specialized metabolites are some of the most crucial target compounds (Gangl et al., 2015). These plant compounds have been used in many areas, including pharmaceuticals, chemicals, food industries, and medicines. Moreover, approximately 50% of all approved medicines are from plant compounds (Lassen et al., 2014). Recently, some researchers and biotechnologies aim to replace many types of plant compounds with microorganisms via genetic technology because various substances are normally found in small amounts in plant, which means that some parts of the plant are wasted biomass. Moreover, there remains an imperfect production of the chemical on an industrial scale for some types of compounds. More recently, microalgae have become fascinating and interesting hosts to produce heterologous isoprenoids, which are high-value plant secondary metabolites. Researchers have strongly suggested that pharmaceutical products, such as terpenoids, are not only produced in plant chloroplasts but also in microalgal chloroplasts (Bock and Warzecha, 2010). Some algae accumulate a large percentage of triacylglycerol (TAGs), which is similar to those found in plant oils (Hu et al., 2008). Unfortunately, some high-value compounds, such as terpenoids, are less expressed in

TABLE 5 | Recent phytochemicals manufactured in microalgae.

Microalgal hosts	Phytochemical productions	Functions	Cultivation modes	References
***Porphyridium* sp.**	• ***Carbohydrates*:** Exopolysaccharides (EPS) • **PUFAs:** Arachidonic acid (AA) • ***Protein-pigment complexes*:** B-phycoerythrin, etc.	High-value bioactive substances (food, medicine, nutrition)	Phototroph, Mixotroph, Heterotroph	Li et al., 2020
Chlamydomonas reinhardtii, Synechococcus elongatus	• ***Cannabinoids*:** delta-9-tetrahydrocannabinoid (Δ9-THC), cannabidiol (CBD), etc.	Treat a wide range of medical conditions (e.g., AIDS, neuropathic pain, spasticity)	Phototroph	Laban, 2019
C. reinhardtii	• ***Hydrocarbons*:** terpenoids	High-value plant secondary metabolites (antioxidant, dietary, supplement, pigment)	Phototroph	Lauersen, 2018
	• ***Metabolites*:** Cytochrome P450 enzymes (P450s) which is involved in the biosynthesis of complex plant metabolites (e.g., paclitaxel accumulation in plant; *Taxus baccata*)	Paclitaxel as a natural source cancer drug	Phototroph	Gangl et al., 2015
***Scenedesmus* sp.**	• ***Pigments*:** β-carotene (red-orange found plants and fruits), Lutein	Health food, dietary, supplements, cosmetics, feed	Phototroph	Chen et al., 2017
***Dunaliella* sp.**	• ***Pigments***: β-carotene, astaxanthin	Food coloring, antioxidant, anti-allergic, anti-inflammatory	Phototroph	Saha et al., 2018; Barkia et al., 2019
***Haematococcus* sp.**	• ***Pigments*:** β-carotene, astaxanthin	Antioxidant, anti-inflammatory		Barkia et al., 2019
***Chlorella* sp.**	• ***Pigments*:** lutein (a large amount of lutein present in marigold flowers) • ***Proteins*:** whole, dried microalgae	Antioxidant, dietary, cosmetic, pigment	Phototroph, Heterotroph	Sun et al., 2016
C. pyrenoidosa	• ***Micronutrients*:** polyphenols (present in diverse plants)	Pharmacological activities, antioxidant	Phototroph	Olasehinde et al., 2017
Neochloris oleoabundans	• ***Fatty acids*:** triacylglycerols (TAGs) (major component of vegetative oils)	Great nutritional, nutraceutical value, edible oils, and industrial purposes.	Phototroph	Chungjatupornchai et al., 2019
Botryococcus braunii	• ***Hydrocarbons*:** alkadiene, botryococcene • ***Metabolites*:** phenolics, carotenoids	high-quality fuel applications, antioxidant, medical values	Phototroph	Cheng et al., 2018; Kempinski and Chappell, 2019
Green algae*, Volvox carteri*	• ***Phytohormones*:** auxin, abscisic acid, cytokinin, ethylene	Plant hormone	Phototroph	Lu and Xu, 2015

E. coli and *Saccharomyces cerevisiae* because those compounds need special localization and post-translational modification (Chemler and Koffas, 2008). In 2018, the invention of producing cannabinoids, which is a phytocompound, in an algae host was presented for a patent. The expression systems and method can convert a fatty acid into a cannabinoid in an algae host (Laban, 2019). Recent studies have shown the ability of microalgal host to express, post-translationally modify, fold, and secrete plant chemicals and proteins (**Table 5**).

Additionally, some studies have attempted to convert autotrophic algae into heterotrophs by using genetic manipulation to adapt microalgae to different growth conditions (Taunt et al., 2018). However, some studies have reported that the yield of *Chlorella* was 200 ng/L to 11.42 mg/L, which is lower than other hosts, including plants (0.1 μg/L to 247 mg/L), mammalian cells (0. 55–80 mg/L), and insect cells (80–300 mg/L). Fortunately, rapid growth of *Chlorella* might gain higher yield (Yang et al., 2016).

CHALLENGES OF MICROALGAL HOSTS

The major challenge is bacterial contamination in heterotrophic microalgal culture and biomass since the faster grow of bacterial populations is a consequence of commercial applications. Thus, sterilization steps are necessary, which cause a higher cost on a large scale due to equipment demands, such as autoclaves, laminar flow cabinets, and boilers. Besides, the use of industrial wastes in the culture medium could be risky for high microbial load. However, lower cost sterilization methods, including sodium hypochlorite usage, are another option to investigate for replacing expensive sterile tools on a large scale (Peiris et al., 2012).

Another major concern is the need for aeration and efficient mixing in the liquid medium for avoiding transfer limitations that can reduce cell biomass and yield (Lopes et al., 2019). In this sense, technological development of bioreactors is required to provide adequate oxygen under gentle stirring at a large scale without the presence of dead zones. Today, the limitations of industrial scale rely on the future development of a bioreactor which can operate in a larger scale (Severo et al., 2019).

Additionally, microalgal hosts, especially under heterotrophic cultivation, are still challenged by some obstacles for phytochemical production. Microalgae recombinant techniques for molecular development, including enhancing transcription, improving translation efficiency, and minimizing post-translational degradation, and process development, such as improving cultivation methods and optimizing scale-up culture, are needed. In the United States and Europe, biopharmaceutical industries are using microbial fermentation and mammalian cells for production. Host system research using microalgae should be encouraged over other hosts. Although, genetically

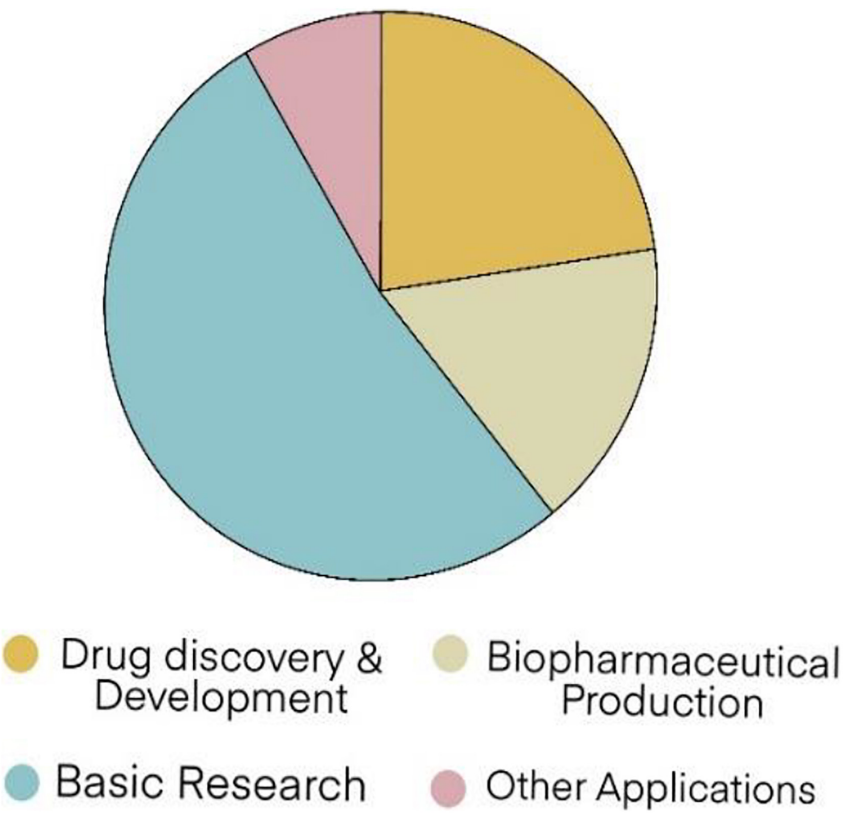

FIGURE 4 | Recombinant protein global market the forecast trends in 2025 (Coherent Market Insights, 2020).

modified microalgae have less of a chance to survive in the environment, it is suggested to analyze the risks before staring industrial production outdoors (Wijffels et al., 2013).

KEY MARKET TRENDS

The growth of valuable protein and compound markets has continually increased in research and development. Therapeutic applications from biopharmaceuticals have become bestsellers for the treatment of many chronic conditions, like diabetes, cancer, psoriasis, multiple sclerosis, rheumatic diseases, and inflammatory bowel diseases. The biopharmaceutical market was valued at approximately US$ 199.7 billion in 2013 and might reach US$ 497.9 billion in 2020; hence, an overall compound growth rate of 13.5% per year (Xu and Zhang, 2014). Many application trends of recombinant proteins in the global market in 2025 are shown in **Figure 4** (Coherent Market Insights, 2020).

Over 50 different biopharmaceuticals have been successfully produced in microalgae (Lauersen et al., 2013). Microalgae represent a third-generation biofuel and an energy source. Moreover, their short life cycle, environmental adaptation, and wide range of distribution serve as good criteria for economic systems. According to global market research, various products

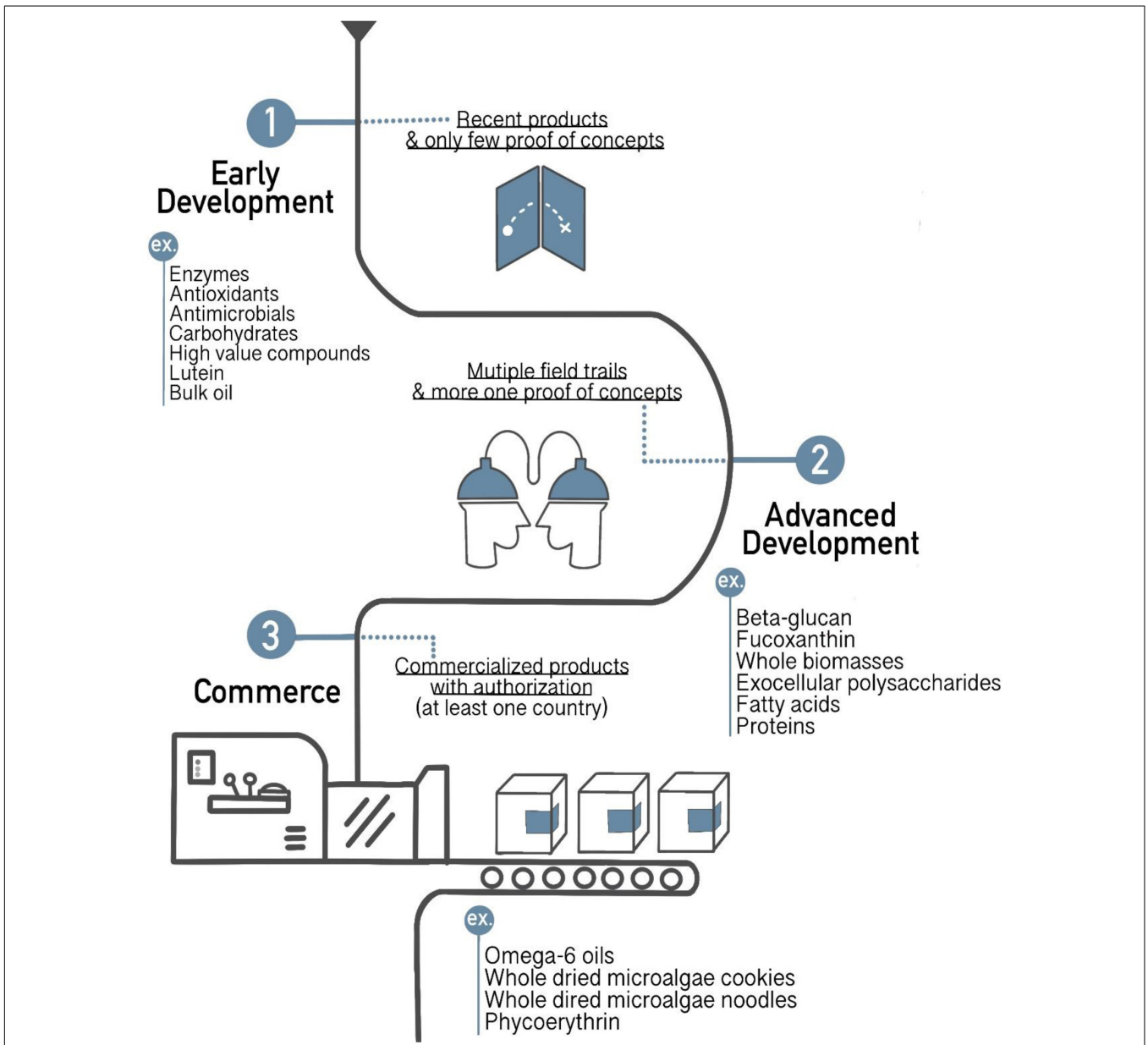

FIGURE 5 | Several new products derived from microalgae in different stages (early development, advanced development, and commerce) of development (adapted from Jacob-Lopes et al., 2019).

from algae are expected to grow at 4.2% annual growth rate from 2018 to 2025. Furthermore, a total market value is more than 3.4 billion US$, while no biopharmaceuticals produced from microalgae have been approved for commercial production (Taunt et al., 2018; Kumar et al., 2020). However, for reasons of high cost and unavailability of genetic information for commercially suitable strains, they have not yet reached industrial maturity and commercial success. So far, a considerable effort has been given to tackle the bottleneck of various methods, including various nutritional-, environmental-, and physiological alteration of cultivation, metabolic and genetic engineering (Pierobon et al., 2018; Chen and Lee, 2019). To meet large market demand, a high technological level and the use of mechanized harvests are required. Exploring the integration of new efficient technology of downstream processes including extraction, concentration, conversion, and purification of recombinant product from microalgae should be considered in future studies.

To date, economic feasibility of some heterologous production will not be achieved with microalgal host, for example, sesquiterpenoid cosmetic and perfume have already been produced by microbial fermentation in the market under the name Clearwood by Firmenich (Lauersen, 2018). However, other productions still have been possibly produced in microalgal host, the return on investment can be achieved in short term. Recently, microalgae productions are continuingly developed in three stages of microalgae-based process developments (**Figure 5**). Commercialized microalgae products are sold on the market with authorization at least one county, such as omega-6 oils, whole dried microalgae cookies, whole dried microalgae noodles, and phycoerythrin. Some of the products are in advanced development which is in the multiple field trails and has more than one proof of concepts, including beta-glucan, fucoxanthin, whole biomasses, exocellular polysaccharides, fatty acids, and proteins. However, most of them are in early development stage that has only few proofs of concepts namely enzymes, antioxidants, antimicrobials, carbohydrates, lutein, bulk oil, and high-value compounds. Furthermore, demanding of high value compounds is increasing. For instant, the high value pigments like β-carotene make a selling price up to US$ 790 per kg (Jacob-Lopes et al., 2019). Recently, the carotenoid market has been reached US$ 1.53 billion until 2021 (Fernandes et al., 2018). Especially, heterotrophic microalgae have much attention for commercial applications because they overcome the difficulties of supplying CO_2 and light compared to autotrophic microalgae (Hu et al., 2017). The cost of dry biomass for heterotrophic cultivation was US$ 2, whereas autotrophic cultivation was around US$ 11. Nevertheless, through the economic aspect, the main costs of heterotrophic cultivation are the set-up, equipment costs, and organic carbon source costs (Lowrey et al., 2015). About 80% of production costs spend to culture medium, so the replacement of alternative organic carbon sources can reduce approximately 40% (Santos et al., 2017). While many species of microalgae can be cultured in wastewater to reduce the costs of carbon source and other nutrition, they can use organic carbon and inorganic N and P from wastewater and also remove heavy metals (Jareonsin et al., 2019). Therefore, researchers are more likely to use wastewater from industrial applications, including livestock, kitchen, or pig wastewater on heterotrophic microalgae to enhance the economic feasibility and sustainability of production (Qin et al., 2019). However, the production of biopharmaceutical products might be challenged by using those wastewaters because of safety concerns.

Owing to biosafety concerns, the way to the world market requires approval of all genetically modified organisms (GMOs). Some organizations, such as European Food Safety Authority (EFSA)[1] and OECD meeting on the Biosafety and Environmental Uses of Micro-Organisms, prepared a guidance protocol for risk assessment of genetically modified microorganisms (OECD, 2015). For instance, the protocols recommended that GM microorganisms should be grown in closed bioreactors, tubular reactors, or polyethylene sleeves, additionally, selection markers should be removed. Once the genetically improved strain is developed, biosafety will define its commercial success.

CONCLUSION AND FUTURE VIEWS

Tremendous breakthroughs in the new discovery of novel expression platforms for producing biopharmaceuticals or phytochemicals are needed. Heterotrophic microalgae are a sustainable and scalable host for recombinant technology. Microalgae share many attributes with higher plants, such as glycosylation patterns and having low risk of contamination by viruses or prions. Unlike higher plants, the closed-system of heterotrophs in fermenters is attractive because of safety aspects for biopharmaceutical products, cost-effectiveness, well-controlled environment, fast growth, and high yield on a large scale, suggesting the use of these organisms as alternative biotechnology. Thus, the genetic tools and design concepts of heterotrophic microalgae should be developed for increasing the number of known microalgae species under heterotrophic conditions.

Microalgae cultivation is well known to be the most profitable business in biotechnological industry since it has less waste. Additionally, the development of other GRAS species that have been grown commercially, such as *Chlorella* sp., *Dunaliella salina*, and *Haematococcus pluvialis,* may provide opportunities for reducing costs and scaling-up; moreover, these promising hosts will help to expand the various applications for recombinant microalgae-based production. Apparently, expanding basic or applied research for the use of autotrophic and heterotrophic microalgae is necessary.

The challenges to meet the economic demand are multifaceted, including quantities, qualities, and cost-effectiveness. Improving yield and product quality in some microalgal hosts remain to be addressed. A small number of microalgal hosts are approaching commercialization as the demand for therapeutics and other production is continually growing. These still remain some limitations for

[1]https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2006.374

being microalgal host, such as difficult engineering due to the lack of a high-efficiency genetic toolbox (especially for heterotrophic microalgae), less-available molecular specific toolkits, short-term stability genetic system, and less efficient manipulation outside laboratory. To counter these limitations of phytocompounds using microalgal host, the basal study of molecular elements, such as identification and cloning of promoters, enhancers, and terminator should be studied up more. The innovation and toolkits for microalgae are also need to be specifically improved. Using industrial or agricultural waste contained with less microbial load should be adapted to medium for sustainability and saving cost for industrial scale. Indeed, fundamental knowledge and research are also necessary, making more research on various cultivation conditions a good option within the next few years.

Many plant chemicals that are of pharmaceutical interest are waiting to be produced by the benefits of genetic engineering of microbial synthesis on an industrial scale. In terms of sustainability, combined with economic, environmental, and short life cycle benefits, hetero- and autotrophic microalgae may reach this goal.

AUTHOR CONTRIBUTIONS

SJ: conceptualization, writing–original draft preparation, writing-reviewing, editing, and investigation–data collection. CP: conceptualization, writing–original draft preparation, writing–reviewing, editing, and supervision. Both authors contributed to the article and approved the submitted version.

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Overexpression of the Transcription Factor *AtLEC1* Significantly Improved the Lipid Content of *Chlorella ellipsoidea*

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Microalgae are considered to be a highly promising source for the production of biodiesel. However, the regulatory mechanism governing lipid biosynthesis has not been fully elucidated to date, and the improvement of lipid accumulation in microalgae is essential for the effective production of biodiesel. In this study, *LEAFY COTYLEDON1 (LEC1)* from *Arabidopsis thaliana*, a transcription factor (TF) that affects lipid content, was transferred into *Chlorella ellipsoidea*. Compared with wild-type (WT) strains, the total fatty acid content and total lipid content of *AtLEC1* transgenic strains were significantly increased by 24.20–32.65 and 22.14–29.91%, respectively, under mixotrophic culture conditions and increased by 24.4–28.87 and 21.69–30.45%, respectively, under autotrophic conditions, while the protein content of the transgenic strains was significantly decreased by 18.23–21.44 and 12.28–18.66%, respectively, under mixotrophic and autotrophic conditions. Fortunately, the lipid and protein content variation did not affect the growth rate and biomass of transgenic strains under the two culture conditions. According to the transcriptomic data, the expression of 924 genes was significantly changed in the transgenic strain (LEC1-1). Of the 924 genes, 360 were upregulated, and 564 were downregulated. Based on qRT-PCR results, the expression profiles of key genes in the lipid synthesis pathway, such as *ACCase*, *GPDH*, *PDAT1*, and *DGAT1*, were significantly changed. By comparing the differentially expressed genes (DEGs) regulated by *AtLEC1* in *C. ellipsoidea* and *Arabidopsis*, we observed that approximately 59% (95/160) of the genes related to lipid metabolism were upregulated in *AtLEC1* transgenic *Chlorella*. Our research provides a means of increasing lipid content by introducing exogenous TF and presents a possible mechanism of *AtLEC1* regulation of lipid accumulation in *C. ellipsoidea*.

Keywords: *Chlorella ellipsoidea*, *AtLEC1*, lipid content, transcriptome, regulation

INTRODUCTION

The sustainable development of biofuels has gained considerable attention in recent years (Koutra et al., 2018). Microalgae biomass and the energy-rich compounds derived from microalgae, such as carbohydrates and lipids, have emerged as the most popular feedstock for the production of biofuels (Wijffels and Barbosa, 2010; Du et al., 2019). The first- and second-generation biofuel feedstock, such as palm, soya beans, rapeseed and wheat, had the disadvantage that the cultivation of these crops might compete for limited arable farmland, which indirectly affects food security and prices (Zhu et al., 2016; Park et al., 2019).

Furthermore, compared with other biofuel feedstocks and terrestrial plants, microalgae are more appropriate for biofuel production because (1) as photosynthetic organisms, microalgae are able to capture solar energy and use water and atmospheric CO_2 with high efficiency to accumulate biomass in the form of organic ingredients, such as lipids (Hu et al., 2008); (2) they could grow in seawater or industrial/domestic wastewaters with a relatively high growth rate (Venkata et al., 2015); and (3) microalgae are environmentally friendly resources for biomass energy, and they could reduce the greenhouse gas effect (Chisti, 2007). However, several factors may limit stable production of microalgae: (1) it is difficult to select appropriate strains that could produce on a large scale and contain high levels of lipids (Xiong et al., 2010); and (2) environmental factors, such as light, temperature, pH, available nutrients, and higher cost of cultivation, restrict microalgae production (Shin et al., 2018).

Some microalgae species, such as *Botryococcus braunii* (57–64%), *Schizochytrium* sp. (50–77%), and *Neochloris oleoabundans* (35–65%), have a high lipid content but slow growth rate and low oil production rates (Rao et al., 2007). However, several other species, such as *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, and *Navicula pelliculosa*, have a high growth rate but a low oil content (<15%) (Hu et al., 2008). Thus, it appears to be difficult to locate the microalgae with simultaneous high cell growth rate and high cellular lipid content. Many efforts have been made to overcome these challenges, such as strain selection (Remmers et al., 2018), the improvement of culture nutrition (especially N, P, and S limitation), and other improvements in growth conditions (temperature, light, and pH) (Guschina and Harwood, 2006; Markou and Nerantzis, 2013; Li-Beisson et al., 2019).

Recently, the rapid development of multiple approaches, including omic analysis, genetic engineering, genome editing, and metabolic pathway engineering, provided efficient ways to increase the lipid content in microalgae. Omics analyses identified complete gene sets encoding fatty acid and triacylglyceride biosynthetic pathways of *Chlorella vulgaris* UTEX 395 (Guarnieri et al., 2018). Compartmentalized genome scale metabolic model iAJ526 was reconstructed with 1,455 reactions, 1,236 metabolites, and 526 genes for *Chlorella variabilis* (Juneja et al., 2016). Proteomic analysis of *C. vulgaris* showed the mechanisms governing lipid accumulation in algae (Guarnieri et al., 2013). Fan et al. sequenced the 56.8-Mbp genome of *C. pyrenoidosa* FACHB-9 to investigate the rapid switch of the intracellular energy storage form from starch to lipids and showed that overexpression of an NAD(H) kinase from *Arabidopsis* increased cellular lipid content by 110.4% (Fan et al., 2015). Chakraborty et al. (2016) found nitrate limitation (1 mM) was suitable for the enhancement of lipids, resulting in the highest yield (48.26% w/w) by using the Taguchi model. Ma et al. (2019) reported that overexpressing ACCase and PEPC genes in a lipid-poor wild strain MC-1 could increase lipid content by 28.6%. In 2014, the CRISPR/Cas9 system was reported to have worked successfully in *C. reinhardtii* (Jiang et al., 2014). Lin and Ng used CRISPR/Cas9 to edit the *fad3* gene and achieved an accumulation of lipid content higher by 46% (w/w) in *C. vulgaris* FSP-E (Lin and Ng, 2020).

Clearly, the first step of the lipid biosynthesis, acetyl-CoA carboxylase (ACCase), plays a vital role in metabolic flux to lipid biosynthesis, since ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first intermediate product in the fatty acid elongation pathway (Kim, 1997; Davis et al., 2000). Next, a series of reactions for fatty acid production are catalyzed by fatty acid synthase (FAS) (Subrahmanyam and Cronan, 1998). However, lipid synthesis and accumulation are controlled by multiple genes. A number of studies show transcription factors (TFs) that regulate multiple genes play an important role in regulating the lipid biosynthesis and metabolic pathways. Overexpression of the soybean TF *GmDof4* significantly enhances the lipid content of *Chlorella ellipsoidea* (Zhang et al., 2014). Kang et al. reported that *Wrinkled1*, a TF of *Arabidopsis*, enhanced lipid production in the microalgae *Nannochloropsis salina* (Kang et al., 2017).

LEC1 is a central regulator that controls many aspects of seed development, including the maturation phase during which seeds accumulate storage macromolecules and embryos acquire the ability to withstand desiccation in *Arabidopsis* (West et al., 1994). The induced overexpression of *LEC1* can affect *ABI3*, *FUS3*, *WRINKLED1* and other TFs and improve the overall level of fatty acid synthesis-related gene expression (Mu et al., 2008). Shen et al. (2010) found that the overexpression of corn *ZwLEC1* gene under embryo-specific weak promoter *EPA1* could significantly increase the oil content of transgenic maize, but plant leaves are reduced to approximately half of the leaves of the wild type. *C. ellipsoidea* is a unicellular eukaryotic organism that has no differentiation of tissues and may be a good receptor of *LEC1* overexpression without lethal or harmful effects to the host cell.

In this study, we investigated the feasibility and the mechanism for improving the lipid content of *C. ellipsoidea* by the overexpression of *AtLEC1*. The results indicated that the lipid content of transgenic *C. ellipsoidea* strains was significantly increased under mixotrophic and autotrophic culture conditions, but the growth rate of the strains was not affected. In addition, RNA-seq data showed that *AtLEC1* significantly regulated 924 genes of *C. ellipsoidea*, and we found the regulation mechanism of *AtLEC1* in *C. ellipsoidea* to have some differences compared with the regulation mechanism in *Arabidopsis*. Our study provided a new route for engineering microalgae to increase the lipid content and help to elucidate the mechanism of lipid accumulation in *C. ellipsoidea* regulated by *LEC1* from a higher plant.

MATERIALS AND METHODS

Strain and Culture Conditions

The *C. ellipsoidea* strains used in this study were grown in Endo medium (Appleyard, 1954) for the mixotrophic culture and in KNOP medium (McLEOD, 1958) for the autotrophic culture in a rotary shaker (DZ-900, Zhongkepusen Co., Ltd., Beijing, China), 200 rpm at 25°C under illumination (100 μmol photons/m^2/s).

AtLEC1 Expression Vector Construction and Transformation of *C. ellipsoidea*

The cDNA of *AtLEC1* was generously provided by Prof. Jianru Zuo (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The *AtLEC1* cDNA was cloned into a T-vector (pEASY-Blunt Cloning Vector, TransGen Biotech. Ltd., Beijing, China), and later inserted at *Spe*I and *Not*I sites of pGreen0029 driven by UBI promoter from maize, which was named pGreen0029-Ubi-AtLEC1-Nos (*pAtLEC1*) (**Figure 1A**). *C. ellipsoidea* was transformed using plasmid *pAtLEC1* according to the previously described method (Bai et al., 2013). Briefly, strains were cultured to the logarithmic phase in Endo medium, mixed with 0.2 M mannitol and 0.2 M sorbitol and kept on ice for 1 h. The resuspended strains were mixed with electroporation buffer (0.08 M KCl, 0.005 M $CaCl_2$, 0.01 M HEPES, 0.2 M mannitol, and 0.2 M sorbitol), a final concentration of 20 μg/mL *pAtLEC1* plasmid, a final concentration of 10 μg/mL plasmid pSoup, and 25 μg/mL salmon sperm DNA. The strains were transformed with a Baekon 2000 (Baekon Co., CA, United States) electroporation device. After electroporation, the strains were screened using SE agar selection medium containing 30 mg/L G418. The selected individual strains were subcultured in SE liquid medium containing 15 mg/L G418.

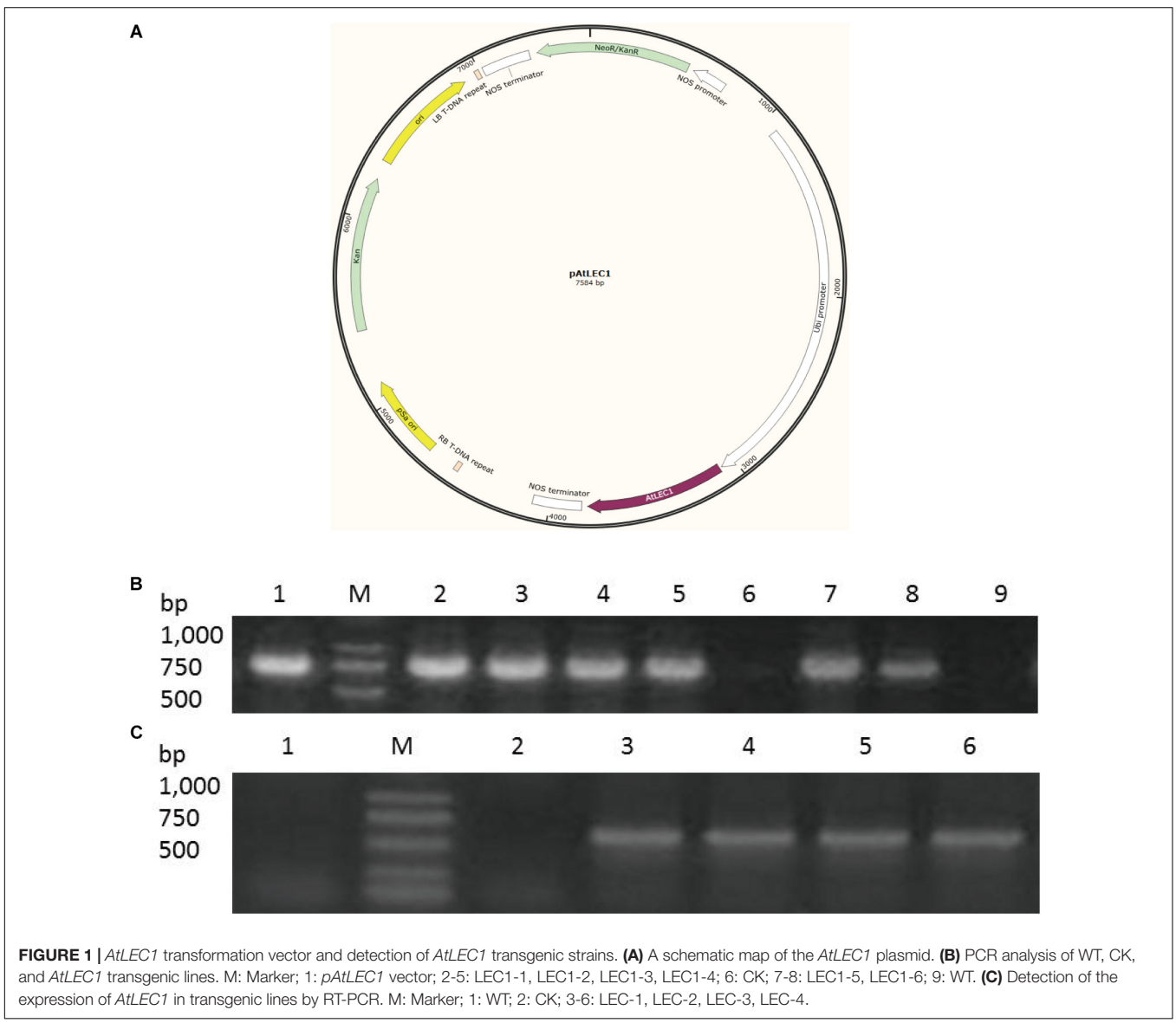

FIGURE 1 | *AtLEC1* transformation vector and detection of *AtLEC1* transgenic strains. **(A)** A schematic map of the *AtLEC1* plasmid. **(B)** PCR analysis of WT, CK, and *AtLEC1* transgenic lines. M: Marker; 1: *pAtLEC1* vector; 2-5: LEC1-1, LEC1-2, LEC1-3, LEC1-4; 6: CK; 7-8: LEC1-5, LEC1-6; 9: WT. **(C)** Detection of the expression of *AtLEC1* in transgenic lines by RT-PCR. M: Marker; 1: WT; 2: CK; 3-6: LEC-1, LEC-2, LEC-3, LEC-4.

Characterization of Transgenic Strains With PCR

Genomic DNA isolation, total RNA isolation, PCR amplification, and cDNA synthesis were performed as previously described (Zhang et al., 2014). All relevant primer sequences used for PCR and RT-PCR are listed in **Supplementary Table 1**. Identification of transgenic strains was carried out using a pair of primers, P1 and P2. The reaction conditions were as follows: denaturation at 95°C for 10 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min followed by final extension at 72°C for 10 min. Total RNA was isolated using an RNA extraction kit (Takara). The reverse transcriptome product was diluted by a factor of 10 for RT-PCR amplification, in which total 20 μL reaction mixtures contained 10 μL 2 × M5 HiPer plus Taq HiFi PCR Mix (Mei5 Biotechnology), 1 μL primer P3, 1 μL primer P4, 1 μL template, and 7 μL ddH_2O. The reaction conditions were as follows: denaturation at 94°C for 5 min, 28 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 20 s, and a final extension step of 72°C for 5 min. RT-PCR products were analyzed by electrophoresis on 1% agarose gel. For the verification of the selected gene expression level, qRT-PCR analyses were performed on a LightCycler® 480. The 20-μL reaction mixtures contained 10 μL EvaGreen 2 × qPCR MasterMix (abmGood.com), 1 μL of each primer (10 μM), 1 μL template, and 7 μL ddH_2O. The reaction conditions were as follows: 1 cycle 95°C for 10 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. To normalize the amount of transcripts in each sample, the relative abundance of 18S rRNA was determined and used as an internal standard control. The gene expression value was the difference (Ct) between the target gene and the reference gene.

Biomass and Everyday Growth Rate Analysis

The biomass of *AtLEC1* transgenic strains and the WT were analyzed under mixotrophic and autotrophic culture conditions at 25°C and illumination (100 μmol photons/m^2/s) in Endo medium and KNOP medium, respectively. The *C. ellipsoidea* biomass concentration (w/v) was equivalent to a specific value of the strain dry weight (DW) that was determined by OD 540 according to the following empirical formula:

$$\mathrm{DW}\left(\mathrm{g/L}\right) = (\mathrm{OD540} + 0.0097)/0.4165 \quad (1)$$

The everyday growth rate (EGR) was calculated according to the equation (White et al., 1991):

$$\mathrm{EGR} = (\mathrm{X2} - \mathrm{X1})/\mathrm{X1} \quad (2)$$

X1 was the biomass concentration on the initial day; X2 was the biomass concentration on the next day.

Measurement of the Soluble Proteins, Carbohydrate, Lipid Content, and the Fatty Acid Composition

To measure the daily growth rate (DGR) and biomass of the *AtLEC1* transgenic and the WT strains, we collected cultured algae after the 1st day (1D), 5th day (5D), and 9th day (9D) under mixotrophic culture conditions and the 5th day (5D), 10th day (10D), and 15th day (15D) under autotrophic culture conditions. Each sample had three biological replicates, and the freeze-dried biomass was collected to measure the soluble proteins, carbohydrate, lipid content, and fatty acid composition. The carbohydrate content was analyzed based on the procedure published by Miao and Wu (2004). Proteins were extracted following the procedure of Rausch (1981) and were quantified using the Bradford method (Bradford, 1976). Lipid extraction was performed by the Soxhlet method that was similar to the procedures reported by Folch et al. (1957). The fatty acid compositions were qualitatively and quantitatively determined using a TurboMass Gas Chromatograph Mass Spectrometer (PerkinElmer, MA, United States) with a capillary column (BPX-70, 30 m × 0.25 mm × 0.25 μm) using the method as previously described (Song et al., 2010). The Nile red staining followed a previous method (Greenspan et al., 1985), which was used to visualize the intracellular lipid bodies as indicators of TAG formation.

Photosynthetic Pigment Content Measurement

Photosynthetic pigment content was measured according to the previous method (Fargasová and Molnárová, 2010). Briefly, 0.02 g of a freeze-dried *Chlorella* powder sample was mixed with 4 mL of 95% (v/v) ethanol in an airtight tube and agitated at room temperature overnight in the dark until the color turned to white. After centrifugation at 5,000 rpm for 5 min, the ethanol phase was removed, and more 95% (v/v) ethanol was added to a volume of 25 mL. Next, the sample was measured with a spectrophotometer at wave lengths of 665 nm (A665), 649 nm (A649), and 470 nm (A470). The 95% ethanol was used as a blank control. The amount of chlorophyll a (Ca) was calculated as Ca = 13.95 × A665 − 6.88 × A649; chlorophyll b (Cb) as Cb = 24.96 × A649 − 7.32 × A665; and carotenoids (Cc) as Cc = (1000 × A470 − 2.05 × Ca − 114.8 × Cb)/245. The total content of chlorophyll per fresh weight was calculated as C = 2 × (Ca + Cb + Cc)/W.

Illumina-Based RNA-Seq Analysis

For the gene expression analysis by RNA-seq, the transgenic *AtLEC1-1* strain and the WT strain were collected on the 5th day of cultivation under mixotrophic conditions. Three independent biological replicates were used for the data analysis. The cDNA library was sequenced on an SE flow cell using Illumina Genome Analyzer IIx (Illumina, San Diego, CA, United States). Finally, 8.08 Gb clean data (total) with more than 90.92% of Q30 were generated from two GAIIx single-end lanes. Using SOAPdenovo with the parameters "-K31–d3–R," 775,293 contigs with an N50 contig size of 2,072 bp were obtained (Li et al., 2009). To detect the differentially expressed genes (DEGs), we first mapped the short reads to the reference genes using the Burrows Wheeler Alignment tool (BWA) program with default parameters. For the validation and annotation of the assembled contigs, a sequence similarity search was conducted against a non-redundant protein database using the BLASTx algorithm with an E-value threshold

of 10^{-3}. The results demonstrated that of 13,566 contigs, 7,559 (55.72%) showed significant similarity to known proteins in the non-redundant (Nr) database. Contigs with a similarity greater than the threshold were annotated using GO, the molecular function, biological process, and cellular component ontologies[1] by the Blast2GO program (Conesa et al., 2005).The RNA-seq data (PRJCA003770) were available in the BIG Data Center[2].

Analysis of Sequence Similarity

To detect protein sequence similarities of LEC1 among different species, a total of 13 homologous genes of *AtLEC1* were selected from *Glycine max*, *Brassica napus*, *Micromonas pusilla*, *Micromonas commode*, *Coccomyxa subellipsoidea*, *Ostreococcus lucimarinus*, *C. reinhardtii*, *Volvox carteri*, *Dunaliella salina*, *Saccharomyces cerevisiae*, *Homo sapiens*, and *Mus musculus*, and the sequence similarity analysis was subsequently performed through software DNAstar v7.1.0. Phylogenetic tree was inferred using the neighbor-joining method and the bootstrap consensus tree inferred from 1,000 replicates in MEGA v7.0 (Kumar et al., 2016).

Statistical Analysis

P-values (means ± SD) were calculated with Student's *t*-test (two-tailed) by Microsoft Excel. *Significance at $p < 0.05$ and ** significance at $p < 0.01$ were used for the comparison with the control based on Student's *t*-test. The experimental replicates, sample size, and significance level of *p*-values are described in the figure legends.

RESULTS

AtLEC1 Expression in *C. ellipsoidea* Does Not Affect Growth

In our study, we transferred an *Arabidopsis* gene *AtLEC1* into *C. ellipsoidea* using the *AtLEC1* expression vector *pAtLEC1* according to a previous method (Bai et al., 2013). A schematic map of the *AtLEC1* plasmid (**Figure 1A**), PCR analysis of *AtLEC1* transgenic strains and detection of the expression of *AtLEC1* in transgenic strains by RT-PCR are presented in **Figures 1B,C**. The primers used in our study are listed in **Supplementary Table 1**. The *AtLEC1* transgenic and wild-type (WT) *C. ellipsoidea* strains were cultured according to a previously described method (Zhang et al., 2014). To detect the effect of *AtLEC1* on *C. ellipsoidea* growth, the DGR and the biomass concentration of transgenic and WT were measured under mixotrophic and autotrophic conditions. The growth curves of *AtLEC1* transgenic strains showed no significant difference compared with the growth curves of WT under mixotrophic culture conditions (**Figures 2A,B**) and autotrophic culture conditions (**Figures 2C,D**). Photosynthetic pigment content was an important growth index in *C. ellipsoidea*. We collected the strains on the 1st day, 5th day, and 9th day of cultivation to measure the chlorophyll content, including chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll content under mixotrophic culture conditions. The result indicated that the total photosynthetic pigment content of WT strains ranged between 9.22 and 9.42 mg/g, and transgenic strain LEC1-1 was 9.69–10.25 mg/g, LEC1-2 was 10.02–10.47 mg/g, and LEC1-3 was 10.10–10.32 mg/g. Except for the chlorophyll a higher in transgenic strains than WT, there were no significant differences between them (**Figure 2E**). In addition, there was no significant difference between *AtLEC1* transgenic strains and WT cultured on the 5th day, 10th day, and 15th day under autotrophic culture conditions (**Figure 2F**). In other words, the *AtLEC1* transformation did not affect the growth and photosynthesis in *C. ellipsoidea*, which established a foundation for further research.

Expression of *AtLEC1* Increases the Lipid Content in *C. ellipsoidea*

Compared with WT, the lipid productivity of transgenic strains was significantly higher than the lipid productivity of WT under mixotrophic and autotrophic culture conditions. Under mixotrophic culture conditions (**Figure 3A**), on the 5th day, the lipid content of WT was 270.64 mg/g, while the lipid content of transgenic strains LEC1-1, LEC1-2, and LEC1-3 was increased by 12.14–22.28%, reaching 303.51–330.95 mg/g. On the 9th day, the lipid content was 287.33 mg/g, while the lipid content of transgenic strains LEC1-1, LEC1-2, and LEC1-3 increased by 22.14–29.91%. Under autotrophic culture conditions (**Figure 3B**), on the 10th day, the lipid content of the WT was 150.96 mg/g, while the lipid content of the transgenic strain was increased by 21.69–30.45%. On the 15th day, the lipid content of transgenic strains was increased by 9.28–22.77%. Gas chromatography/mass spectrometry (GC/MS) analysis indicated that the main types of fatty acids of *AtLEC1* transgenic strains and WT were not changed, but the content of total fatty acids C18:1 (oleic acid) and C18:2 (linoleic acid) increased significantly in the transgenic strains under both mixotrophic and autotrophic culture conditions (**Figures 3C,D**). Under mixotrophic culture conditions, on the 5th day, the C18:1, C18:2 and total fatty acid content of the transgenic strains increased by 22.70–41.25, 19.07–26.14, and 13.19–18.16%, respectively. On the 9th day, the C18:1, C18:2 and total fatty acid content of the transgenic strains increased by 36.40–64.58, 20.67–23.25, and 24.20–32.65%, respectively (**Figure 3A**). Under autotrophic culture conditions, on the 5th, 10th, and 15th days of cultivation, the total fatty acid content of the transgenic strains increased by 8.29–39.61, 18.88–23.13, and 24.40-28.87%, respectively (**Figure 3B**). The lipid increase in transgenic strains could also be clearly observed by Nile red staining (**Figure 3E**). Oil droplet fluorescence was measured on a Varian 96-well plate spectrofluorometer, and the results showed that transgenic strains can accumulate more oil droplets than WT strains. At the same time, the carbohydrate content of the three transgenic strains was not significantly different (**Figures 4B,D**), while the protein content was significantly decreased (**Figures 4A,C**) compared to the protein content of the WT. In brief, under mixotrophic culture on the 9th day, the protein content in WT was 288.53 mg/g, the protein content in LEC1-1 was

[1] http://www.geneontology.org

[2] https://bigd.big.ac.cn/databases

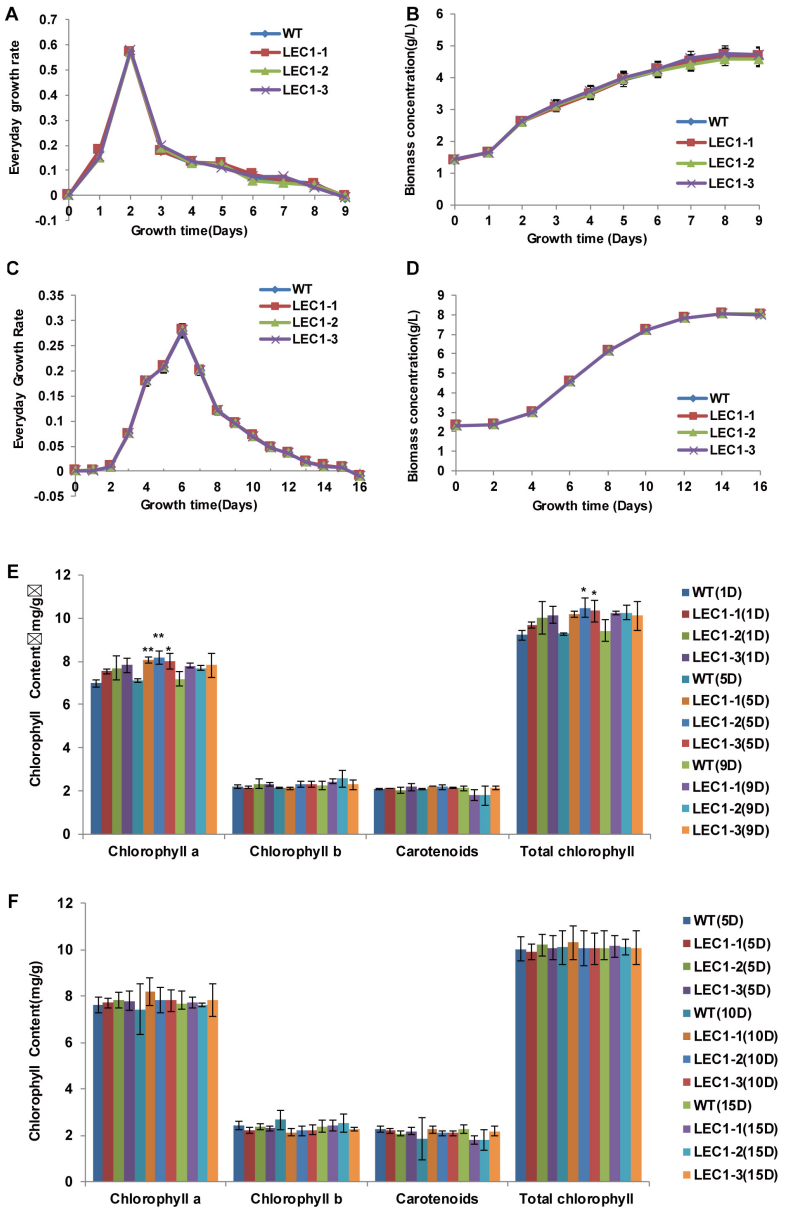

FIGURE 2 | Growth characterization of *AtLEC1* transgenic *Chlorella ellipsoidea*. **(A)** Every growth rate (EGR) of *AtLEC1* transgenic strains under mixotrophic culture conditions for 9 days. **(B)** The biomass of *AtLEC1* transgenic strains under mixotrophic culture conditions. **(C)** The EGR of *AtLEC1* transgenic strains under autotrophic culture conditions for 16 days. **(D)** The biomass of *AtLEC1* transgenic strains under autotrophic culture conditions. **(E)** The chlorophyll content of *AtLEC1* transgenic strains under mixotrophic culture conditions. **(F)** The chlorophyll content of *AtLEC1* transgenic strains under autotrophic culture conditions. WT, wild type; LEC1-1, LEC1-2, LEC1-3, three *AtLEC1* transgenic strains. The values are the means of three biological replicates. Asterisks indicate statistically significant differences, Student's *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$ compared with WT under the same conditions.

decreased by 18.23%, reaching 235.93 mg/g, the protein content in LEC1-2 was decreased by 19.67%, reaching 231.77 mg/g, and the protein content in LEC1-3 was decreased by 21.44%, reaching 226.66 mg/g. Under autotrophic culture for 15 days, the protein content in WT was 404.13 mg/g, in LEC1-1 was decreased by 12.28% reaching 354.50 mg/g, in LEC1-2 was decreased by 14.99% reaching 343.53 mg/g, and in LEC1-3 was decreased by 18.66% reaching 328.71 mg/g. These results demonstrated that the overexpression of *AtLEC1* significantly increased oil production and decreased the protein content in *C. ellipsoidea*.

Transcriptome Analysis of *AtLEC1* Strains

Transcriptome analyses for transgenic *AtLEC1-1* and WT strains were obtained using the Illumina GAIIx platform. The RNA sample was collected at the stage of mixotrophic culture

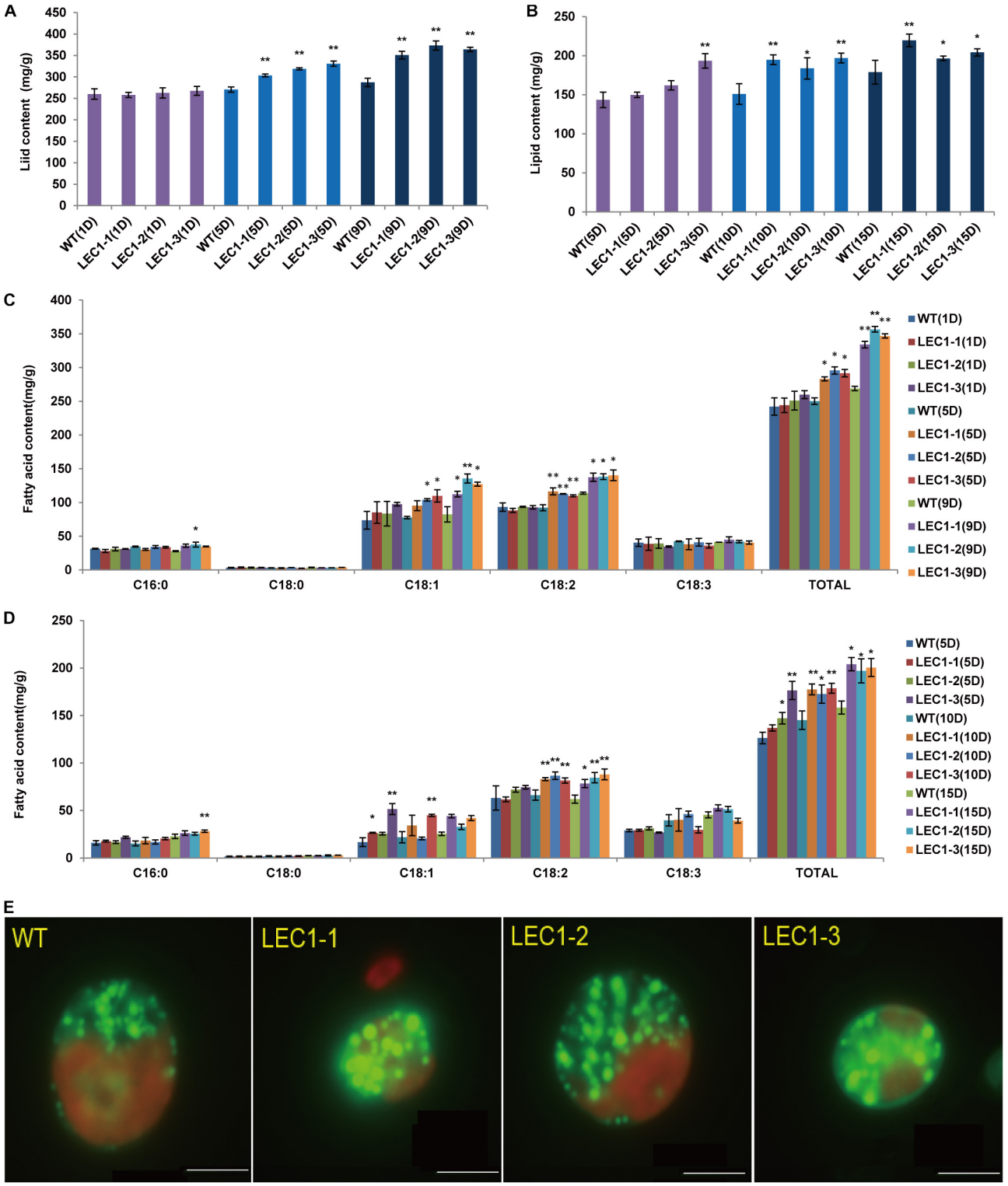

FIGURE 3 | Effect of *AtLEC1* on *Chlorella* fatty acid composition and lipid content. **(A)** The lipid content in *AtLEC1* transgenic strains under mixotrophic culture conditions. **(B)** The lipid content in *AtLEC1* transgenic strains under autotrophic culture conditions. **(C)** The fatty acid composition and total fatty acids in *AtLEC1* transgenic strains under mixotrophic culture conditions. **(D)** The fatty acid composition and total fatty acid content in *AtLEC1* transgenic strains under autotrophic culture conditions. **(E)** Observation and determination of oil droplets in *C. ellipsoidea* and WT by Nile red staining. WT, wild type; LEC-1, LEC-2, LEC-3, three *AtLEC1* transgenic strains; Bars = 5 μm. The values are the means of three biological replicates. Asterisks indicate statistically significant differences, Student's *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$ compared with WT under the same conditions. 1D, 5D, 9D, 10D, and 15D were the 1st, 5th, 9th, 10th, and 15th days, respectively.

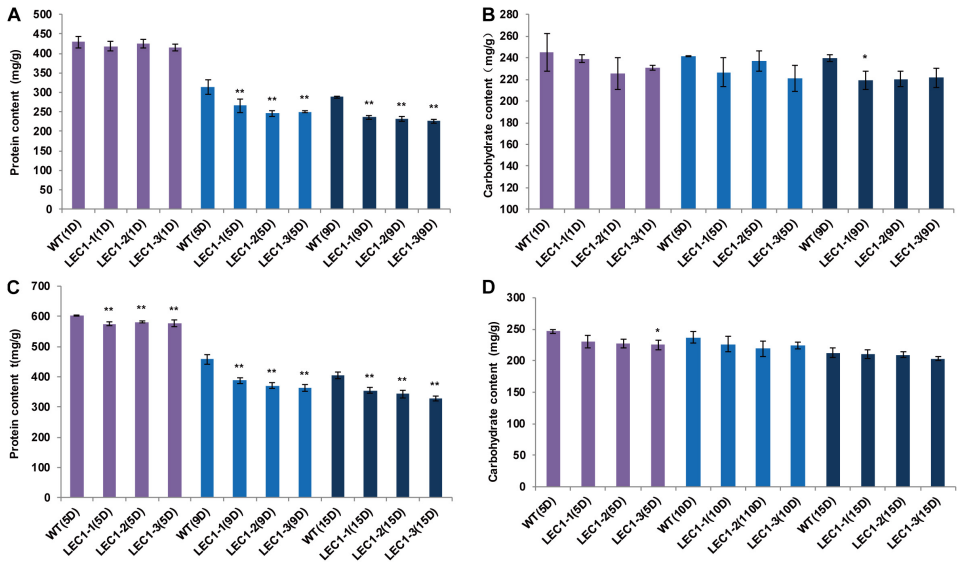

FIGURE 4 | Effect of *AtLEC1* on protein content and carbohydrate content. **(A)** The protein content in *AtLEC1* transgenic strains under mixotrophic culture conditions. **(B)** The carbohydrate content in *AtLEC1* transgenic strains under mixotrophic culture conditions. **(C)** The protein content under autotrophic culture conditions. **(D)** The carbohydrate content in *AtLEC1* transgenic strains under autotrophic culture conditions. 1D, 5D, 9D, 10D, and 15D were the 1st, 5th, 9th, 10th, and 15th days, respectively.

conditions for 5 days. After the quality control, 38,967 unigenes with an average length of 791 bp were obtained, 7,788 unigenes of which can be annotated by public databases such as COG, GO, KEGG, Swiss-Prot, NR, and pfam. We found expression of 10,823 genes was changed, including 5,413 upregulated genes and 5,410 downregulated genes (**Supplementary Table 2**). To explore the functional information of unigene transcription, MapMan v3.5[3] was used to classify the metabolic pathways. According to the criteria to determine differential expression of genes [false discovery rate (FDR) $\leq$ 0.01] (Ness et al., 2011), 924 DEGs were identified, including 360 significantly upregulated genes and 564 significantly downregulated genes (**Figure 5A**), and 471 DEGs were annotated by different public databases (**Supplementary Table 2**). Compared with the transcriptome data of *Arabidopsis* with overexpression of *AtLEC1*, 269 of 924 DEGs in *C. ellipsoidea* showing protein sequence similarity with 245 genes from *Arabidopsis* were classified into 28 known pathways (**Figure 5B** and **Supplementary Figure 1**). Compared with WT, DEGs relative to five pathways were significantly upregulated in *AtLEC1* strains, including the minor CHO metabolism, the glycolysis, the fermentation, the metal stress and the secondary metabolism (**Supplementary Figure 1**). In contrast, DEGs relative to six pathways were significantly downregulated, including the gluconeogenesis/glyoxylate cycle, the oxidative pentose phosphate, the amino acid metabolism, the cofactor metabolism, the tetrapyrrole synthesis, and the nucleotide metabolism (**Supplementary Figure 1**). The transcriptome indicated that some of these genes were significantly changed in protein, RNA metabolism and transporter in the *AtLEC1* transgenic strains (**Supplementary Table 3**). Therefore, these genes may be involved in the regulation of lipid accumulation in *AtLEC1* transgenic *Chlorella*.

Verification of the Regulatory Function of *AtLEC1* by qRT-PCR

The relative expression level of 15 significantly regulated genes associated with lipid and fatty acid metabolism were analyzed by qRT-PCR (**Figure 6**). The WT and transgenic LEC1-1, LEC1-2, and LEC1-3 strains were collected on the 1st day, 5th day, and 9th day of cultivation under mixotrophic conditions. Three independent transgenic strains were analyzed. The results indicated that the expression levels were the highest on the 5th day (logarithmic stage) and decreased on the 9th day (plateau stage). The results (**Figures 2B**, **3A**) suggested that the accumulation of biomass and lipid content reached its maximum value on the 9th day, indicating that the gene expression was earlier than the lipid accumulation. Therefore, the strain growth on the 5th day may be a vital period for lipid accumulation in *C. ellipsoidea*. Furthermore, we observed that in transgenic *AtLEC1* strains on the 5th day, the expression levels of some genes related to lipid synthesis were higher compared with the genes in WT, such as *ACC* (*Ce.101511* and *Ce.91597*), *GPDH* (*Ce.61185*, *Ce.81049*, and *Ce.82444*), *PDAT* (*Ce.67794*), *DGAT* (*Ce.70246*), and *NF-Y* (*Ce.NF-YA*) (**Figure 6**). Interestingly, the expression level of *DGAT1* (*Ce.70246*) in transgenic strains was $2^{6.85}$–$2^{8.5}$ times higher than the expression level in WT. *DGAT* was the rate-limiting enzyme of TAG synthesis in the Kennedy pathway (Lehner and Kuksis, 1996). In addition, we found there were no significant differences for partial genes of *ACCase*, *FAS*, and *GPDH*, such as *ACCase* (*Ce.56171*, *Ce.80365* and *Ce.71421*), *FAS* (*Ce.86271*), and *GPDH* (*Ce.78368*), in transgenic *AtLEC1-1* and

[3]http://mapman.gabipd.org/home

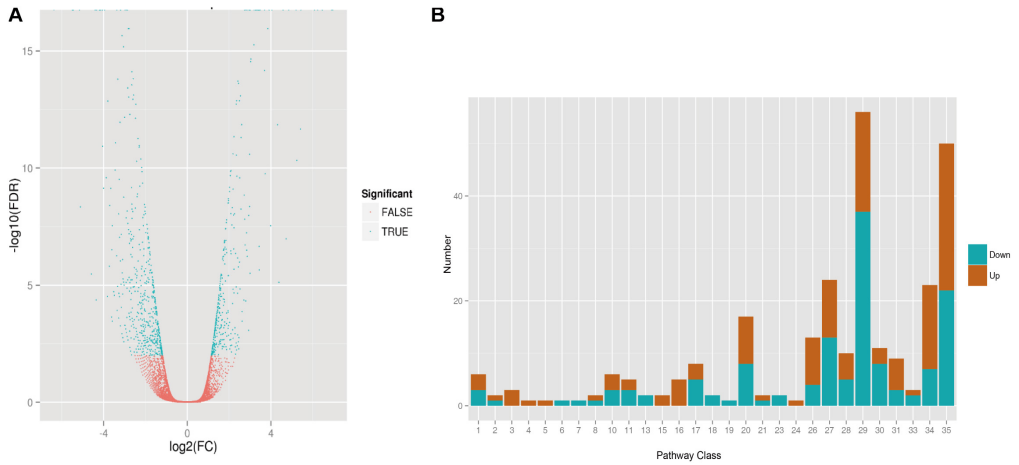

FIGURE 5 | RNA-seq in transgenic *AtLEC1-1 Chlorella* under mixotrophic culture conditions. **(A)** Volcano plot showing the differential expression gene analysis of transgenic *AtLEC1-1* and wild-type strains. **(B)** Differential expression analysis of *AtLEC1-1* strains involved in metabolic regulation. Notes: 1. Photosynthesis; 2. Major carbohydrate metabolism; 3. Minor CHO metabolism; 4. Glycolysis; 5. Fermentation; 6. Gluconeogenese/glyoxylate cycle; 7. Oxidative Pentose Phosphate; 8. TCA/org conversion; 10. Cell wall synthesis; 11. Lipid synthesis; 13. Amino acid handling factor; 15. Metal stress; 16. Secondary metabolism; 17. Hormone synthesis; 18. Cometabolism; 19. Pyrrole metabolism; 20. Coercion; 21. Redox regulation; 23. Nucleotide synthesis; 24. Biodegradation of xenobiotics; 26. Others; 27. RNA; 28. DNA; 29. Protein; 30. Signal; 31. Cells; 33. Developmental diversity; 34. Carrier protein; 35. Not classified.

WT, which may function diversely. These results suggested that *AtLEC1* in *Chlorella* could increase the expression level of such genes as *ACC* and *GPDH*, especially *DGAT*, thereby helping to explain the increasing lipid accumulation in *Chlorella*.

AtLEC1 Regulates the Protein and Carbohydrate Metabolic Networks

To investigate whether the protein and carbohydrate were involved in *AtLEC1* metabolism regulation, WT and transgenic LEC1-1, LEC1-2, and LEC1-3 strains were cultured on the 1st day, 5th day, and 9th day under mixotrophic conditions. The candidate genes related to protein and carbohydrate metabolism were selected (**Supplementary Table 4**), and the expression level of these genes was detected by qRT-PCR to verify the regulation function of *AtLEC1* (**Figure 7**). On the 5th day of culture, the expression level of protein biosynthesis-related genes, such as *Ce.59398* (nitrogen assimilation regulatory), *Ce.75003* (nitrite transporter), *Ce.6021* (ribosomal protein 60S subunit L23), *Ce.6463* (TCP-1/cpn60 chaperonin family protein), *Ce.3024* (TCP-1/cpn60 chaperonin family protein), and *Ce.6962* (germin-like protein), were downregulated. The expression level of storage protein-related genes in WT, such as *Ce.6962* and *Ce.3951* (RmlC-like cupin superfamily protein), was approximately 16- and 4-fold, similar to the expression level of storage protein-related genes in LEC1-1, respectively. These results may explain why the protein accumulation significantly decreased in the *AtLEC1* transgenic strain compared with the protein accumulation in WT. Among six selected genes that were related to carbohydrate metabolism (*Ce.3222*, *Ce.7504*, *Ce.8451*, *Ce.4421*, *Ce.6877*, and *Ce.5786*), the expression levels of *Ce.3222* and *Ce.7504* genes were significantly increased in *AtLEC1* transgenic strains on the 5th day. *Ce.3222* and *Ce.7504* may be involved in Rubisco function in the dark reaction of photosynthesis, and their increased expression could increase the raw materials for *de novo* synthesis of fatty acids.

Among eight selected genes that were related to signal transduction (*Ce.8227*, *Ce.7158*, *Ce.1278*, *Ce.8956*, *Ce.1245*, *Ce.8398*, *Ce.7337*, and *Ce.6996*), the expression level of *Ce.1245* and *Ce.6996* was upregulated in *AtLEC1* transgenic strains on the 5th day of culture. Among 7 selected genes that were related to material transport (*Ce.6313*, *Ce.3018*, *Ce.4615*, *Ce.5277*, *Ce.3824*, *Ce.6325*, and *Ce.16975*), the expression level of five genes was downregulated in *AtLEC1* transgenic strains, except for *Ce.4615* and *Ce.16975*. The expression levels of the genes encoding short-chain fatty acid dehydroreductase, *Ce.19209* and *Ce.1132*, were downregulated in *AtLEC1* transgenic strains.

DISCUSSION

AtLEC1 Increased the Lipid Content but Did Not Affect the Growth in *C. ellipsoidea*

With the development of biotechnology and molecular biology, multiple approaches have provided insight into the mechanisms of lipid synthesis and accumulation in microalgae. Based on the transcriptome and lipidome of *C. reinhardtii* under heat stress, a phospholipase A2 homolog and the DAG acyltransferase gene *DGTT1* were identified (Légeret et al., 2016). Cecchin et al. (2020) sequenced the nuclear and organelle genomes of *C. vulgaris 211/11P* by combining next-generation sequencing and optical mapping of isolated DNA molecules and identified 10,724 nuclear genes, 121 chloroplast genes and 48 mitochondrial genes. *LEC1* is an important TF, and its function in microalgae has not been elucidated. In our study, the total fatty acid content and total lipid content in *AtLEC1*

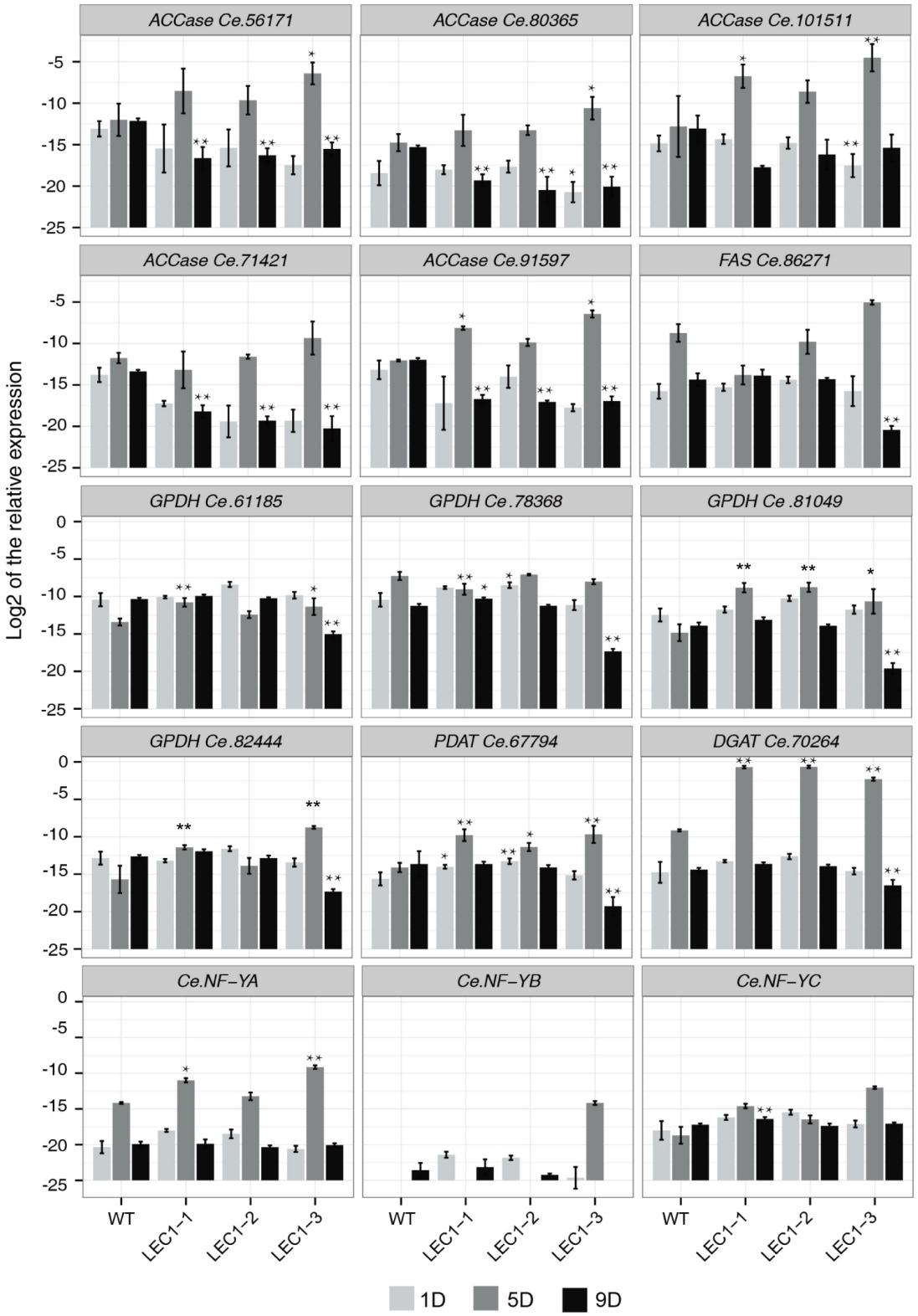

FIGURE 6 | Expression of lipid accumulation-related genes in *AtLEC1* transgenic strains. WT, wild type; LEC-1, LEC-2, LEC-3, three *AtLEC1* transgenic strains; 1D, 5D, and 9D were sampled on the 1st, 5th, and 9th days under mixotrophic culture conditions. The relative abundance of 18S rRNA was used as an internal standard control. The values are the difference (Ct) between the target gene and the reference gene. The values are the means of three biological replicates. Asterisks indicate statistically significant differences, Student's *t*-test: $*p < 0.05$, $**p < 0.01$ compared with WT under the same conditions.

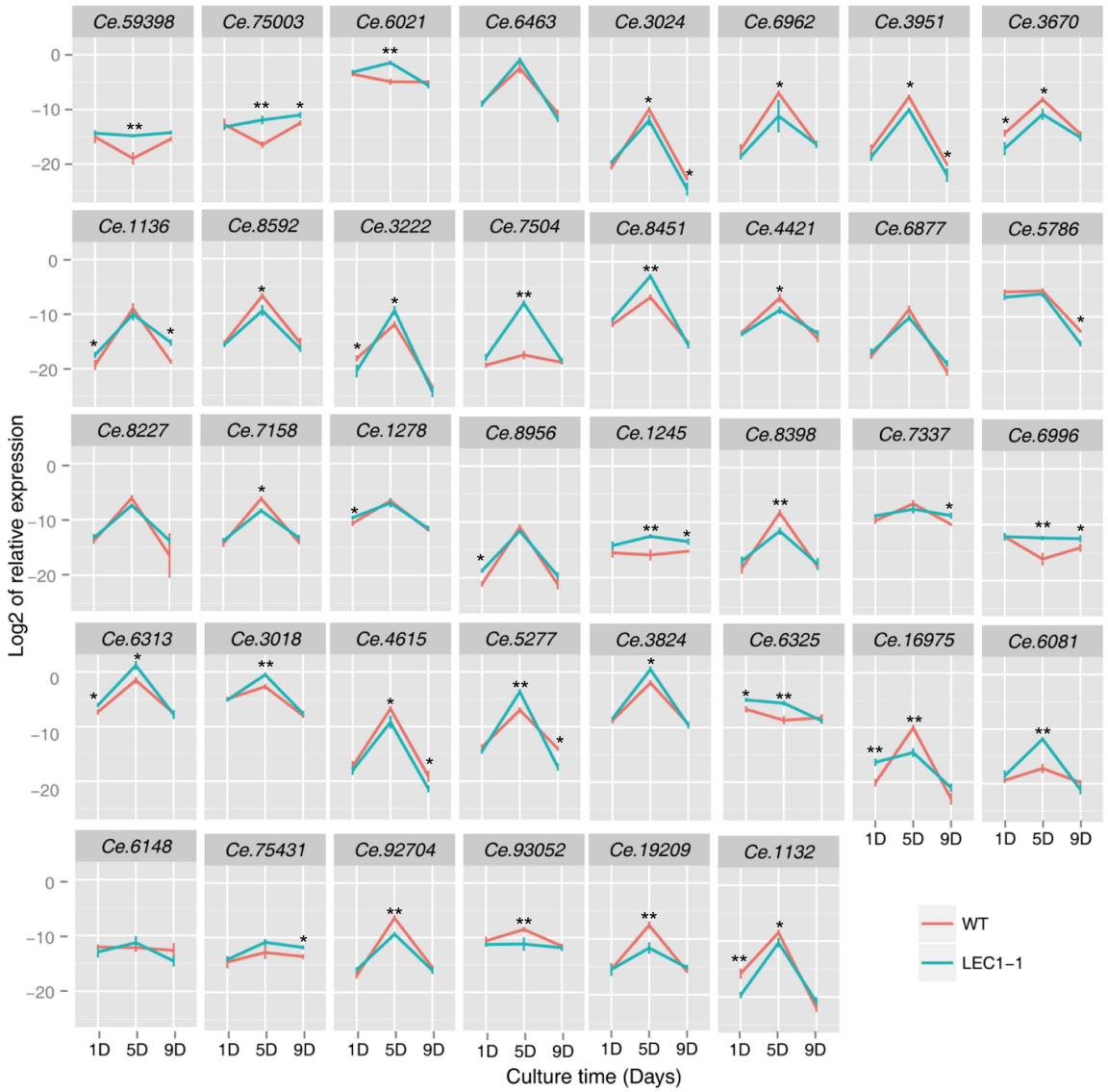

FIGURE 7 | Expression of protein and carbohydrate metabolism-related genes in *AtLEC1* transgenic strains. WT, wild type; *LEC-1*, *AtLEC1* transgenic strain; 1D, 5D, and 9D were sampled on the 1st, 5th, and 9th days under mixotrophic culture conditions. The relative abundance of 18S rRNA was used as an internal standard control. The values are the difference (Ct) between the target gene and the reference gene. The values are the means of three biological replicates. Asterisks indicate statistically significant differences, Student's *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$ compared with WT under the same conditions.

transgenic *C. ellipsoidea* strains were significantly increased by 24.2–32.65 and 22.14–29.91% under mixotrophic culture conditions, respectively. Under autotrophic conditions, the total fatty acid content and total lipid content were significantly increased by 24.4–28.87 and 21.69–30.45%, respectively. Notably, the overexpression of *AtLEC1* did not affect the growth rate of strains, but the protein content significantly decreased. In higher plants, *LEC1* is expressed primarily in embryonic tissues and plays an important biological function in controlling late embryonic development and cotyledon formation (Kwong et al., 2003; Wang and Perry, 2013). Loss-of-function *Lec1* mutations cause phenotypically abnormal embryos (Meinke, 1992), defects in storage protein and lipid accumulation (Santos-Mendoza et al., 2008), and etiolation-related phenotypes in early seedlings in *Arabidopsis* (Huang et al., 2015). The overexpression of *BnaLEC1* under a seed-specific promoter (*Napin A*) from *B. napus* caused the *Arabidopsis* transgenic plants to be abnormal after germination with complete death or sterility. However, when the promotor activity is only 18% of the original, plant growth and propagation are normal with a remarkable improvement in lipid content (Tan et al., 2011). Our results suggested that *AtLEC1* can improve microalgae oil productivity and that it has advantages for improving *Chlorella* lipid content over that of higher plants, which can eliminate the unfavorable characteristics caused by the different tissue and organ differentiation of higher plants.

AtLEC1 Regulated the Gene Expression of *C. ellipsoidea*

LEC1 served as a key regulator to coordinate the expression of fatty acid biosynthetic genes. In our study, key genes related to lipid synthesis were identified, including *ACCase* (*Ce.101511* and *Ce.91597*), *GPDH* (*Ce.61185, Ce.81049,* and *Ce.82444*), *PDAT1* (*Ce.67794*), and *DGAT1* (*Ce.70246*). Their expression levels were higher in the transgenic strains than in the WT. *ACCase* activity is directly related to fatty acid accumulation (Leyva et al., 2014). *DGAT* is the rate-limiting enzyme of TAG synthesis in the Kennedy pathway (Lehner and Kuksis, 1996). Our results showed that *AtLEC1* could enhance the expression of lipid biosynthesis-related genes to improve lipid content in *C. ellipsoidea*. *LEC1* encodes a nuclear factor YB (NF-YB) subunit of NF-Y (or the CCAAT box–binding factor HAP3), which is a heterotrimer consisting of three subunits (NF-YA, NF-YB, and NF-YC) and is highly conserved in all eukaryotic organisms (Edwards et al., 1998; Lotan et al., 1998; Mu et al., 2013). Although the homolog of AtLEC1 was not detected in our RNA-seq, three members of the NF-Y gene family, *CeNF-YA*, *CeNF-YB*, and *CeNF-YC*, were found. Only the expression of *CeNF-YA* was enhanced in the transgenic cells. The results suggested that the expression of one of the NF-Y members can be affected by *AtLEC1* and may contribute to the increase in lipid accumulation.

Several studies have indicated that *LEC1* is directly involved in regulating photosynthesis and chloroplast function during seed development in higher plants (Willmann et al., 2011; Allorent et al., 2015). *LEC1* controls distinct gene sets at different developmental stages, *LEC1* binding alone does not appear to be sufficient to regulate gene expression, and *LEC1* function is partially dependent on *ABSCISIC ACID INSENSITIVE3 (ABI3)*, *FUSCA3 (FUS3)*, and *WRINKLED1 (WRI1)* in the regulation of fatty acid biosynthesis in higher plants (Mu et al., 2008; Pelletier et al., 2017; Jo et al., 2020). However, there were no members of the ABI3VP1/B3 family in our transcriptomic database; therefore, the homologous genes of *FUS3*, *ABI3* or *LEC2* were not found. *WRI1* belongs to *AP2-EREBP/ERF*, which plays an important role in fatty acid biosynthesis in higher plants (Focks and Benning, 1998). In our study, 10 TF genes (*AP2-EREBP/ERF* family) were selected, namely, *Ce.7138*, *Ce.4614*, *Ce.1006*, *Ce.3182*, *Ce.6245*, *Ce.5515*, *Ce.5831*, *Ce.2945*, *Ce.11419*, and *Ce.2372,* for their expression detection in WT, and *AtLEC1* transgenic *Chlorella* grown under mixotrophic culture for 1, 5, and 9 days was analyzed by qRT-PCR. Surprisingly, the expression of these genes was not detectable (data not shown). The differential regulatory patterns due to the great genomic diversity between higher plants and unicellular green algae need to be studied further.

Difference Between the Genes Regulated by *AtLEC1* in *Arabidopsis* and in *Chlorella*

To analyze the differences in the regulatory network affected by *AtLEC1* between *Chlorella* and higher plants, the transcriptomes of *AtLEC1 Chlorella* and *AtLEC1* transgenic *Arabidopsis* (GSE12137) were analyzed. As in previous studies, in *AtLEC1* transgenic *Arabidopsis*, approximately 425 genes were significantly upregulated and 262 genes were significantly downregulated in transgenic seedlings when *AtLEC1* was induced by estradiol, and over 58% of known enzyme-coding genes were upregulated in the plastidial fatty acid synthetic pathway (Mu et al., 2008). In our study, according to the annotation, 220 regulated *Chlorella* genes were annotated (134 genes were upregulated and 86 genes were downregulated) by 180 genes (97 genes were upregulated and 83 genes were downregulated) from *Arabidopsis* genes (**Supplementary Table 5** and **Supplementary Figure 2**). Among these genes, 60 genes in *Arabidopsis* and 72 genes in *Chlorella* were upregulated, such as *Ce. 6624* (*CAC3*), *Ce. 6305* (*BCCP2*), and *Ce. 1815* (*MOD1/ENR1*), which were key genes controlling fatty acid biosynthesis flux. Approximately 59% (95/160) of genes related to lipid metabolism annotated by MapMan were upregulated in the *AtLEC1* transgenic *Chlorella* strain compared with the WT. In transgenic *Arabidopsis* and *Chlorella*, overexpression of *LEC1* downregulated the expression of 39 genes in *Arabidopsis* and 45 genes in *Chlorella*, such as *Ce.59398* and *Ce.75003*, relative to protein biosynthesis, which were key genes controlling nitrogen metabolism (**Supplementary Table 2**).

Compared with overexpression of *LEC1* in *Arabidopsis*, some homologous genes had different changeable trends in *Chlorella*. The 41 genes in *LEC1* overexpression *Arabidopsis* were upregulated but not their 43 homologous genes in *AtLEC1* transgenic *Chlorella*, and their functions were involved in protein biosynthesis, vitamin metabolism and tetrapyrrole synthesis (**Supplementary Table 2**). The 26 genes were downregulated in *LEC1* overexpression *Arabidopsis,* but their homologous genes in *AtLEC1* transgenic *Chlorella* were upregulated (**Supplementary Table 2**). However, only 6 genes of *Chlorella* had the same regulation model with their homologous genes of *Arabidopsis* with the overexpression of *AtLEC1*. For example, *Ce.3222* and *Ce.7504* were significantly downregulated and could interact with Rubisco in the dark reaction of photosynthesis, whereas the other four genes *Ce.6081* (related to cell development), *Ce.7764* (ethanol dehydrogenase), *Ce.4276* (ATPase), and *Ce.6877* (aldehyde dehydrogenase) were significantly upregulated. The functions of the protein-modified related genes *Ce.1480* and *Ce.4858*, encoding Golgi body localization proteins, were contrary to their regulation in *Arabidopsis*. Thus, it would be of a great interest to dissect the differences in *AtLEC1* function in *Chlorella* and *Arabidopsis*.

We did not find *AtLEC1-Like* sequences in *C. ellipsoidea.* In the alignment of protein sequences between *AtLEC1* and other species, 14 homologs of *AtLEC1* were screened, and the similarities among them were 34.9–78.3%. The selected genes/proteins include *AtLEC1-like* from algae (MpuLEC1L, CsuLEC1L, MspLEC1L, OluLEC1L, CreLEC1L, VcaLEC1L, and DsaLEC1L), and higher plants (GmLEC1, BnLEC1, and AtL1L), and several NF-Y subunits (ScHAP323, HsNF-YB24, MmNF-YB38, and CeNF-YB) (**Supplementary Table 6**). All of these genes contain a conserved HFD domain (Nardone et al., 2017) (**Supplementary Figure 3**). *LEC1* in higher plants derived only by a suitable promoter can be used to improve the seed oil content (Mu et al., 2008; Tan et al., 2011). However, homologs of *LEC1*

from the algae may improve the oilseed crop without abnormal agricultural traits through the genetic engineering due to no function differentiation for specific tissues as in higher plants, which warrants further investigation.

The *AtLEC1* could significantly increase the lipid content and decrease the protein content of *C. ellipsoidea* without affecting the growth rate of strains and biomass under mixotrophic culture and autotrophic culture conditions. Transcriptome sequencing analysis showed that *AtLEC1* could promote expressions of 59% of the genes related to the lipid biosynthesis in *C. ellipsoidea*, but the differences of mechanism of *AtLEC1* in regulating lipid accumulation in *C. ellipsoidea* and *Arabidopsis thaliana* warrants further investigation. In general, our research provides a new means of improving the lipid content in *Chlorella* and may help to elucidate the mechanism governing lipid accumulation in *Chlorella*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material.**

AUTHOR CONTRIBUTIONS

ZH and CF designed and supervised the study. XaL, DZ, and JZ performed the experiments. XaL, DZ, XuL, and CF analyzed the data. XaL and DZ wrote the manuscript. ZH, YC, YH, and CF revised the manuscript. RR-CW helped to direct the graduate research, interpreted and discussed data, and revised the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.626162/full#supplementary-material

Supplementary Figure 1 | *AtLEC1* regulated metabolism pathway.

Supplementary Figure 2 | Venn diagram showing the differentially expressed genes in *AtLEC1* transgenic *C. ellipsoidea* and *A. thaliana*.

Supplementary Figure 3 | Sequence analysis of LEC1 from different species.

Supplementary Table 1 | Primers used for gene cloning, detection of genes by PCR and quantitative real-time PCR.

Supplementary Table 2 | All the differentially expressed genes based on the transcriptomic analysis between the LEC1-1 strain and the wild-type strain.

Supplementary Table 3 | Genes related to protein metabolism, RNA metabolism and transport in *AtLEC1* transgenic *C. ellipsoidea*.

Supplementary Table 4 | *AtLEC1* regulated candidate genes related to protein and carbohydrate metabolism in the metabolic network.

Supplementary Table 5 | Differentially expressed genes in *AtLEC1* transgenic *C. ellipsoidea* and *A. thaliana*.

Supplementary Table 6 | Protein sequence homologous alignment of *AtLEC1* from different species.

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Automated Online Flow Cytometry Advances Microalgal Ecosystem Management as *in situ*, High-Temporal Resolution Monitoring Tool

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Microalgae are emerging as a next-generation biotechnological production system in the pharmaceutical, biofuel, and food domain. The economization of microalgal biorefineries remains a main target, where culture contamination and prokaryotic upsurge are main bottlenecks to impair culture stability, reproducibility, and consequently productivity. Automated online flow cytometry (FCM) is gaining momentum as bioprocess optimization tool, as it allows for spatial and temporal landscaping, real-time investigations of rapid microbial processes, and the assessment of intrinsic cell features. So far, automated online FCM has not been applied to microalgal ecosystems but poses a powerful technology for improving the feasibility of microalgal feedstock production through *in situ*, real-time, high-temporal resolution monitoring. The study lays the foundations for an application of automated online FCM implying far-reaching applications to impel and facilitate the implementation of innovations targeting at microalgal bioprocesses optimization. It shows that emissions collected on the FL1/FL3 fluorescent channels, harnessing nucleic acid staining and chlorophyll autofluorescence, enable a simultaneous assessment (quantitative and diversity-related) of prokaryotes and industrially relevant phototrophic *Chlorella vulgaris* in mixed ecosystems of different complexity over a broad concentration range (2.2–1,002.4 cells $\cdot$ μL^{-1}). Automated online FCM combined with data analysis relying on phenotypic fingerprinting poses a powerful tool for quantitative and diversity-related population dynamics monitoring. Quantitative data assessment showed that prokaryotic growth phases in engineered and natural ecosystems were characterized by different growth speeds and distinct peaks. Diversity-related population monitoring based on phenotypic fingerprinting indicated that prokaryotic upsurge in mixed cultures was governed by the dominance of single prokaryotic species. Automated online FCM is a powerful tool for microalgal bioprocess optimization owing to its adaptability to myriad phenotypic assays and

its compatibility with various cultivation systems. This allows advancing bioprocesses associated with both microalgal biomass and compound production. Hence, automated online FCM poses a viable tool with applications across multiple domains within the biobased sector relying on single cell–based value chains.

Keywords: *Chlorella vulgaris*, phenotypic fingerprinting, online flow cytometry, microalgae, prokaryotes

INTRODUCTION

Cellular agriculture and along with it renewable biobased materials relying on single-cell biorefineries as, for instance, those associated with yeasts, bacteria, and microalgae, are gaining momentum. Microalgae have attracted attention as a sustainable means of a next-generation biotechnological production system for the food, feed, pharmaceutical, nutraceutical, and biofuels sector. They are of emerging interest owing the sustainable notion of their connected value chains. Microalgal biomass is characterized by a beneficial composition with protein contents of up to 65%, depending on the species employed or lipid contents with a beneficial ratio of ω6- to ω3-polyunsaturated fatty acids. Industrial products extracted from microalgae, for instance, include β-carotene, lipids, polysaccharides, and vitamins such as vitamin B_{12}, proteins, or phycocyanin (Hyka et al., 2013; Caporgno and Mathys, 2018; Canelli et al., 2020; Rischer et al., 2020).

The economization of microalgal bioprocesses remains a main target, which comprises optimizing the productivity and reproducibility of microalgal biomass and compound production. Flow cytometry (FCM) poses a viable technology for improving the feasibility of the bioprocesses associated with microalgal biorefineries. Microalgae are a diverse group of microorganisms differing in their morphology, ecology, physiology, and biochemistry. FCM enables a rapid and accurate discrimination and quantification of different cells, as well as a depiction of physiological states based on their inherent cell characteristics. So far, FCM has been applied for the monitoring of bioprocesses associated with, for example, astaxanthin, oil, or glucose production (Hyka et al., 2013). The development of automated tools adjunctive to FCM that enable online and inline culture monitoring further perpetuates the application of the technology for single-cell bioprocess management. Automated online FCM enables spatial and temporal landscaping, as well as investigations of rapid processes on a quantitative and phenotype-related base harnessing intrinsic cell features *in situ*, at real-time, and at high-temporal resolution.

An important aspect in optimizing the feasibility of microalgal bioprocesses in terms of reproducibility and productivity is associated with the management of culture ecologies. Phototrophic microalgae production as monocultures is not a realistic scenario on industrial scale. Additionally, culture contamination, for example, caused by the extraneous invasion of parasitic prokaryotic species or microalgal grazers, was reported as a primary bottleneck to impairing microalgal productivities. Culture contaminations can lead to biomass and consequently economic losses (Enzing et al., 2014). A real-time detection and quantification tool allows taking immediate countermeasures as a response to microbial disturbances caused by such contaminants or to the upsurge of prokaryotic counts during culture. Thus, it could contribute to the stability, reproducibility, and consequently productivity of microalgal feedstock production. FCM is advantageous over traditional techniques, such as plating, which are often laborious and fail to reflect complex ecosystems, as it allows for a fast and reproducible detection and enumeration of cultivable and non-cultivable microorganisms (Hammes and Egli, 2005). Microalgae can be easily distinguished from prokaryotic organisms or abiotic particles based on their size and granularity, i.e., forward (FSC) and sideward (SSC) scattered light intensities, respectively (Haberkorn et al., 2019). The nucleic acid content or pigment autofluorescence provide additional, distinctive features (Hammes and Egli, 2010; Hyka et al., 2013; Prest et al., 2013; Besmer et al., 2014).

These phenotypic properties reflecting inherent cellular features allow establishing FCM data analysis approaches that enable a community characterization beyond a detection and purely quantitative assessment. Props et al. (2016) established a data analysis approach relying on phenotypic fingerprinting that enabled the assessment of prokaryotic community dynamics in aquatic ecosystems. In combination with automated online FCM, they demonstrated a detection of contaminations based on alterations in the phenotypic fingerprint and thus α-diversity of the prokaryotic community *in situ* and in real-time. Establishing such approaches for microalgal cultures could contribute to contamination management or also support ecological engineering approaches. These insights could contribute to optimizing microalgal bioprocess feasibility by supporting the development of technological innovations for improved upstream performance. For instance, Haberkorn et al. (2021) showed that nanosecond pulsed electric field processing (nsPEF) could aid in fostering the upstream performance of microalgal feedstock production. Progress in implementing nsPEF on an industrial scale in non-axenic cultures has so far been hampered by a lack in understanding microalgal-bacterial interactions and the underlying intracellular treatment mechanisms. Automated online FCM combined with data analysis relying on phenotypic fingerprinting could perpetuate the understanding of the underlying microbial community responses. It provides real-time data on community diversity and insights into intrinsic cell responses following nsPEF treatments, by, for instance, depicting alterations in pigment, protein, and lipid content (Le Chevanton et al., 2013; Shurin et al., 2013; Cho et al., 2015; Zhang et al., 2018).

Automated online FCM is also a viable option for bioprocess optimization related to microalgal compound production. FCM

Abbreviations: FCM, flow cytometry; FSC, forward scattered light intensities; HFL3, higher red fluorescence emission; HNA, high nucleic acid; LNA, low nucleic acid; SSC, sideward scattered light intensities; TCC, total cell concentration.

is adaptable to myriad phenotypic assays and is, together with the automation module, compatible with various cultivation systems. Hence, it enables monitoring microalgal compounds, including proteins, lipids, or pigments *in situ* and at real-time (Hyka et al., 2013). Hence, automated online FCM could also aid in perpetuating microalgal production from monocultures through a quantification of microalgal counts in real-time, e.g., as response to external treatment stimuli. The assessment of compositional alterations in real-time, such as those related to pigment, lipid, or protein content, poses another option (Gao et al., 2020). The application of online FCM-based monitoring has yet been limited to the assessment of prokaryotes in aqueous ecosystems. Consequently, procedures and protocols for microalgal cultures are lacking. Thus, the study aimed to assess the feasibility of automated online FCM as an *in situ*, high-temporal resolution monitoring tool for the assessment of population dynamics in non-axenic *Chlorella vulgaris* cultures. The present study (1) provides a staining protocol and gating strategy that allow a simultaneous assessment (quantitative and diversity-related) of prokaryotes and microalgae in mixed ecosystems. It highlights the applicability harnessing industrially relevant phototrophic *C. vulgaris* in coculture with indigenous prokaryotes as the case study. (2) As a proof of concept, dynamic microbial events were tracked using *C. vulgaris* in five different ecosystems of defined and undefined cocultures with prokaryotes. (3) The study is the first to demonstrate the applicability of basic (detection and quantification) and advanced (phenotypic fingerprint) data analysis combined with automated online FCM to microalgal cultures.

MATERIALS AND METHODS

Axenic *C. vulgaris* Culture

Axenic *C. vulgaris* SAG 211-12 was originally obtained from the culture collection of algae at Goettingen University, Germany. Cultures were maintained on modified diluted seawater nitrogen (DSN) medium agar plates (1.5% agar) using nitrate (141.65 g L^{-1} $NaNO_3$) as nitrogen source under ambient conditions (30.3 μmol · photons · m^{-2} · s^{-1}, 24 ± 1°C, ambient CO_2 = 400 ppm) (Pohl et al., 1987; Haberkorn et al., 2020). For experiments, axenic cultures were grown by transferring single *C. vulgaris* colonies in 150-mL cultivation volume of sterile, modified DSN medium using 500-mL Erlenmeyer flasks. Cultures were stored in a shaking incubator (Multitron Pro; Infors AG, Bottmingen, Switzerland) applying cultivation conditions described by Haberkorn et al. (2019) for 7 days.

Cocultures

Tistrella mobilis TH-33 (KF783213.1), *Pseudomonas pseudoalcaligenes* CLR9 (KF478199.1), and *Sphingopyxis* sp. AX-A (JQ418293.1) were maintained at −80°C in 80% vol/vol glycerol (80% vol/vol in dH_2O). For experiments, all prokaryotic cultures were streaked out onto separate tryptic soy broth (TSB) agar plates (3% TSB, 1.5% agar) and incubated (30°C, 5 days). Subsequently, liquid cultures were prepared by transferring single bacterial colonies into 35 mL liquid TSB (3% in dH_2O) and incubating at 30°C for 36 h.

For the experimental cultures, prokaryotic cells in the early exponential growth were used. Therefore, prokaryotic cell counts were quantified in the liquid cultures by manual FCM. Cultures were diluted with filtered (0.1-μm, Millex-GP, Millipore; Merck KGaA, Darmstadt, Germany) water (Evian; Danone, Paris, France) to a total cell concentration (TCC) below than 2.0×10^5 cells · mL^{-1}. Samples were stained with a SYBR® Green I solution (working solution: 1:100 in 0.1-μm filtered dimethyl sulfoxide; Life Technologies, Eugene, OR, United States; final stain concentration: 1:10,000), incubated for 10 min at 37°C in the dark, and manually assessed on the flow cytometer and cell counts determined.

Based thereon, cultures were standardized to 10^7 cells · mL^{-1} and washed three times in 35 mL modified DSN (10,000 × *g*; 5 min) to remove excess TSB. Subsequently, cultures were stored in 35-mL cultivation volume using sterile, modified DSN, and 100-mL Erlenmeyer flasks in a shaking incubator (Multitron Pro; Infors AG) at 30°C, 150 rpm, 70% relative humidity, 400 ppm CO_2, and 36 μmol · photons · m^{-2} · s^{-1} until coculture establishment. Cocultures with *C. vulgaris* were established 16 h (*Tistrella* sp., *Sphingopyxis* sp.) or 4 h (*Pseudomonas* sp.) following standardization. See **Table 1** for inoculation ratios of experimental cultures. Cocultures with three prokaryotic strains were established such that equal shares of each prokaryotic strain were obtained. The samples were cultivated in a shaking incubator (Multitron Pro; Infors AG) at 30°C, 150 rpm, 70% relative humidity, 400 ppm CO_2, and 36 μmol · photons · m^{-2} · s^{-1} for 3 days. Technical constraints of the sampling robot allowed to assess one culture by automated online FCM. As the study did not encompass a fully ecological scope, but rather aimed at demonstrating the feasibility of automated online FCM for microalgae–prokaryote cocultures, each coculture experiment was conducted once.

Flow Cytometry

All samples were measured on a BD Accuri™ C6 Plus flow cytometer (BD Accuri Cytometers, San Jose, CA, United States) equipped with a 20-mW laser emitting at a wavelength of 488 nm. This allowed a collection of signals related to (1) FSC and (2) SSC light intensities, (3) green (533 ± 30 nm; FL1 channel), and (4) red fluorescence intensity (> 670 ± 25 nm; FL3 channel). The collection of those signals allowed to quantify (A) cell size, (B) cell granularity, (C) nucleic acid content (by SYBR® Green I staining), and (D) chlorophyll autofluorescence, respectively. Before each experiment, the calibration of the flow cytometer was assessed with calibration beads (BD™ CS&T RUO Beads; BD Biosciences, San Jose, CA, United States).

Manual flow cytometer measurements were always conducted with an analyzed volume of 50 μL, a flow rate of 66 μL · min^{-1}, and a lower threshold of 800 on the FL1-H channel. Automated online FCM was conducted with a fully automated sampling, staining, and incubation robot (OC-300; onCyt Microbiology AG, Zurich, Switzerland) combined with the BD Accuri™ C6 Plus flow cytometer. Samples were taken continuously at 25-min intervals throughout the entire experiment until termination on

TABLE 1 | Coculture combinations of *C. vulgaris* with the prokaryotic strains *Sphingopyxis* sp., *Tistrella* sp., and *Pseudomonas* sp. assessed by automated online FCM, as well as corresponding initial and final cell concentrations [cells · μL^{-1}].

	Coculture	Initial cell concentration [cells · μL^{-1}]		Final cell concentration [cells · μL^{-1}]	
		C. vulgaris	Prokaryotes	*C. vulgaris*	Prokaryotes
1	*C. vulgaris–Sphingopyxis* sp.	46.5	2.4	49.0	37.0
2	*C. vulgaris–Tistrella* sp.	27.2	2.2	48.0	28.4
3	*C. vulgaris–Sphingopyxis* sp., *Tistrella* sp., *Pseudomonas* sp. I	43.6	2.6	39.1	47.6
4	*C. vulgaris–Sphingopyxis* sp., *Tistrella* sp., *Pseudomonas* sp. II	66.4	126.6	66.7	1002.4
5	*C. vulgaris*–undefined; spontaneous, fortuitous contamination	45.6	2.2	49.2	39.9

Cocultures were established and assessed as single cocultures (n = 1).

day 3 and measured using the same standard flow cytometer settings described for manual FCM. For each measurement point, a single sample was collected, diluted 1:100 with 0.1-µm filtered water (Evian; Danone), stained with SYBR® Green I (working solution: 1:5,000 in 0.22-µm filtered 10 mM TRIS buffer, pH 8.0 containing 50 mM sodium thiosulfate; final stain concentration: 1:10,000) and incubated (10 min, 37°C). Subsequently, the sample was pumped to the flow cytometer and measured for 90 s (equivalent to an analyzed volume of approximately 61 µL). Between sampling, all internal tubing, the syringe pump, and the incubation/mixing chamber were rinsed with sodium hypochlorite solution (1% active chlorine), sodium thiosulfate solution (100 mM), and ultrapure water (Besmer et al., 2014, 2016, 2017a,b; Besmer and Hammes, 2016).

Staining Protocol Validation

The operating principle of the staining robot for automated online FCM comprises first a sampling step from the culture, followed by an incubation with the stain, and subsequently a measurement step on the FCM, which was adopted for staining protocol validation. Additionally, the nucleic acid staining of prokaryotic communities harnessing SYBR® Green I (37°C, 10 min) was shown to provide sensitive and reproducible quantitative data and phenotypic fingerprints on prokaryotes during automated online FCM (Besmer and Hammes, 2016; Besmer et al., 2016, 2017a,b; Props et al., 2018). As the study aimed at establishing a staining protocol for the simultaneous assessment of prokaryotes and microalgae, the feasibility of applying the staining protocol for the assessment of *C. vulgaris* was investigated. Six discreet subsamples of axenic *C. vulgaris* SAG 211-12 were stained with SYBR® Green I. Each sample was measured manually and individually on the flow cytometer in quintuplicates applying the same conditions as described in section "Flow Cytometry" assessing cells at a staining temperature of 37°C for 5, 8, 10, and 15 min. Additionally, the effect of the staining temperature was investigated by staining cells for 10 min at 4, 37, and 40°C. Negative controls were analyzed using SYBR® Green I in filtered water only, following the same staining protocol.

Gating

Gate establishment for microalgae first encompassed assessing fresh and axenic *C. vulgaris* culture. Aliquots of the same axenic *C. vulgaris* sample were diluted with filtered (0.1-µm, Millex-GP, Millipore; Merck KGaA) water (Evian; Danone) to obtain a cell concentration below 2.0×10^5 cells · mL^{-1}. Subsequently, six discrete sub-samples were stained (37°C, 10 min) with SYBR® Green I (working solution: 1:5,000 in 0.22-µm filtered 10 mM TRIS buffer, pH 8.0 containing 50 mM sodium thiosulfate; final stain concentration: 1:10,000) in the dark and measured in quintuplicate (Besmer et al., 2017b). Samples were measured manually and separately on the flow cytometer. Negative controls were analyzed using SYBR® Green I in filtered water only, following the same staining protocol. The microalgal gate was established based on green (FL1) and red (FL3) fluorescent intensities. Microalgal gates were validated for their fit throughout all samples obtained in this study (**Figures 1C–F**). Gates for assessing prokaryotes in coculture with *C. vulgaris* were initially adopted from Prest et al. (2013) and included prokaryotic regions for low (LNA_p) and high nucleic acid content (HNA_p) organisms (**Figures 1A–F**). Prokaryotic gates were validated for their fit throughout all samples by first assessing axenic prokaryotic cultures, followed by applying the gates to prokaryotes in coculture with *C. vulgaris*. No compensation was applied.

Data Analysis

Raw data were collected with the BD Accuri™ C6 software (v1.0.1; BD Accuri Cytometers). Each measurement point generated a single FCS file, which was exported to the R statistical environment (R-Studio, v1.1.456). Data and statistical assessment were performed using the functionalities offered by the *flowCore* (v1.38.2) and *Phenoflow* (v1.1.2) packages. Virtual gating was applied following the gating strategy described in *Staining Protocol Validation*.

For data obtained from automated online FCM of the established cocultures, basic FCM data analysis (detection and quantification) was conducted for both *C. vulgaris* and prokaryotic (HNA_p gate) communities. Data analyses relying on phenotypic fingerprints, and based thereon the phenotypic diversity index, were established for the prokaryotic community employing the HNA_p domain of the multispecies assemblages and the undefined culture to assess shifts in the prokaryotic community relating to phenotypic diversity-based alterations. Therefore, the *Diversity_rf* function was used (number of bootstraps, $n = 3$), employing an adapted analytics approach

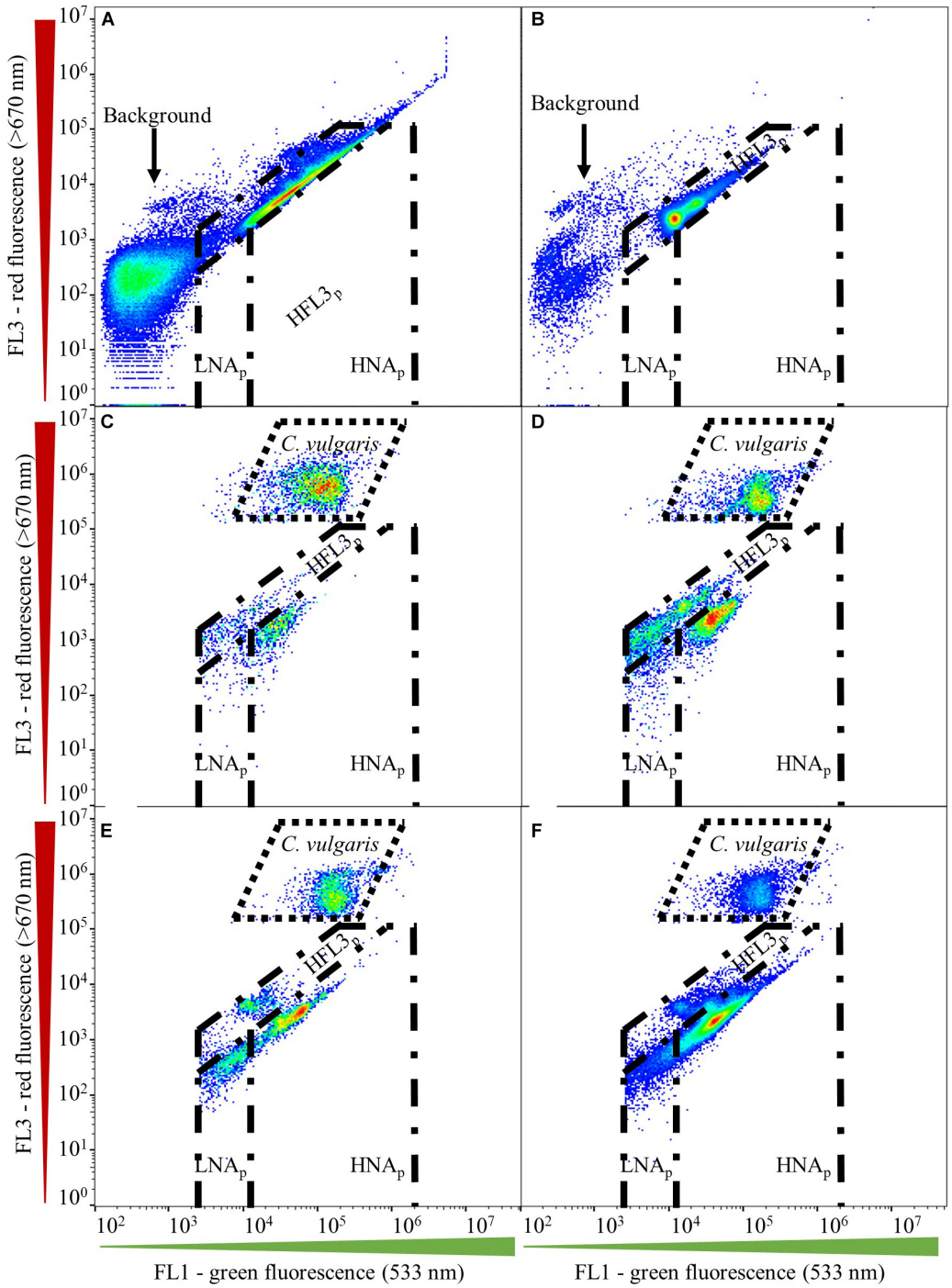

FIGURE 1 | Gating strategy established using dual-density-plots on the green (FL1) and red (FL3) fluorescent channels. Gates, including microalgal, HNA_p, LNA_p, and $HFL3_p$ domains, are shown for **(A)** axenic *Tistrella* sp., **(B)** axenic *Sphingpyxis* sp., **(C)** *Tistrella* sp.–*C. vulgaris* coculture, **(D)** *Sphingopyxis* sp.–*C. vulgaris* coculture, and *Sphingpyxis* sp., *Tistrella* sp., *Pseudomonas* sp.–*C. vulgaris* coculture inoculated to a lower **(E)** and higher **(F)** initial prokaryotic concentration than *C. vulgaris*.

initially suggested by Props et al. (2018) for prokaryotes in aquatic ecosystems. Briefly, the function performs bivariate kernel density estimation on selected phenotypic traits (FL1-A, FL3-A, FSC-A, and SSC-A) and concatenates the obtained values to a one-dimensional feature vector, the phenotypic fingerprint. The phenotypic fingerprint then serves for calculating the phenotypic diversity index. In analogy to taxonomy, i.e., relative abundance-based α-diversity, the phenotypic diversity index

resembles the "effective number of phenotypic states" in a microbial community. Following Props et al. (2018), the Hill-diversity metric of order two was employed as α-diversity measure to put equal weight on the richness and evenness.

A *t* test was performed to statistically assess data obtained from gate establishment. A non-significant Shapiro–Wilk test ($P > 0.05$) and *F* test ($P > 0.05$) indicated normal distribution and homogeneity of variances of the obtained data, respectively. A Wilcoxon rank-sum test was conducted to assess statistical significances of data collected from staining protocol validation, as the data were not normally distributed.

RESULTS

Gate Definition

Applying the microalgal gate based on the emission spectra of SYBR® Green I and of chlorophyll (autofluorescence) provided a valid and reproducible approach to assess microalgal cell counts in fresh *C. vulgaris* samples. No significant difference was found between the datasets collected on the FSC/SSC and FL1/FL3 channels (*t* test; $t = -2.09$, $df = 10$; $P = 0.06$; $n = 30$). Quantitative assessment revealed a relative standard deviation of 3.1% and 3.2% of counts collected on the FSC/SSC and FL1/FL3 channels, respectively, indicating low intrasample variation. Counts collected on the FL1/FL3 channels were shown to represent *C. vulgaris* biomass yields, indicating that the staining protocol (SYBR® Green I; 37°C, 10 min) and subsequent count determination employed in this study represented *C. vulgaris* cell counts and biomass yields well (Haberkorn et al., 2019). No shift was observed in microalgal nucleic acid or chlorophyll content throughout the cocultures assessed, which was indicated by a 100% coverage within the gate established on the FL1/FL3 fluorescent channels.

In the prokaryotic domain, the emission collected on the FL1/FL3 fluorescent channels revealed the presence of different clusters (**Figures 1A–F**). Gates for assessing prokaryotic populations were initially adopted from Prest et al. (2013), who proposed a discrimination of prokaryotic regions characterized by low (LNA) and high nucleic acid (HNA) content. Albeit axenic prokaryotic cultures located in the HNA domain suggested by Prest et al. (2013), coculture with *C. vulgaris* resulted in a shift of the localization of prokaryotes on the FL1/FL3 channels toward lower emission on the green (FL1) and red (FL3) fluorescent channels (**Figures 1A–D**). Additionally, prokaryotes in the multispecies assemblage located at the intersection of the initial LNA and HNA domains proposed by Prest et al. (2013) prevented a clear discrimination of the two populations (**Figures 1E,F**). Hence, LNA_p and HNA_p gates for assessing prokaryotes in coculture with *C. vulgaris* required adaptation toward lower emission on the green (FL1) (1.5×10^4) and red (FL3) fluorescent spectrum. For a majority of cocultures, counts collected in the LNA_p gate were negligible and might have been associated with background scattering.

Cocultures, such as those established with *Sphingopyxis* sp. (**Figure 1D**) and three prokaryotic strains (**Figures 1E,F**), indicated the presence of an additional prokaryotic cluster that emitted higher on the red fluorescence (FL3) channel, resulting in the establishment of a third gate denoted as $HFL3_p$. However, signal collected within the $HFL3_p$ gate could not be confirmed for all cocultures. In fact, counts obtained within the $HFL3_p$ gate could quantitatively negligible during coculture. Some studies describe those signals collected in the $HFL3_p$ domain to be associated with background noise or scattering (Hammes et al., 2008; Hammes and Egli, 2010; Prest et al., 2013). In addition, the presence of a $HFL3_p$ domain yet remains unreported for microalgal and prokaryotic aquatic ecosystems. Hence, diversity assessment of prokaryotic communities including HFL3p fractions would be speculative. Thus, the HFL3p fraction was excluded from subsequent community diversity analysis.

Indistinct signal at fluorescent intensities lower than those proposed for the LNA_p domain on the green (FL1) fluorescent channel, as well as at higher fluorescent intensities on the red (FL3) fluorescent channel, was associated with background scattering and thus excluded from further analysis (**Figures 1A,B**; Berney et al., 2008).

Staining Protocol Validation

No significant difference was observed between a staining time of 10, 5, 8, or 15 min (at 37°C) on the FL1/FL3 fluorescent or on the FSC/SSC channels. Significantly higher ($P < 0.05$) counts were obtained when staining cells at 37°C (10 min) than at 4°C, whereas no difference was observed when increasing the staining temperature to 40°C (**Figure 2**).

Online Monitoring of Culture Dynamics

Establishing gates for prokaryotes and *C. vulgaris* based on the intensities collected on the green (FL1) and red (FL3) fluorescent channels enabled the discrimination of microalgal and prokaryotic populations and thus a simultaneous quantitative and diversity-related (multispecies assemblage, undefined coculture) assessment during automated online FCM.

Sphingopyxis sp. showed a 15.1-fold count increase from 2.4 to 37.0 cells · μL^{-1} with an initial lag phase lasting the first 72.5 h of cultivation and subsequent exponential growth phase, not outnumbering *C. vulgaris* throughout the entire cultivation period (**Figure 3A** and **Table 1**). Visual inspection of FL1/FL3 fluorescent intensities indicated the presence of a prokaryotic cluster that was emitting higher on the red (FL3) fluorescent channel (**Figure 1D**). *Tistrella* sp. counts increased 13.1-fold during cultivation but did not surpass *C. vulgaris* cell counts (**Figure 3B** and **Table 1**). An initial lag phase lasted approximately 50 h followed by an accelerated growth phase until the end of the cultivation period. Most of the counts were collected in the HNA_p domain. With continuing cultivation, the share of cells located in the $HFL3_p$ gate increased, leading to a maximum of 8 cells · μL^{-1}. However, visual inspection of density plots obtained on the FL1/FL3 fluorescent channels did not show distinct clusters or patterns that would substantiate the presence of *Tistrella* sp. in the $HFL3_p$ gate.

Inoculating the multispecies assemblage of *C. vulgaris* and *Tistrella* sp., *Sphingopyxis* sp., and *Pseudomonas* sp. to a lower concentration than *C. vulgaris* resulted in an 18.7-fold

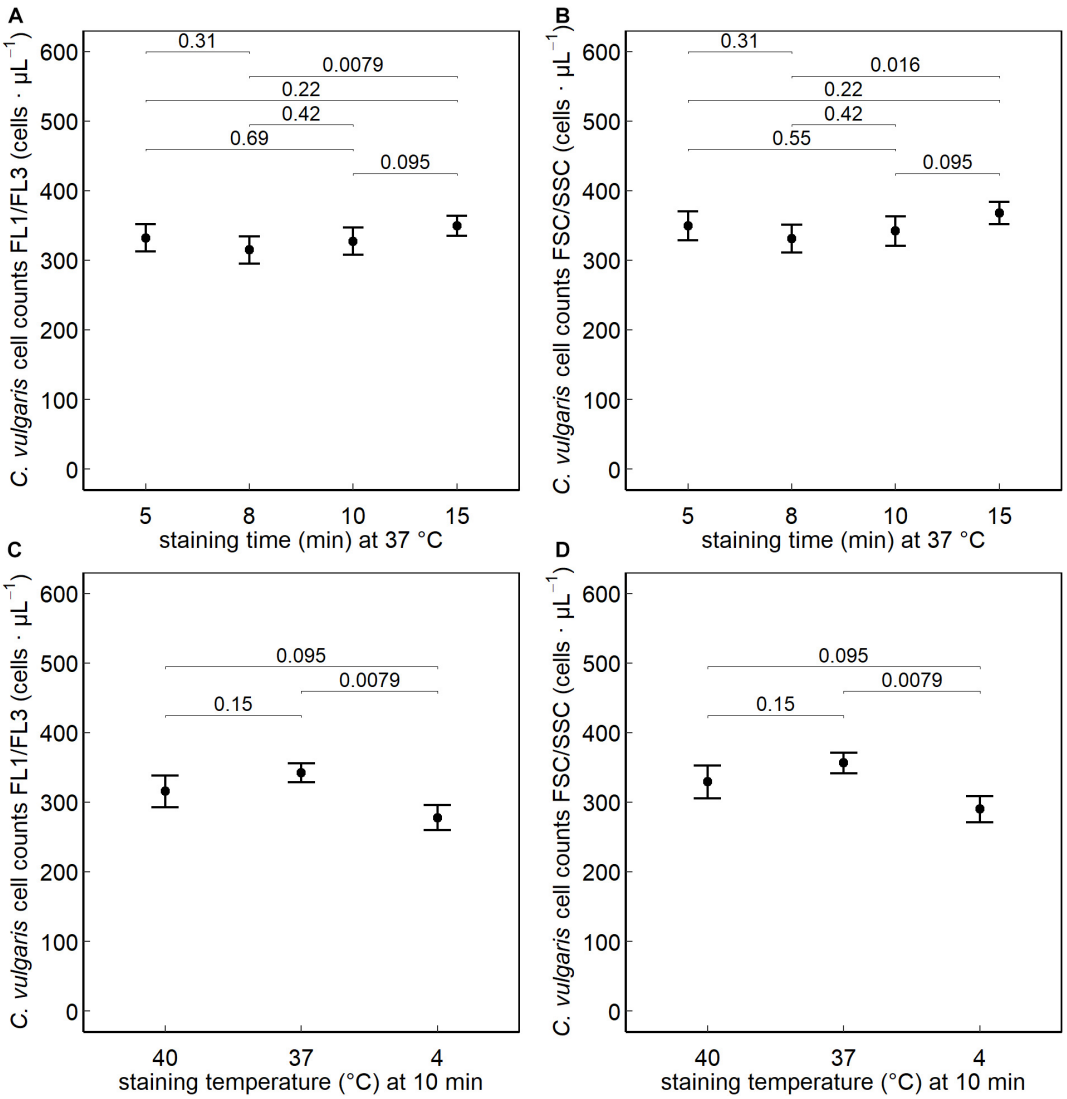

FIGURE 2 | *Chlorella vulgaris* cell counts collected on the green (FL1) and red (FL3) fluorescent and FSC/SSC channels after nucleic acid staining with SYBR® Green I. The effect of varying the staining times (min) at 5, 8, 10, and 15 min at a staining temperature of 37°C **(A,B)** and the effect of the different staining temperatures 40°C, 37°C, and 4°C at a staining time of 10 min **(C,D)** were assessed ($n = 30$).

prokaryotic count increase with an initial lag phase followed by an exponential growth phase that started approximately 50 h after inoculation (**Figure 3C** and **Table 1**). During the initial lag phase, clusters within the $HFL3_p$ gating domain were observed, which remained at constant 12.8 ± 2.2 cells · μL^{-1} throughout the entire cultivation period. During culture of the multispecies assemblage, the phenotypic diversity index increased by 59.7% from initial 1,465.5 ± 89.1 a.u. to a maximum of 2,341.1 ± 66.6 a.u. approximately 26 h after inoculation followed by a decline of 28.2% to 1,680.5 ± 19.3 a.u. at the end of the cultivation period (**Figure 4A**). Inoculating the multispecies assemblage to a higher concentration than *C. vulgaris* resulted in an immediate incidence of exponential prokaryotic growth for the first 37.5 h of cultivation, with a clear dominance of counts collected in the HNA_p gating domain (**Figure 3D** and **Table 1**). Although the maximum observed prokaryotic cell concentration amounted to 1,305.7 cells · μL^{-1} (37.5 h after inoculation), the overall prokaryotic count increase was only 10-fold. After reaching a maximum of 1,305.7 cells · μL^{-1}, prokaryotic counts decreased 1,002.4 cells · μL^{-1}. Cells collected in the $HFL3_p$ gating domain were high at a constant concentration of 47.6 ± 9.2 cells · μL^{-1} throughout the cultivation period. The phenotypic diversity index increased by approximately 17.1% from initial 1,131.4 ± 21.7 a.u. to a maximum of 1,324.5 ± 6.0 a.u. (**Figure 4B**).

The protocol was also applicable to prokaryotes in the coculture based on short-term spontaneous fortuitous contamination. Prokaryotes initially showed decelerated growth, followed by an exponential increase at the end of the cultivation period leading to a maximum cell concentration of

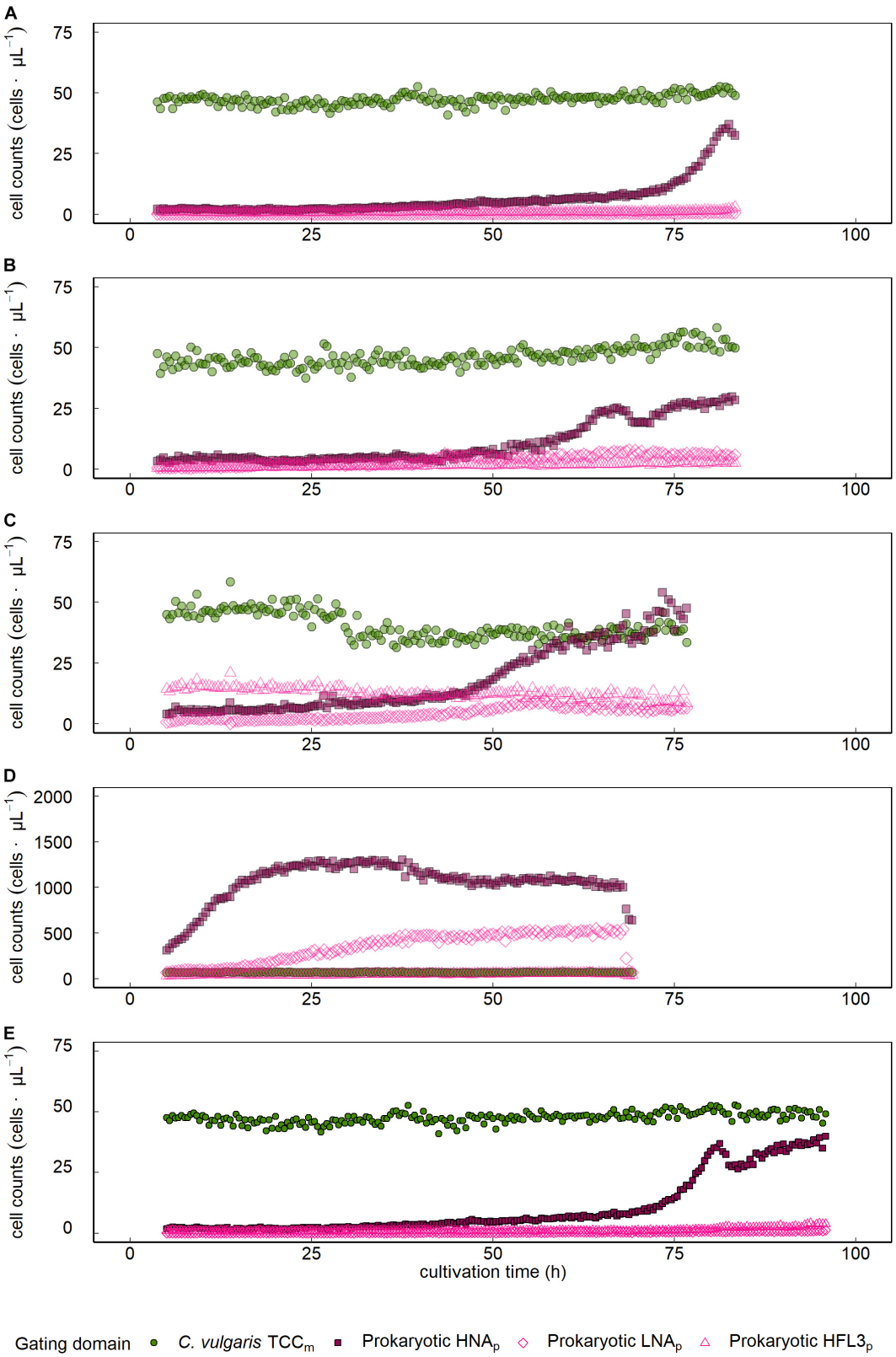

FIGURE 3 | Growth dynamics of *C. vulgaris* and prokaryotes in mixed ecosystems. Data is presented for single cocultures that were established from *C. vulgaris* with **(A)** *Sphinogpyxis* sp., **(B)** *Tistrella* sp., *Sphinogpyxis* sp., *Tistrella* sp., and *Pseudomonas* sp. inoculated to a **(C)** lower or **(D)** higher initial concentration than *C. vulgaris*, and **(E)** a coculture based on spontaneous, fortuitous contamination of axenic *C. vulgaris* cultures. FCM assessed *C. vulgaris* total cell concentration (TCC_m).

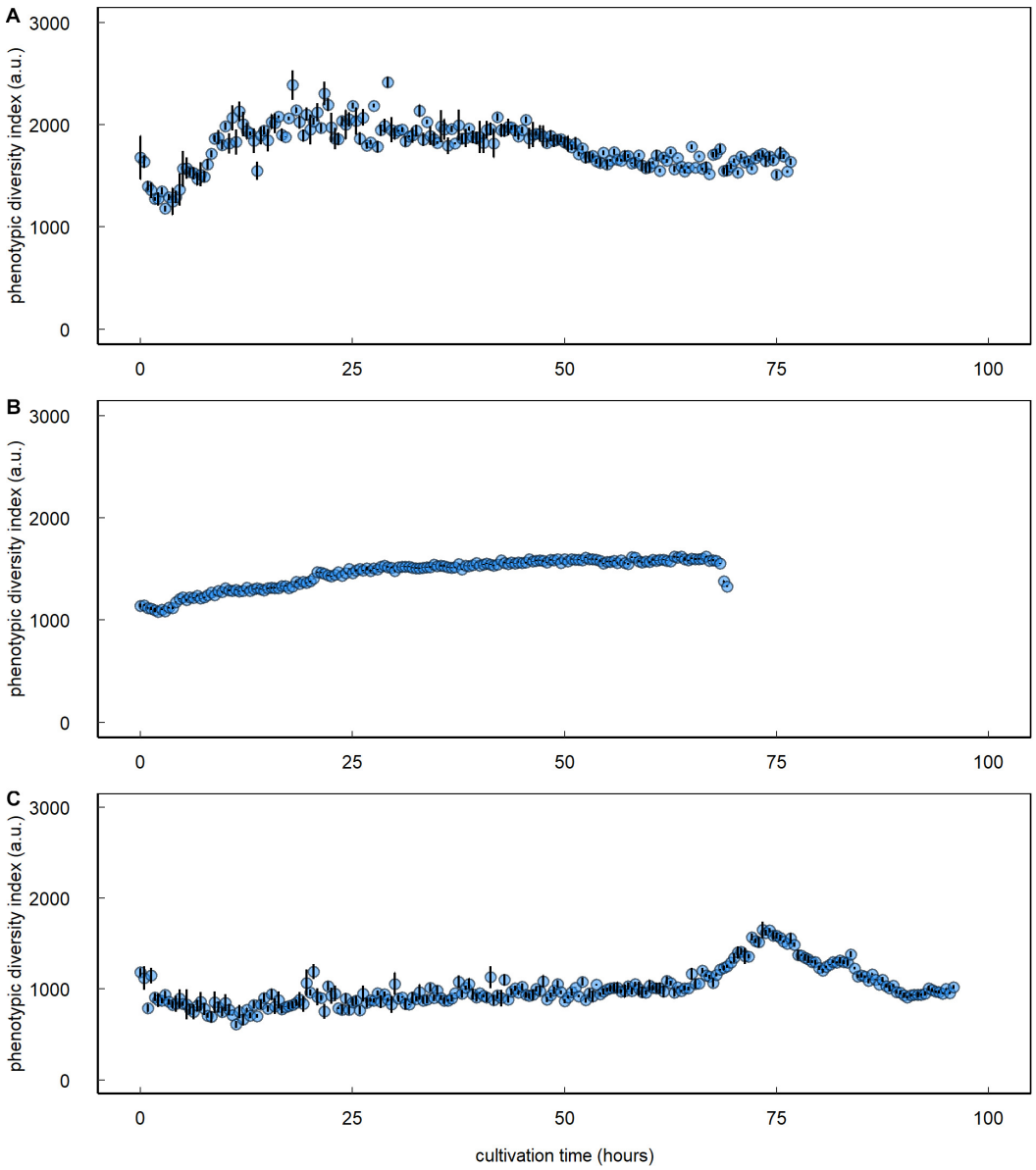

FIGURE 4 | Prokaryotic phenotypic diversity index of *C. vulgaris*–prokaryote mixed ecosystems. Data are presented for single cocultures that were established from *C. vulgaris* with *Sphingopyxis* sp., *Tistrella* sp., and *Pseudomonas* sp. inoculated to **(A)** a lower or **(B)** higher initial concentration than *C. vulgaris* and **(C)** a coculture based on spontaneous, fortuitous contamination of axenic *C. vulgaris* cultures. Error bars denote bootstrap errors (n = 3).

39.9 cells · μL^{-1}, where the HNA_p fraction clearly dominated (**Figure 3E** and **Table 1**). The phenotypic diversity index initially showed no decisive pattern fluctuating between 2,000 and 3,000 a.u. but revealed a distinct peak with a maximum of 1,553 ± 39.0 a.u. 78.4 h after inoculation followed by a decline to 944.1 ± 10.2 a.u. at the end of the cultivation period (**Figure 4C**).

DISCUSSION

Because microalgae have emerged as a next-generation biotechnological production system for the biobased domain, delivering feedstock and high-value components, the economization of their connected value chains remains a main target. Important for optimizing the reproducibility and productivity of microalgal feedstock production are stable cultures, which can be supported by the *in situ*, real-time monitoring and management of culture ecologies. The study showed that the protocol employed harnessing chlorophyll autofluorescence and nucleic acid staining based on SYBR® Green I in conjunction with automated online FCM provided a rapid and sensitive approach for microalgal culture assessment.

A clear advantage of microalgae is their naturally occurring pigments and size, which allows distinguishing them from

abiotic particles, other microalgal species, or bacteria. In this study, harnessing the autofluorescence of chlorophyll enabled discriminating *C. vulgaris* from bacteria and abiotic particles using the red fluorescent (FL3, > 670 nm) channel and thus facilitated gating microalgae during coculture. Microalgal pigments, including chlorophyll, are gaining increasing relevance as bioproducts for the industrial exploitation of microalgae. Chlorophyll, for example, has applications in the cosmetics, food, pharmaceutical, and nutraceutical domain (da Silva Ferreira and Sant'Anna, 2017). The feasibility of microalgal bioproduct, i.e., pigment assessment harnessing their naturally occurring autofluorescence, opens promising applications. Automated online FCM could be employed as an online and inline monitoring and management tool for optimizing bioprocesses associated with microalgal pigment production. Industrially relevant microalgae, such as *Dunaliella salina*, *Haematococcus pluvialis*, and *Scenedesmus almeriensis*, which lack in chlorophyll, are producers of other commercially exploited, high-value pigments, such as carotenoids including astaxanthin (excitation: 488 nm; emission: 675 nm) (Ukibe et al., 2008; Enzing et al., 2014). Harnessing the autofluorescence of those pigments by FCM allows circumventing chemical or toxic staining, extraction, and analysis protocols, whereas automated online FCM provides the additional advantage of real-time data acquisition for bioprocess management.

Microalgal pigment content does not necessarily correlate with biomass yields but responds to variations in light, temperature, and nutrient availability, which impedes its use as a measure for biomass yield quantification (da Silva Ferreira and Sant'Anna, 2017). To enable a quantitative assessment of cocultures, cells were stained with SYBR® Green I (37°C, 10 min), which was shown to enable a sensitive quantification of prokaryotes by FCM with limits at cell concentrations as low as 0.1–1 cells $\cdot\ \mu L^{-1}$ (Prest et al., 2013; Besmer and Hammes, 2016; Besmer et al., 2017a,b). In this study, no significant difference was observed between *C. vulgaris* counts collected on the FSC/SSC and FL1/FL3 fluorescent channels. Hence, the nucleic acid staining protocol employed (SYBR® Green I, 37°C, 10 min) served as a valid and reproducible approach for quantifying microalgal and prokaryotic counts simultaneously during coculture by automated online FCM. Accordingly, Haberkorn et al. (2019) showed that axenic *C. vulgaris* counts collected on the FL1/FL3 channels following a staining with SYBR® Green I corresponded well with those collected on the FSC/SSC channels and actual *C. vulgaris* biomass yields.

The staining protocol employed enabled a sensitive, rapid quantification of microalgal and prokaryotic populations at different concentrations and of different complexity. Automated online FCM enabled discriminating different growth phases of prokaryotes, as well as fluctuations and concentration peaks at high-temporal resolution and within a broad concentration range (2.2–1,002.4 cells $\cdot\ \mu L^{-1}$). Prokaryotic growth in coculture was characterized by lag phases lasting up to or longer than 2 days, while other cultures showed an immediate incidence of exponential growth. Individual cocultures and the multispecies assemblage inoculated to a lower concentration than *C. vulgaris* yielded higher prokaryotic counts than the multispecies assemblage that was inoculated to a higher concentration than *C. vulgaris*. Inoculating prokaryotes to a higher concentration than *C. vulgaris* resulted in an immediate incidence of exponential prokaryotic growth followed by an 8-fold prokaryotic count increase. Conversely, inoculating prokaryotes to a lower concentration than *C. vulgaris* resulted in an extended lag phase and an 18.7-fold prokaryotic count increase. Oligotrophic environments were reported as being dominated by slow-growing prokaryotic populations (Klappenbach et al., 2000). The high salt content and the absence of organic carbon sources in the initial DSN medium suggest a classification of the environment as oligotrophic promoting slow-growing prokaryotes. Both bacteria and microalgae were shown as being capable of releasing dissolved organic carbon into the environment in coculture providing a carbon source for growth, which might have supported prokaryotic growth even under oligotrophic conditions (Cho et al., 2015). The ability of automated online FCM in conjunction with the established staining protocol (SYBR® Green I, 37°C, 10 min) to depict prokaryotic community dynamics *in situ*, in real-time, and at high-temporal resolution covering different concentration ranges yields promising applications of the technology as an online and inline monitoring tool during microalgal culture. Hence, incorporating automated online FCM into microalgal feedstock production could support culture management, as it enables taking immediate countermeasures in case of contamination or prokaryotic upsurge.

In the prokaryotic gating domain, the emissions collected on the FL1/FL3 fluorescent channels revealed the presence of different clusters. SYBR® Green I is sensitive toward nucleic acids, including DNA and RNA (Proctor et al., 2018). Assuming a complete penetration of the stain into the cells, shifts in the green (FL1) fluorescence intensity can occur because of alterations in the nucleic acid content, for instance, that observed for different prokaryotic communities owing to their difference in genome size or during different growth stages of prokaryotes (Prest et al., 2013; Buysschaert et al., 2018). Various studies have described in depth and also characterized prokaryotic HNA and LNA fractions in aquatic ecosystems, resulting in a gating approach as suggested by Prest et al. (2013) (Lebaron et al., 2002; Bouvier et al., 2007; Besmer et al., 2014, 2017a; Props et al., 2018; Zhao et al., 2018). Initial gate establishment for prokaryotic communities encompassed adopting those gates suggested by Prest et al. (2013) to enable discriminating the different clusters. However, employing the same gating strategy did not allow for a clear discrimination of HNA and LNA prokaryotic fractions on the FL1/FL3 fluorescent channels. Instead, prokaryotes showed lower emission on the green fluorescence channel (FL1) in coculture with *C. vulgaris* and thus located at the fringe of the suggested border separating HNA and LNA domains demanding an adaptation tailored to the *C. vulgaris* cocultures investigated in this study. HNA and LNA fractions appear as two domains separated by their fluorescence intensity on the green fluorescent channel (FL1) after staining with SYBR® Green I. The required shift of the gating domains might relate to a deceleration of prokaryotic metabolic activity in coculture with *C. vulgaris* and consequently lower fluorescence intensity on the green

fluorescence (FL1) channel. Hyka et al. (2013) reported that the nucleic acid content of microalgae can also fluctuate, depending on the phase of the cell cycle, but no alterations were observed in this study. Additionally, prokaryotic presence or elevated growth did not affect the nucleic acid content of *C. vulgaris*, which was indicated by 100% coverage in the established gate and the applicability of the same gate to *C. vulgaris* in axenic and non-axenic cultures. However, future studies employing automated online FCM for assessing microalgal dynamics over longer cultivation periods or the impact of processing on microalgal physiology might consider adapting the proposed gate toward higher or lower emission on the green fluorescent (FL1) channel. Most prokaryotic counts were collected in the established HNA_p gating domain. LNA_p fractions, on the other hand, were characterized by low counts. These observations are in accordance with other studies showing that the majority of read counts in aquatic, prokaryotic ecosystems are associated with the HNA domain (Prest et al., 2013; Besmer et al., 2016; Proctor et al., 2018). In turn, the presence of prokaryotes characterized by LNA contents was confirmed by several studies for aquatic ecosystems. However, LNA_p domains yet remain unreported for microalgal ecosystems. Although the presence of LNA content prokaryotes in this study cannot be excluded, a clear identification of LNA prokaryotes remains challenging for two reasons. First, the LNA_p fraction was characterized by low counts. The corresponding gating domain might have also captured counts from background scattering. Hence, it remains questionable whether the counts captured in the LNA_p domain might have been affiliated with background noise or LNA-content prokaryotes. Second, a partially unclear allocation of LNA_p counts, i.e., a location at the fringe of the gating border, to the gating domain further impeded a clear identification of prokaryotic clusters that might have been associated with an LNA_p domain. An unclear allocation of counts into the gating domain supports the assumption that counts collected in the LNA_p gating domain were related to background scattering. Furthermore, an unclear allocation could relate to changes in the metabolic state of cells that lead to a shift in their location on the density plots obtained from FCM. For instance, bacterial sporulation, such as that reported for species of the order *Bacillales*, increases the level of dye uptake, resulting in higher emission on the respective channel (Zhang et al., 2020). Members affiliated with the order of *Bacillales* were also reported for microalgal cultures (Steichen et al., 2020). To study those interactions, fluorescence-activated cell sorting would allow separating the different prokaryotic populations of interest. Combining the sorting with taxonomic assessments based on, for instance, 16S rDNA amplicon sequencing would allow identifying the populations of interest. Subsequently, more complex interactions between selected prokaryotic species of each fraction with microalgae could be studied employing engineered cocultures.

Hence, an LNA_p domain as suggested by Prest et al. (2013) was not applicable in the cocultures assessed in this study and was thus excluded from further diversity analysis. However, future studies investigating more complex microalgal ecosystems might relate back to an LNA_p gating domain for a prokaryotic diversity assessment or contamination monitoring, as several studies have shown the existence of prokaryotes in complex aquatic ecosystems to locate in the LNA_p domain (Proctor et al., 2018). Cocultures, such as those established with *Sphingopyxis* sp. (**Figure 1D**) and three prokaryotic strains (**Figures 1E,F**), indicated the presence of an additional prokaryotic cluster that emitted higher on the red fluorescence (FL3) channel, which was denoted as $HFL3_p$ domain. Interestingly, higher emittance on the red fluorescence (FL3) channel was observed for prokaryotes, including *Sphingopyxis* sp. in individual coculture with *C. vulgaris*, as well as in both multispecies assemblages. Certain members affiliated with *Sphigomonadaceae* were reported being capable of pigment formation, involving carotenoids, such as asthaxanthin or bacteriochloropohyll a, which can cause an increase of the red fluorescence (FL3) intensity (Rosenberg et al., 2014). The rise-time periods of the $HFL3_p$ fraction observed during those cocultures could thus relate to an induction of pigment formation or to a count increase of cells forming those pigments during coculture. However, the presence of prokaryotes located in the $HFL3_p$ domain could not be confirmed for all cocultures. In fact, counts obtained within the $HFL3_p$ were quantitatively negligible during coculture. Some studies describe those signals collected in the $HFL3_p$ domain to be associated with background noise or scattering (Hammes et al., 2008; Hammes and Egli, 2010; Prest et al., 2013). Although low counts might not serve as sole exclusion criterion of the $HFL3_p$ cluster, a lack in stable occurrence throughout all cocultures combined with an open affiliation of the cluster to prokaryotic organisms and the resultant potential of bias through background noise inclusion led to the exemption of the $HFL3_p$ cluster from the diversity analysis in this study.

Combining automated online FCM with data analysis relying on phenotypic fingerprinting based on inherent cell characteristics provides a powerful tool for detecting, tracking, and quantifying prokaryotic disturbances or contaminations and could also pose a viable option for microalgal cultures (Buysschaert et al., 2018; Props et al., 2018). In this study, diversity assessment based on prokaryotic phenotypic fingerprints did not allow for a discrimination of different prokaryotic strains, which could relate to similarities in the phenotypic parameters assessed. But phenotypic fingerprinting indicated that the differences in prokaryotic growth patterns were associated with a dominance of one or two strains within the multispecies assemblage. For the multispecies assemblage with prokaryotes inoculated to a lower concentration than *C. vulgaris*, the phenotypic diversity index increased 1.6-fold within the initial 26 h of cultivation. Conversely, the phenotypic diversity index only gradually increased for the multispecies assemblage with higher initial prokaryotic counts leading to a maximum 1.2-fold increase at the end of the cultivation period. An increase in the phenotypic diversity index relates to an increase in the evenness component and thus equalization of the different community members (Props et al., 2018). This equalization could relate to an assimilation of phenotypic characteristics among community members. Hence, phenotypic fingerprinting indicated that during coculture of the multispecies assemblage inoculated to higher prokaryotic than *C. vulgaris* concentration,

one or two of the three strains dominated throughout the entire cultivation period that governed the growth performance and resulted in overall decelerated prokaryotic growth. The information obtained by automated online FCM, combined with data analysis relying on phenotypic fingerprinting, poses a powerful tool that could improve not only the understanding of population dynamics underlying complex ecosystems but also their response to external events. For instance, the implementation of emerging processing technologies, such as nsPEF in single cell–based biorefineries, remains hampered by a lack of understanding the underlying ecosystem responses or treatment mechanisms (Buchmann and Mathys, 2019; Haberkorn et al., 2021). This situation could be overcome by implementing automated online FCM in combination with data analysis approaches relying on phenotypic fingerprinting to assess responses in real-time. Additionally, Helisch et al. (2020) highlight the importance of long-term stability of non-axenic microalgae-based ecosystems as crucial to establish life-support systems for long-term space exploration, which demands *in situ* monitoring tools, such as automated online FCM, which provide data at high-temporal resolution for optimal process control.

CONCLUSION

Automated online FCM poses a powerful technology for improving the feasibility of microalgal feedstock production through providing data on culture dynamics *in situ* and at high-temporal resolution. Harnessing emissions collected on the FL1/FL3 fluorescent channels, obtained by nucleic acid staining and chlorophyll autofluorescence, enables a simultaneous assessment of prokaryotes and *C. vulgaris* in artificially engineered and natural cultures over a broad concentration range (2–1,002 cells $\cdot$ μL^{-1}). Automated online FCM in combination with data analysis relying on phenotypic fingerprinting provides information on quantitative and diversity-related community dynamics. Simultaneously, the study highlights different prokaryotic community fractions in microalgal cultures. Differences in the nucleic acid content and pigmentation could allow distinguishing them by FCM. In that context, characterizing non-axenic *C. vulgaris* cultures beyond phenotypic assessments proposed in this study on a taxonomic base could further advance automated online FCM by identifying populations of interest. Such assessments provide a better understanding of the underlying microbial network interactions. The study lays the foundations for an application of automated online FCM implying far-reaching applications to impel and facilitate the implementation of innovations targeting at microalgal bioprocesses optimization.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IH and CO: data acquisition. IH: manuscript drafting. LB, MB, and AM: final approval of the manuscript. All authors: conceptualization, study design, technical support, analysis, data interpretation, and critical revision.

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REVIEW
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Microalgal Biodiesel Production: Realizing the Sustainability Index

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Search for new and renewable sources of energy has made research reach the tiny little tots, microalgae for the production of biodiesel. But despite years of research on the topic, a definitive statement, declaring microalgae as an economically, environmentally, and socially sustainable resource is yet to be seen or heard of. With technological and scientific glitches being blamed for this delay in the progress of the production system, an assessment of the sustainability indices achieved so far by the microalgal biodiesel is important to be done so as to direct future research efforts in a more coordinated manner to achieve the sustainability mark. This article provides a review of the current economic, environmental, and social status of microalgal biodiesel and the strategies adopted to achieve them, with suggestions to address the challenges faced by the microalgal biodiesel production system.

Keywords: microalgae, biodiesel, sustainability, economic, environmental, social

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INTRODUCTION

Living in an age where life revolves around energy in all forms, a crisis of sustainability is indeed indispensable. With the continued consumption of fossil fuels by the expanding populations, maintaining economic, environmental and social sustainability is a difficult proposition. Hence, strong abatement practices and policies to encourage research on renewable energy resources are being developed. It is in this context that energy in the form of biofuels is being produced from renewable resources of plant origin. Although various other alternatives like geothermal, wind and solar energy are being surveyed, bioenergy is looked at as a strong resource of energy in the coming years.

In such a scenario, the presence of objectionable facts such as issues of food security and energy balance in the first- and second-generation biofuels and the desire for new, sustainable energy resources has brought into limelight, a garden pond nuisance, microalgae, as a promising renewable fuel feedstock. Reports of its high oil yields, dramatic GHG savings, faster growth rate, more harvesting cycles and higher carbon fixation rates, all devoid of any negative effects on farming are reasons of its sudden popularity (Balat and Balat, 2010).

Research on microalgae as a source of energy were extensively carried out in the 1970s, in the United States, but shortage of adequate funding and shift of focus to other feedstocks and technologies gradually brought an end to the research program (Demirbas, 2011). However,

Abbreviations: ARRA, American Recovery and Reinvestment Act; BECCS, bioenergy for carbon capture and sequestration; BTU, British thermal unit; CO_2, carbon dioxide; EROI, energy return on investment; FER, fossil energy ratio; GGE, gallons of gasoline equivalent; GHG, greenhouse gas; ILO, International Labor Organization; IPCC, intergovernmental panel for climate change; ITUC, International Trade Union Confederation; LCA, life-cycle assessment; LCIA, life-cycle impact assessment; MJ, mega joules; NEB, net energy balance; NER, Net energy ratio; POP, pathways out of poverty; UNEP, United Nations Environment Programme.

with the spurt of concerns today, regarding climate change, food vs. fuel feud, land use change, etc., resulting due to the use of first- and second-generation biofuel feedstocks, the need for search of alternative energy sources has aroused and reawakened interest in microalgae. Although microalgae possess several advantages as compared to first- and second-generation biodiesel feedstocks and are being experimented on different aspects worldwide, sustainable microalgal biodiesel production appears to be a difficult target to reach with regard to its economic, environmental and social positioning. This review extends its scope to identifying the sustainability indices achieved through microalgal biodiesel production and addressing the knowledge gaps in this area for focused research and innovations.

SUSTAINABLE BIOFUELS

Definition

The term 'sustainability' has been rightfully defined by the World Commission on Environment and Development as "the development that satisfies the needs of the present generations without compromising the ability of the future generations to meet their own needs." Sustainable development comprehends economic, social, and ecological standpoints of conservation and change (**Figure 1**) (UNCED, 1992).

Despite the widespread use of the term, 'sustainability,' human beings fail to cater to the basic requirements for a sustainable society which is clearly reflected through their activities of environmental degradation, overconsumption, population growth and their quest for indefinite economic growth in a closed system.

Parameters to Be Considered for Sustainable Biofuel Production

For achieving economic sustainability, low-cost production strategies with greater output to input ratio is imperative besides being available at affordable market rates. At times, the need to maximize returns from investments overlooks the environmental considerations giving rise to negative implications. Additionally, the demand for economic gains affects food production and availability, creating adverse impacts on the society. Hence, to balance between economic and environmental sustainability, higher productivity must be targeted.

Environmental sustainability can be assessed through use of environment friendly, renewable sources of energy along with use of chemicals and machines during the production process with minimum negative environmental impact. These assessments are done with the help of some indicators which can either be global (GHG emissions, renewable energy) or regional (water management, soil and resource depletion, local pollution, etc.). Moreover, the government and private led directives, schemes and initiatives for spreading awareness and activity, also contribute toward environmental sustainability in a major way (Afgan, 2008).

Social sustainability can be ascertained through implementations of certification schemes, scorecards and regulations for mitigating the negative impacts such as child labor, minimum wage, compensation for lost land and resources etc (Haye and Hardtke, 2009). However, evidences of implementation of these measures in reality, has been very limited, suggesting lower degrees of interest or awareness for establishing social sustainability. Low social and political participation and contrasting social norms have been few of the many reasons for this debacle. Hence a participation of society and resources for a collaborative effort toward social and economic development and sustainability should be planned.

The basic criteria and indicators for production of sustainable biofuels have been clearly stated by Silva Lora et al. (2011) (**Table 1**). The report also mentions that for assessing the sustainability of biofuels, parameters like life cycle impact assessment, quantification of substituted fossil energy, energy allocated for co-product development and changes in soil utilization should be importantly considered.

MICROALGAL BIODIESEL PRODUCTION: THE SUSTAINABILITY CHECK

Algae biodiesel industry is starting to take off. Algae projects see an emerging trend in the production of algal-based *drop in* fuels and various high-value products. In a bid to realize the sustainability index, extensive research efforts are being carried out by researchers, academicians and industrialists worldwide to improve the economic and environmental benefits from algal biodiesel through improvement in upstream and downstream processes. Despite such widespread research activities in the field, many questions still remain unanswered. What exactly is the sustainability index? How far are we from reaching the sustainability mark? Is the same sustainability index applicable to all nations and societies? Although the sustainability index is a concept as vague as, 'to each his own,' in this world of expanding problems and populations, to ensure continued efforts in the right direction and at the right pace, a sustainability check of the microalgal biodiesel production system is indispensable.

Economic Sustainability

Biodiesel production is an energy intensive process. All the processes during biodiesel production, starting from procurement of raw materials to processing, manufacturing, storing and marketing, contribute toward the product's economic feasibility. The decision to use a particular biodiesel initially depends on its cost competence. So research efforts to bring down the cost of microalgal biodiesel to comparable rates with conventional petroleum diesel are being focused on. Hence, for ensuring cost effectiveness of the microalgal biodiesel, few important strategies are generally followed, such as (i) increasing the amount of energy captured from the atmosphere, (ii) increasing the amount of energy harvested from the microalgal biomass, (iii) increasing the biomass yield of the resource, (iv) increasing the number of co-products produced, (v) decreasing the energy input during downstream processes, and (vi) increasing the ability of the product to be stored for a longer period of time.

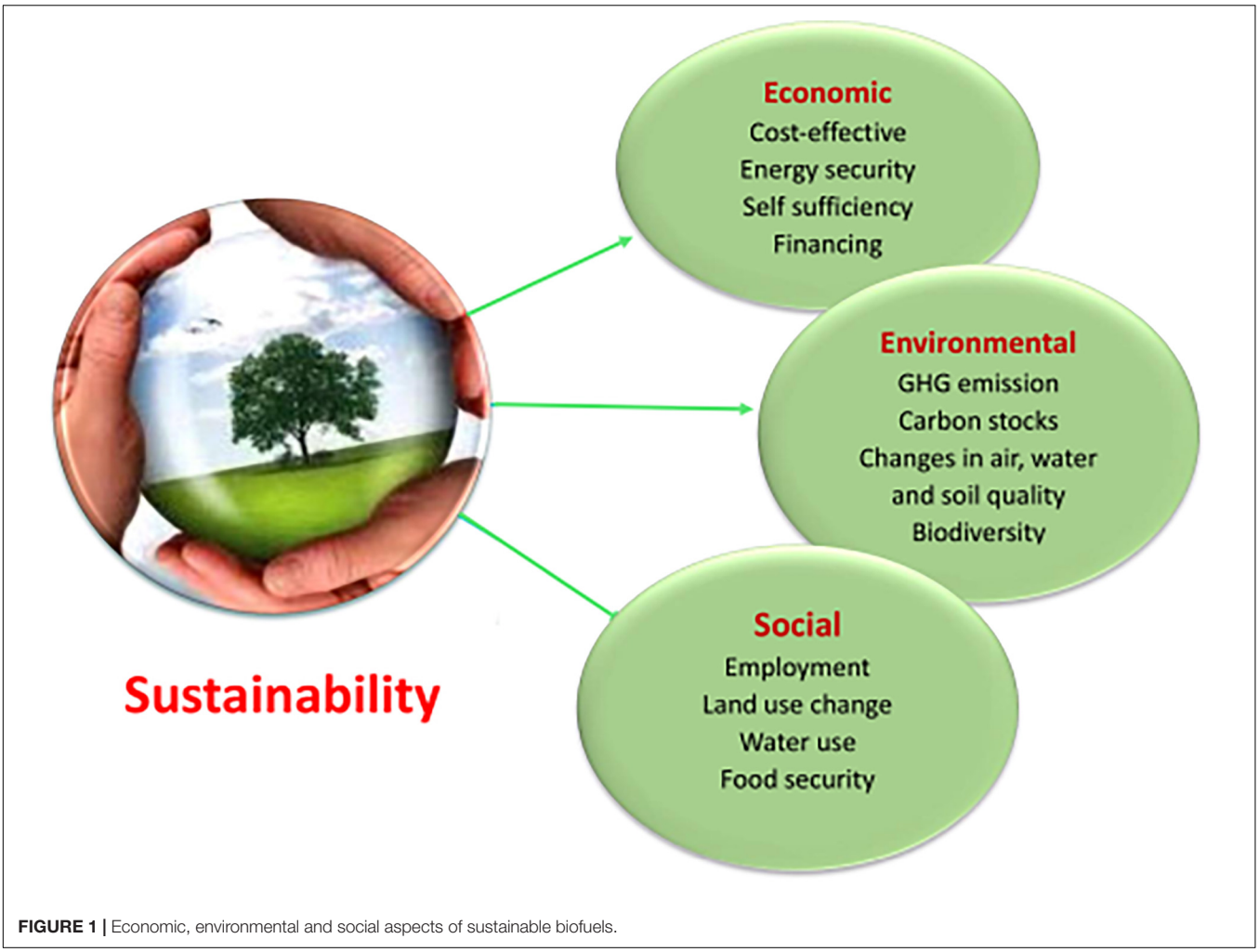

FIGURE 1 | Economic, environmental and social aspects of sustainable biofuels.

TABLE 1 | Criteria and sustainability indicators for sustainable biofuel production (Silva Lora et al., 2011).

Criteria	Sustainability indicators
(i) Should be carbon neutral in terms of GHG emissions.	(i) Economic indicators (cost of production).
(ii) Should have no negative water footprint and land use change problems.	(ii) Output/input ratio (net energy analysis).
(iii) Should not challenge food security.	(iii) Substituted fossil fuel per hectare.
(iv) Should be economically affordable by the society.	(iv) Avoided GHG emissions (CO_2 savings).
(v) Should not disturb the biodiversity.	(v) Environmental impacts evaluation using impact categories indicators.
	(vi) Carbon emissions due to land use changes.
	(vii) Renewability indicators (energy accounting).

Over the past few years, remarkable advancement has been achieved in the microalgal biodiesel production systems, with respect to technological and economic development. While way back in 2010–2011, the microalgal biodiesel was produced at more than $100/GGE (Gallons of Gasoline equivalent) in a paddle- wheel driven microalgal pond cultivation system (National Research Council [NRC], 2012), over the years, through technological advancement, cost of algal biodiesel production has been lowered to $7.50/GGE (National biodiesel board [NBB], 2009) is further estimated to come down to $3.00/GGE by 2030 (Office of Energy Efficiency and Renewable Energy [EERE], 2017). This cost cut can be attributed to modifications in cultivation, strain selection, harvesting and extraction technologies and co-product development, all of which determine the final cost of the product, i.e., biodiesel. Various organizations and companies have proposed different cost reduction strategies. Few examples include the use of jet mixer technology for direct extraction of lipids from wet algal biomass by researchers from University of Utah (Mohanty, 2019), use of patented harvesting and algae oil extraction systems by *Missing Link Technology* and *Algae Venture Systems* (Lane, 2014), and use of patented quantum fracture technology for efficient

and innovative single step oil extraction from microalgae by Origin Oil Co., (Eckelberry and Eckelberry, 2008). Interestingly, this invention by Origin Oil Co., claims to reduce microalgal biodiesel cost to $2.00/GGE. In addition to inventions in the production and conversion process, various companies like Sapphire Energy, Muradel, Solazyme, Algae.Tec, Cellana and Neste Oil, BioProcess Algae and Algenol are setting up large-scale production units for attaining commercial feasibility of algal oil (European Technology and Innovation Platform Bioenergy [ETIP], 2014). Through co-ordinated research and development activities, microalgal biodiesel production is gradually moving from economic uncertainity to economic feasibility. Specifics of few selected strategies adopted for reducing the cost of microalgal biodiesel production have been listed in **Table 2**.

Although microalgal biodiesel production is a topic being researched worldwide, reports on detailed cost analysis of the final product (as an effect of the entire production system), is limited, nevertheless production models with cost reduction calculations anticipating a competitive market for microalgal biodiesel, substitute the limitation (Richardson et al., 2010; Harun et al., 2011). Microalgal biodiesel is gradually moving toward being more cost effective but complete economic parity with petroleum diesel is yet to be realized. With diesel currently costing $3.08/gal (Diesel Prices, 2021) on an average throughout the world, to make it comparable with algal diesel an equivalent market price is inevitable. For this to be achieved, algal biomass yields (given that all integrated systems based on algal biomass processing are constrained by high cultivation variability) will have to be increased approximately from 12 to >30 gdw/m^2/day on a sustained basis, the energy-return-on-investment (EROI) for harvesting algae from ponds ideally would need to be >20, i.e., no more than 5% of the energy content of the algae should be spent during harvesting and the lipid extraction and conversion efficiency to biodiesel should be improved so as to ensure minimum expenses in the defined process (Stephens et al., 2010; Olivieri et al., 2013; Barry et al., 2015; Barsanti and Gualtieri, 2018). Additionally, further lessening of microalgal biodiesel prices can be accomplished by focusing on maximizing lipid content in high biomass yielding microalgal strains and valorization of the algal biomass, as it results in more substantial cost reduction (Bellou et al., 2014; Zhu, 2015). Photobioreactors are also known to be very effective for producing high biomass and lipid productivities, but given the construction and operation challenges such as overheating, fouling, improper gas exchange etc., this option appears less sustainable for commercial use. A study by Veeramuthu and Ngamcharusrivichai (2020) demonstrated algal biodiesel cost to be $20.53 and $9.84 per gallon using a PBR and open raceway pond cultivation method, respectively. Reports in support of this claim have also been published by various other researchers and companies like Weissman et al. (1988), Craggs et al. (2011), Efroymson et al. (2020), Ganesan et al. (2020), Jo et al. (2020) including National renewable energy laboratory (Davis, 2017) and the Solix (Kanellos, 2009). In **Table 3** below, different strategies being followed in the last 5 years over and above the ones mentioned in **Table 2** above, with an anticipation to reduce the final selling price of microalgal biodiesel, are highlighted.

Reduction in algal biodiesel costs through coupling of economic and environmental sustainability, is yet another emerging potential strategy for the future. Use of fossil fuel releases CO_2 into the atmosphere which is sequestered back by microalgae for growth and product development. This technology called the BECCS has been rated as the most technologically and economically potential solution for mitigating the impact of GHG emissions, by the IPCC (National biodiesel board [NBB], 2009). Additionally, tax credits and/or carbon credit policies provide further cost reductions by incentivising carbon capture for bioenergy production (Sayre,

TABLE 2 | Strategies adopted for reducing the cost of microalgal biodiesel production.

Sl No.	Estimated cost of microalgal biodiesel ($/gallons of gasoline equivalent)	Strategy adopted	References
(1)	$7.50	Use of a newly discovered microalgal strain, *Chlorella* sp. DOE1412, with a robust ability to accumulate high quantity of lipid under variety of conditions when grown in a self-designed open pond cultivation system, the Aquaculture Raceway Integrated Design (ARID) with an integrated temperature control mechanism, and harvested through electrocoagulation (EC), a low-energy harvesting method for subsequent oil extraction and upgradation to biodiesel without using any solvents in a hydrothermal liquefaction chamber.	National Alliance for Advanced Biofuels and Bioproducts [NAABB], 2014
		Simultaneous lipid extraction and transesterification in a mixer containing methanol and sodium hydroxide through ultrasonication of the harvested microalgal biomass with an annual average productivity of	Nagarajan et al., 2013
(2)	$2.68	30 g/m^2/day using CO_2 from flue gas	
	$1.58	60 g/m^2/day using CO_2 from flue gas	
	$3.67	30 g/m^2/day using pure CO_2	
	$2.11	60 g/m^2/day using pure CO_2.	
(3)	$4.35	Use of a high lipid containing (41% dcw) microalgal strain with an annual average productivity of 30 g/m^2/day processed through hydrothermal liquefaction technique and purified for biodiesel production while simultaneously utilizing the spent biomass for production of other value-added products such as bioethanol and methane. Re-circulation and re-use of water and solvents through the biorefinery system has also been applied.	Davis et al., 2011

TABLE 3 | Research efforts in the last 5 years with an anticipation to reduce the final selling price of microalgal biodiesel.

Sl. no.	Targeted step	Adopted strategy	References
(1)	Strain improvement	Fluorescence-activated cell sorting to analyze single-cell fluorescence and sort cells with high fucoxanthin and lipid productivities.	Gao et al., 2021
(2)	Strain improvement	Post-treatment processing using H_2SO_4 + Ca $(OH)_2$ for enhanced ethanol production from algae.	Seon et al., 2020 Südfeld et al., 2021
(3)	Strain improvement	Use of coral inspired 3D materials for higher biofuel production by increasing the photon resident time for enhanced light absorption by algal cells.	Wangpraseurt et al., 2020
(4)	Strain improvement	Strain improvement through high-throughput screening platforms i.a. involving single-cell methodologies such as fluorescence-activated cell sorting (FACS) for the identification and isolation of better-performing strains by combining qualitative staining of lipid bodies using the fluorophoric dye BODIPY with FACS methodology.	
(5)	Strain improvement	Use of broad range and wide variety of carbon sources for enhancing growth and lipid accumulation in algae.	Patnaik and Mallick, 2019
(6)	Strain improvement	Researchers at Tokyo Institute of Technology have identified an enzyme belonging to the glycerol-3-phosphate acyltransferase (GPAT) family as a promising target for increasing biofuel production from the red alga *Cyanidioschyzon merolae*.	Fukuda et al., 2018
(7)	Strain improvement	Researchers at Los Alamos National Laboratory, with colleagues at NREL and the University of Georgia report that a freshwater production strain of microalgae, *Auxenochlorella protothecoides* UTEX 25, is capable of directly degrading and utilizing non-food plant substrates, such as switchgrass, for cell growth. In addition, the use of plant substrates increases lipids production.	Vogler et al., 2018
(8)	Strain improvement	Rapid screening of high lipid accumulating microalgal strains through droplet microfluidics based screening platform.	Kim et al., 2017
(9)	Strain improvement	Doubling of lipid content while sustaining growth using CRISPR-Cas 9 for modulating a transcriptome regulator in *Nannochloropsis gaditana*.	Ajjawi et al., 2017
(10)	Strain improvement	Discovery of an algal photoenzyme that converts algal fatty acids to alkanes and alkenes under low-light driven conditions.	Sorigué et al., 2017
(11)	Microalgal cultivation and valorization	Use of iron oxide nanoparticles for improved growth and biogas production in algae.	Rana et al., 2020
(12)	Microalgal cultivation	Use of tannery wastewater for growth and biofuel production from green microalgae through bioremediation.	Nagi et al., 2020
(13)	Microalgal cultivation	Outdoor open pond batch production of green microalga *Botryococcus braunii* for high hydrocarbon production using different salinity concentrations.	Ruangsomboon et al., 2020
(14)	Microalgal cultivation	Use of iron and magnesium addition for improving population dynamics and high value product formation in microalgae grown in anaerobic liquid digestate.	Ermis et al., 2020
(15)	Microalgal cultivation	A simplistic approach of algal biofuels production from wastewater using a Hybrid Anaerobic Baffled Reactor and Photobioreactor (HABR-PBR) System.	Khalekuzzaman et al., 2019
(16)	Microalgal cultivation	Use of 40,000L closed raceway ponds for algal growth and lipid accumulation under biphasic nitrogen starved conditions.	Bagchi et al., 2019
(17)	Microalgal cultivation	Biomimetic light dilution using side-emitting optical fiber for enhancing the productivity of microalgae reactors. This technique enables homogeneous illumination of large reactor volumes with high optical density eventually increasing the rate of reproduction by 93%.	Wondraczek et al., 2019
(18)	Microalgal cultivation	Multi-bandgap Solar Energy Conversion via Combination of Microalgal Photosynthesis and Spectrally Selective Photovoltaic Cell for higher biomass production.	Cho et al., 2019
(19)	Microalgal cultivation and product extraction	Discovery of a new mechanical algal milking technique for extracellular production of polysaccharides and phycobilliproteins.	Uchida et al., 2020
(20)	Product extraction	Pulsed Electric Fields-Assisted Extraction of Valuable Compounds From *Arthrospira Platensis*.	Carullo et al., 2020
(21)	Product extraction	Electroporation as a Solvent-Free Green Technique for Non-Destructive Extraction of Proteins and Lipids From *Chlorella vulgaris*.	Eleršek et al., 2020
(22)	Microalgal harvesting	Effective harvesting of *Nannochloropsis* microalgae using mushroom chitosan	Chua et al., 2020
(23)	Microalgal harvesting and valorization	Induction of flocculation and photobiological hydrogen production under anaerobic conditions using an engineered chemoenzymatic cascade system.	Chen et al., 2020
(24)	Microalgal cultivation and harvesting	Use of a Tris-Acetate-Phosphate-Pluronic (TAPP) medium that undergoes a thermoreversible sol-gel transition to efficiently culture and harvest microalgae clusters without affecting the productivity as compared to that in traditional culture in a well-mixed suspension.	Estime et al., 2017

(Continued)

TABLE 3 | Continued

Sl. no.	Targeted step	Adopted strategy	References
(25)	Microalgal harvesting	Use of pine bark, a natural substrate for immobilization of microalgae grown in wastewater for easy and cost-effective separation of algal cells.	Garbowski et al., 2020
(26)	Microalgal harvesting	Use of cellulose nanofibrils for cost-effective microalgal harvesting through encapsulation of microalgal cells by nanofibrous structure formation.	Yu et al., 2016
(27)	Microalgal valorization	To develop a thin-layer artificial biofilm technology for sustainable and long-termethylene photoproduction, where recombinant *Synechocystis* sp. PCC 6803 cells holding ethylene forming enzyme (Efe) from *Pseudomonas syringae* are entrapped within the natural polymer matrix, thus forming the thin-layer biocatalytic structure.	Vajravel et al., 2020
(28)	Microalgal valorization	*Chlorella vulgaris* extract as a serum replacement that enhances mammalian cell growth and protein expression.	Ng et al., 2020
(29)	Microalgal valorization	Researchers were able to increase hydrogen production by combining unicellular green alga called *Chlamydomonas reinhardtii* with *Escherichia coli* bacteria. The teamwork of the algae and bacteria resulted in 60% more hydrogen production than they are able to produce if algae and bacteria work separately.	Fakhimi et al., 2019
(30)	Microalgal valorization	Use of algal protein from the de-oiled biomass as a replacement of the commercially available fish meal under an algal refinery approach.	Patnaik et al., 2019
(31)	Microalgal valorization	Microalgal Protein Extraction From *Chlorella vulgaris* FSP-E Using Triphasic Partitioning Technique With Sonication.	Chia et al., 2019
(32)	Microalgal valorization	Mild Fractionation of Hydrophilic and Hydrophobic Components From *Neochloris oleoabundans* Using Ionic Liquids.	Desai et al., 2019
(33)	Microalgal valorization	Synthesis of benzene, an elementary petrochemical, along with other hydrocarbons.	Pingen et al., 2018
(34)	Downstream processing	A synthetic protocol to the fixation of carbon dioxide by converting it directly into aviation jet fuel using novel, inexpensive iron-based catalysts.	Yao et al., 2020
(35)	Downstream processing	The use of jet mixer technology for direct extraction of lipids from wet algal biomass by researchers from University of Utah.	Mohanty, 2019
(36)	Downstream processing	Low-temperature catalyst based Hydrothermal liquefaction of harmful Macroalgal blooms, and aqueous phase nutrient recycling by microalgae.	Kumar et al., 2019
(37)	Downstream processing	Bleaching, deoxygenation and hydroisomerization of crude extracted algal lipids to renewable diesel.	Kruger et al., 2017
(38)	Downstream processing	Establishment of axenic cultures of armored and unarmored marine dinoflagellate species using density separation, antibacterial treatments and stepwise dilution selection.	Lee et al., 2021
(39)	Technique and technology advancement	A simple and non-destructive method for chlorophyll quantification of *Chlamydomonas* cultures using digital image analysis for easy and fast assessment of growth.	Wood et al., 2020
(40)	Technique and technology advancement	Metabolomics as a tool for understanding the molecular basis for these metabolic and physiological changes, and for early detection of stress in freshwater alga *Poterioochromonas malhamensis* exposed to silver nanoparticles.	Liu et al., 2020
(41)	Technique and technology advancement	Development of a pVEC peptide-based ribonucleoprotein (RNP) delivery system for genome editing using CRISPR/Cas9 in *Chlamydomonas reinhardtii*.	Kang et al., 2020
(42)	Technique and technology advancement	Development of a species-specific transformation system using the novel endogenous promoter calreticulin from oleaginous microalgae *Ettlia* sp.	Lee et al., 2020

2010). In the following section, we consider details of the suggested strategy for achieving environmental sustainability while taking care of the cost effectiveness that is consequent to the entire process.

Environmental Sustainability

The impact that the microalgal biodiesel production process has on the environment during its entire life cycle decides its environmental sustainability. Starting from the choice of the cultivation area to use of nutrients for growth and lipid accumulation enhancement, to use of different energy intensive harvesting techniques followed by extraction of lipids using different extracting solvents and then conversion of the extracted lipid to biodiesel, all contribute toward the environmental sustainability of the product. This sustainability index can be verified by the use of certain indicators such as GHG emissions, energy security, water management, soil and resource depletion, local pollution, etc. Tools such as Life Cycle Impact Assessment (LCIA) are used for measuring these indicators. LCAs can highlight areas of concern and focus the future research efforts on aspects of the supply chain that carry the largest environmental burden (US Environmental Protection Agency [EPA], 2010).

Global warming due to increasing concentrations of greenhouse gases in the atmosphere is a daunting environmental challenge in today's world. Of the different greenhouse gases present, CO_2 is majorly responsible for this problem. CO_2

is naturally present in the atmosphere, but activities such as burning of forests, mining and burning coal increase their concentrations to dangerous levels in the atmosphere by converting the carbon stored in the solid state to gaseous state (Sayre, 2010). Microalgae are widely known for being potential sequesters of large amounts of CO_2 from the atmosphere thus lowering GHG emissions relative to petroleum diesel. Additionally, their ability to recycle the released CO_2 from the different stages of the microalgal biodiesel process within their own system, categorizes them as an environmentally sustainable resource. Many researchers have reported that algal biodiesel has the ability to reduce the GHG emissions by half (55,400 g of CO_2 equivalent per million BTU) as compared to what is emitted by low sulfur diesel fuel (101,000 g of CO_2 equivalent per million BTU) (Brune et al., 2009; Gude et al., 2012). This is further confirmed by the United States Environmental protection agency as per which algal/microalgal biodiesel has the potential to meet the Renewable Fuel Standard requirement 2007 by reducing 50% of GHG emissions as compared to petroleum diesel (Sissine, 2007). With petroleum diesel having GHG emissions about 90 g CO_2 eq/MJ of fuel, for warranting minimum negative impact on the environment, several strategies have been developed and adopted worldwide in order to reduce CO_2 emissions, details of which have been listed in **Table 4**.

Negative emissions signify an outlet of CO_2 from the atmosphere whereas reduced emissions signify a reduced inlet of CO_2 into the atmosphere. Both have their own respective benefits, but with the OECD Environmental Outlook 2050 at the 2011 United Nations Climate Change Conference, suggesting achieving CO_2 concentration targets at lower than 450 ppm by the Bioenergy for Carbon Capture and Storage Technology (BECCS), negative emissions should be critically pursued (National biodiesel board [NBB], 2009).

Microalgae are environmentally sustainable resources emitting green house gases during biodiesel production in quantities lower than that emitted during petroleum diesel production. This can be justified from the **Table 4** above, which shows GHG emissions from microalgal biodiesel production systems lesser than 90 g CO_2 eq/MJ of fuel, recorded for petroleum diesel. But this may not always be true, as microalgal biodiesel production systems in certain cases emit more than 2–10-fold higher greenhouse gases as compared to petroleum diesel (Zaimes and Khanna, 2013). The reason for this variation lies in differences in operational and input parameters of the microalgal biodiesel production process and the interplay between them and the use to which the produced biodiesel is put to. Additionally, the emissions and NER value may also vary from place to place depending on the government regulations and policies and the co-products produced, as highlighted by a research study on corn ethanol by Farrell et al. (2006). On the mention of the operational and input parameters, it is important to note that there are some influencing factors which decide the GHG emission values of the production chain (**Figure 2**). The factors primarily are cultivation > harvesting > drying > oil extraction/conversion > transport of feedstock > final fuel product, with a decreasing order of importance as regards to their contribution to the final GHG emission figures. Although the figures look promising, yet excessive reliance on few assumptive data sets of selective parameters in some analyses, make way and arouse the need for more elaborate research on the details of the influencing parameters.

Today's distressing circumstances require that the world emit a total of no more than 1,200 gigatonnes of carbon by the end of this century. That is about 30 years' worth of carbon emissions at existing levels. But these situations also anticipate absorption of upto 1,000 gigatons of carbon through the above-mentioned merger of bioenergy and carbon capture and storage (CCS), a combination known by the abbreviation BECCS (Azadi et al., 2014). This would then lead to an increase in the total positive emissions (emissions that can be recirculated among the biological system without causing any negative impact on the atmosphere) from 1,200 to 2,200 gigatonnes. Other options such as afforestation, storage of carbon in the soil, and direct air capture of carbon also exist, but are dependent on certain interlinked factors such as land use change and chopping down of trees which transform them from carbon sequestering to carbon releasing strategies. On the other hand, carbon stored in the soil is constantly at the risk of being disturbed. Direct air capture technologies like artificial trees and scrubbing towers are remarkably gee-whiz and show great promise, but are years away from commercialization, currently even more expensive than already very expensive CCS, and we shouldn't forget that they have a voracious energy appetite themselves. Other possibilities such as the geoengineering techniques of ocean fertilization or enhanced weathering of natural or artificial minerals remain unproven at scale and are already raising hackles amongst some environmentalists. And these are not prominent in any of the considered scenarios (US Environmental Protection Agency [EPA], 2010). As a result, BECCS remains the top bet in the GHG emissions sweepstakes.

Another key metric often considered in microalgal biodiesel analysis is NEB which is defined as the difference between the energy value of the output fuel and the total primary energy consumed in producing the fuel (Zaimes and Khanna, 2013). As such a positive NEB is one important criterion for an environmentally sustainable transportation fuel, because it indicates that more energy is produced than is consumed via the system. EROI and NER are two other energy metrics and represent the ratio of the energy of the final fuel to the direct and indirect primary energy required for its production (Stephenson et al., 2010). Thus, if EROI and NER values are less than unity, then the system has a negative NEB. A variation of EROI known as FER or $EROI_{fossil}$ considers only the consumption of primary fossil energy throughout the fuel supply chain and thus measures how much fuel product is generated per unit investment of primary fossil resources. As such $EROI_{fossil}$ values provide a surrogate measure for the renewability of the biofuel. Accordingly, $EROI_{fossil}$ values more than unity are desirable, because more energy is produced via the biofuel than the fossil energy consumed throughout the supply chain (Vasudevan et al., 2012).

With an energy content of 5–8 kWh/kg (18,000–28,800 kJ/kg dry cell weight), feasibility of microalgal biodiesel production

TABLE 4 | GHG emissions by microalgal biodiesel production system and strategies adopted for their reduction.

Sl no.	GHG emissions (g CO_2eq/MJ of biodiesel)	Strategy adopted	References
(1)	−11.4	In this Well to Wheel (WTW), *Scenedesmus dimorphous* with an annual biomass productivity of 13 g/m^2/day was cultivated in open raceway ponds fed with fertilizer grade N, P, K and industrial flue gas as carbon source. The entire process chain moved from harvesting of the biomass using bio-flocculation and dissolved air floatation followed by centrifugation to use of hydrothermal liquefaction for further processing to bio-oil. The energy expenses and GHG emissions were balanced by recycling of nutrients present in the aqueous phase from the HTL unit, bypassing the need of drying and the co-product credits of the combustible gases emitted from the hydrothermal system for improving the energetics of the biodiesel production process.	Bennion et al., 2015
(2)	71	In this Well to Wheel (WTW), green microalgae with an annual biomass productivity of 25 g/m^2/day and lipid content of 25% (dcw) was cultivated in open raceway ponds fed with recirculated growth media from the liquid digestates and biogas as carbon source. The entire process chain moved from harvesting of the biomass using bio-flocculation and dissolved air floatation followed by centrifugation to lipid extraction from wet biomass using *n*-hexane and transesterification using methanol. The energy expenses and GHG emissions were balanced by water and 66% nitrogen and 90% phosphorous recycling and the co-product credits of biogas produced during anaerobic digestion and processed through combined heat and power technique for use on-site during the biodiesel production process.	Yuan et al., 2015
(3)	28.50	In this Well to Wheel (WTW) Life cycle analysis, an algal biomass productivity of 20 g/m^2/day and lipid content of 30% (dcw) was assumed in open raceway ponds fed with nutrients from a wastewater source. The entire process chain moved from harvesting of the biomass through bio-flocculation and gravity clarifiers to use of solar dryers for drying the harvested biomass for lipid extraction by hexane and transesterification to biodiesel by using methanol. The energy expenses and GHG emissions were balanced by 89% of nutrient and solvent recycling and the co-product credits of glycerine produced during transesterification and biogas generated from anaerobic digesters which were used in providing electricity through the entire production process.	Woertz et al., 2014
(4)	35.2	In this Well to Wake (WTW) Life cycle analysis, algae with an annual biomass productivity of 20 g/m^2/day and a lipid content of 14% (dcw) was cultivated in open raceway ponds fed with nutrients from a wastewater treatment plant (WWTP). The entire process chain moved from harvesting of the biomass using settling tanks followed by centrifugation to processing in a hydrothermal liquefaction unit for bio-oil production and transportation to a refinery for upgradation of the extracted oil eventually transporting it to the airport for use as jet fuel. The energy expenses and GHG emissions were balanced by nutrient recycling of the aqueous phase of the hydrothermal liquefaction unit, but a major contribution to GHG emission neutralization was brought about by the integration of the hydrothermal liquefaction unit to the algal cultivation and dewatering system in the WWTP instead of integrating it in the refinery along with the upgradation unit thus bypassing the extra energy lost in transporting the extracted oil to the refinery for upgradation.	Fortier et al., 2014
(5)	41	In this Well to Wheel (WTW), green microalgae with an annual biomass productivity of 22 g/m^2/day and lipid content of 30% (dcw) was cultivated in open raceway ponds fed with fertilizer grade N, P, K and waste flue gas as carbon source. The entire process chain moved from harvesting of the biomass using gravity clarifiers followed by centrifugation to lipid extraction from wet biomass using n-hexane and transesterification using methanol. The energy expenses and GHG emissions were balanced by the co-product credits of biogas and methane generated through anaerobic digestion and hydrothermal gasification, respectively, eventually producing heat and electricity through combined heat and power technique for use during the biodiesel production process.	Azadi et al., 2014
(6)	50	In this Well to Wheel (WTW), *C. vulgaris* with an annual biomass productivity of 23.5 g/m$^{2/}$day and lipid content of 25% (dcw) was cultivated in open raceway ponds fed with fertilizer grade N, P, K and waste flue gas as carbon source. The entire process chain moved from harvesting of the biomass using aluminum sulfate flocculation followed by centrifugation to use of waste heat dryer for drying the wet biomass for lipid extraction using *n*-hexane and transesterification using methanol. The energy expenses and GHG emissions were balanced by water and 75% nutrient recycling and the co-product credits of glycerine produced during transesterification, and heat and electricity produced from the residual de-oiled biomass processed through combined heat and power technique for use during the biodiesel production process.	Zaimes and Khanna, 2013
(7)	−46.92	In this Cradle to Grave (CTG) life cycle analysis, microalgae with an annual biomass productivity of 25 g/m^2/day and lipid content of 30% (dcw) was cultivated in open raceway ponds fed with nutrients from sea water and industrial flue gas as carbon source. The entire process chain moved from harvesting of the biomass using chemical-hydraulic flocculation with aluminum sulfate and filtration followed by drying within a thermal dryer for lipid extraction using hexane and transesterification to biodiesel using methanol. The energy expenses and GHG emissions were balanced by nutrient and water recycling after lipid extraction and transesterification steps	Pardo-Cárdenas et al., 2013

(Continued)

TABLE 4 | Continued

Sl no.	GHG emissions (g CO_2eq/MJ of biodiesel)	Strategy adopted	References
(8)	−53	In this Well to Pond (WTP) life cycle analysis, microalgae with an annual biomass productivity of 25 g/m^2/day and lipid content of 25% (dcw) was cultivated in open raceway ponds fed with fertilizer grade N, P, K and waste flue gas as carbon source. The entire process chain moved from harvesting of the biomass using settling and dissolved air floatation followed by centrifugation to processing through hydrothermal liquefaction technique for bio-oil production. The energy expenses and GHG emissions were balanced by nutrient recycling from the hydrothermal liquefaction technique and by production of electricity by passing the waste gaseous elements from the hydrothermal chamber to the combined heat and power unit.	Frank et al., 2013

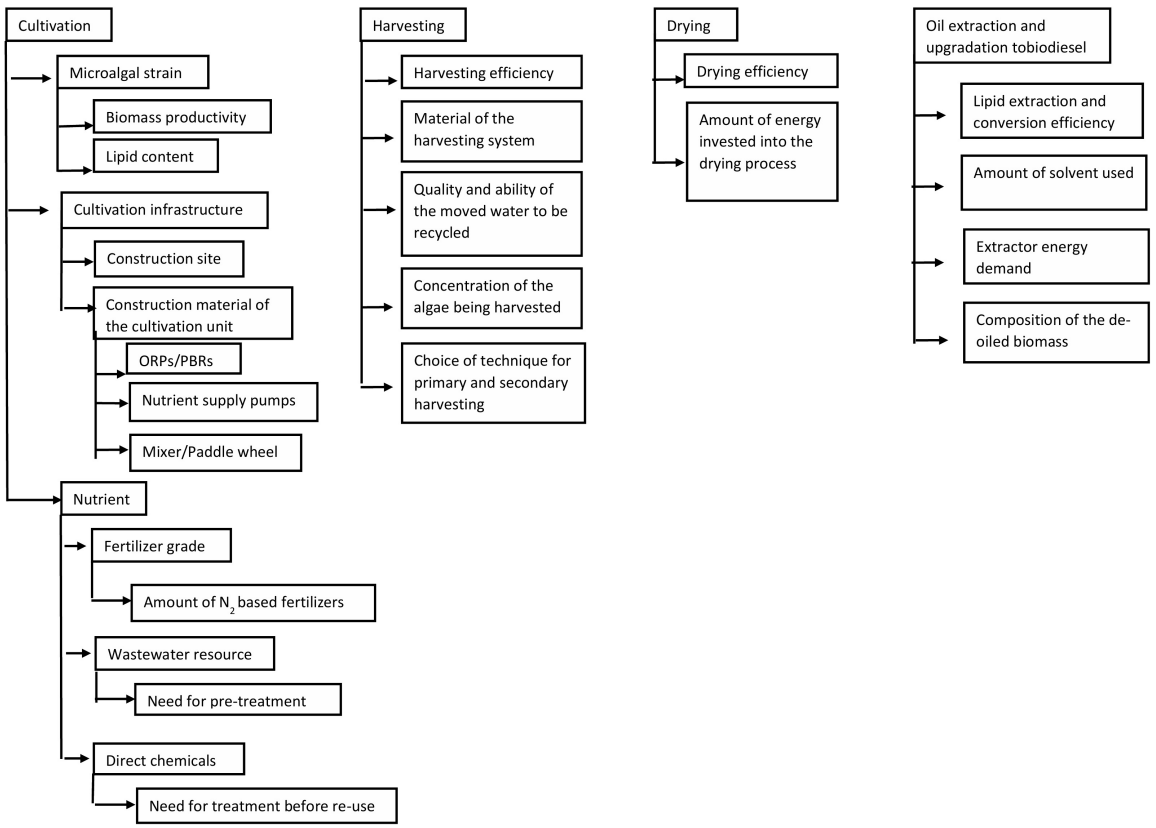

FIGURE 2 | Factors influencing the GHG emissions from the microalgal biodiesel production system.

with respect to energy security, can be ascertained if the amount of energy required to produce and process the microalgal biodiesel is found to be lower than the energy contained per dry weight of the alga (Yuan et al., 2015). In current day scenario, petroleum diesel has an $EROI_{fossil}$ of 4.64 but the $EROI_{fossil}$ of microalgal biodiesel as per published reports is less than unity (Brentner et al., 2011). Various strategies are currently in progress to raise the $EROI_{fossil}$ values with some achieving an $EROI_{fossil}$ of 1.88 through use of energy efficient harvesting and drying techniques and use of the produced electricity through combined heat and pressure technique for powering the entire production process (Chowdhury et al., 2012) and some others achieving an $EROI_{fossil}$ of 2.01 through integration of the microalgal biodiesel system with a wastewater treatment plant (Zaimes and Khanna, 2013). Some researchers are with the belief that microalgal biodiesel can have an $EROI_{fossil}$ of 8 (Stephenson et al., 2010), but with current research techniques, for improving the desirability of the microalgal biodiesel, achieving minimum EROI values of 3 is suggested (Marzochella et al., 2010; Vasudevan et al., 2012).

$EROI_{fossil}$ and GHG emissions are indirectly proportional to each other with an increase in the value of one parameter bringing about a decrease in the other and vice versa. Hence strategies to reduce the GHG emissions from the microalgal biodiesel production process eventually raise the $EROI_{fossil}$ values, thus

producing an environmentally sustainable biofuel. EROI is not an absolute indicator of sustainability, but it does help to indicate where a particular source fits in with regional, national and global energy markets. In that context, a competitive EROI for algae biodiesel provides support for a national energy policy that replaces petroleum.

Social Sustainability

The ability of a product to be sustained for use by the society and for the society, decides its social sustainability. This social dimension of microalgal biodiesel sustainability is decided by its ability to positively impact rural development, poverty reduction and inclusive growth (Elbehri et al., 2013). To judge the impact of the biodiesel production system on the abovementioned indices, factors such as, land ownership rights, local stewardship of common property resources and labor rights are mostly looked into Mohr and Linda (2013). Land being a limited resource, the decision/interest of people holding rights over the land to earn value from it through wealth generation or greening of the environment greatly affects the social sustainability of the microalgal biodiesel. Similarly, stewardship of local common property resources such as community forests, common grounds, threshing grounds, rivers and riverbeds by the co-owners/stewards of the property is another influencing factor as their agreement to the proposal of utilizing the common property resources for bioenergy production at the cost of their dependence on these properties at the time of need is highly essential. The ability of the microalgal biodiesel to generate rural employment and welfare by increasing inflow of capital, fertilizers, infrastructure and technologies to the agricultural/farm sector thus creating new employment opportunities, higher wages and increased self-sufficiency in terms of access to electricity and pumped portable water without causing any negative impact is another unavoidable factor to be considered while deciding the social sustainability of the microalgal biodiesel (Levidow, 2013).

In the market of bioenergy, microalgal biodiesel is like a new born baby waiting to be nurtured and groomed. In such a scenario implementation of social certification schemes, rules, laws or acts for ensuring its social sustainability is too early to be true. Under such circumstances, with the Renewable Fuel Standard, 2007 in United States and Renewable Fuel Quality Directive, 2008 in European Union, mandating a substantial portion of renewable fuel in the transportation sector by 2040, countries all over the world are gearing up with microalgae as a source of biodiesel and encouraging its use by their people through grants to companies equipped for their production (GCC, 2016).

With United States leading the world in microalgal research, majority of research efforts in the field are concentrated here (Gude et al., 2012). Hence the search for the first steps in ensuring social sustainability of microalgal biodiesel can be traced in this nation. The Department of Energy, United States, as part of the nation's energy strategy had announced ∼$25 million funding to reduce the price of algal biodiesel below $5/GGE by 2019. This funding is believed to support creation of green jobs, innovations, improvement in environment and national energy security. The funding has been partitioned to two phases with the first phase concentrating on valuable co-products development from microalgae besides biodiesel production and the second phase concentrating on carbon capture technologies for improved yields of microalgal biomass (Casey, 2014). Microalgal biodiesel companies with an intention to form strategic partnerships to attract private investments are leveraging co-operative agreements of the Energy Department. For, e.g., Sapphire Energy, an algae based green crude producer and awardee of the DOE funding has signed two commercial contract agreements with Phillips 66 and Tesoro (one being an integrated energy manufacturing and logistics company and the other being an independent refiner and marketer of petroleum products) to upgrade its biodiesel to on-spec diesel which can be used in existing diesel fuel tanks (Liu et al., 2013). Similarly, contract agreements between United States DOE and Hawaii Bioenergy, New Mexico State University and California Polytechnic State University to demonstrate algal biodiesel yields greater than 2,500 gallons per acre with a funding of $ 16.5 million have also been entered into (Office of Energy Efficiency and Renewable Energy [EERE], 2014). In addition to these the United States Government has effectively implemented the Clean Power Plan of the Environmental Protection agency, and has been hailed successful by the Algae Biomass Organization for maximum carbon capture by microalgae, setting federal guidelines for states to reduce carbon emissions by 32% before 2,030 to regulate the concentration of CO_2, an environmental pollutant, in the atmosphere (Kommers, 2013). Such strategic actions have also been taken by companies in Canada and the European Union and various other parts of the world European biofuels technology platform (EBTP, 2016; NRCC, 2013).

Employment generation by microalgal biodiesel production is a statistic yet to be derived but with the emergence of numerous companies interested in working for biodiesel production from microalgae, employment of laborers in large numbers is expected. Statistics of job creation from biodiesel production in 2011 (first and second-generation biodiesel) shows a support of 39,027 jobs and more than $ 2.1 billion in household income in the United States (national biodiesel board) (National biodiesel board [NBB], 2009). These jobs created by using economic and environmentally sustainable means (biodiesel), are categorized as 'Green Jobs' and are more clearly defined by the UNEP as a job in any field of work be it agriculture, manufacturing, R&D etc., that contributes substantially to the preservation and restoration of environmental quality. It is a joint initiative by the UNEP, the ILO, and the ITUC in the year 2007 (United Nations Environmental Programme [UNEP], 2008).

With a rush for jobs by the skilled and educated masses, unemployment among the unskilled rises to alarming levels. In order to balance this difference, programs like Pathways out of Poverty (POP), a national workforce training program by the United States government's ARRA of 2009 trains individuals living below or near poverty level with skills needed to enter the green job market, focusing primarily on the energy efficiency and renewable energy industries. The training programs focus on teaching basic literacy and job readiness skills in addition to providing supportive assistance with

childcare and transportation to overcome barriers to employment (Universidad, 2009).

CHALLENGES AND AVENUES FOR FUTURE RESEARCH

Microalgal biodiesel production has been initiated on a pilot scale at various places, but a discussion on their ability to profoundly displace petroleum diesel, has been mostly ignored. In today's market condition, microalgal biodiesel is more expensive than petroleum diesel as the improved economics of production are inadequate for environmentally sustainable production let aside the oblivion of social sustainability. A retrospection of the different research studies on microalgal biodiesel production system highlights few major challenges in the production of biodiesel from microalgae eventually hindering its commercialization. A few essentials are explicitly addressed.

1. The different stages of microalgal biodiesel production continue to be highly energy intensive impeding attainment of economic and environmental sustainability.
2. A low-cost arrangement for water, nutrients and CO_2 with minimum negative impact on the environment and microalgal culture quality still appear to be challenging.
3. Maintaining a monoculture inside the raceway ponds continues to be difficult to achieve.
4. Unsuitability of non-native algae to a new ecosystem creates risks of microalgal spills.
5. Scaling up of microalgal culture is a big problem with high degrees of uncertainity about the replication of functional characteristics in the scaled up cultivation system.
6. Huge variation in GHG emissions and EROI data from different research studies question the efficacy of the strategies being adopted.
7. Lack of faster and efficient tools for screening of oleaginous microalgal strains slows down progress in the field.
8. Lack of complete biochemical and molecular profiling of oleaginous microalgae restricts informations and innovations.
9. Lack of detailing of the cultivation and operational parameters used in the microalgal biodiesel production system, hinders complete sustainable development of the production system.
10. Routes for recuperating energy from the microalgal biomass left after oil extraction are required for attaining a net positive energy balance during the production of microalgal biodiesel.
11. Lack of sufficient genetic and metabolic engineering in the field of microalgal biodiesel confines exploration of genes that control the production of lipid in microalgae.
12. Wasted energy from captured photons during photosynthesis is a major challenge in mass algal cultivation.
13. Uncertainities about policy support and competition from other fuels further adds to the plight.

With a focus and determination to defy the pessimistic view of a group of research scientists who claim that microalgal biodiesel can never outcompete petroleum diesel, research organizations, institutions and individuals are working with hastened speed to address the challenges mentioned above. Although fortunately there has been some success in achieving some near-term goals as has been mentioned in the previous sections, there still remains enough work to be done in future, details of which have been mentioned below.

1. With microalgal cultivation requiring huge inputs of nitrogen and phosphorous, recycling of nutrients with special emphasis on the quality and quantity of nutrients being recycled can be focused on.
2. With reports of 100% nutrient recycling raising the cost of microalgal biodiesel by $2/Gal as compared to 0% recycle (Davis et al., 2017), alternative wastewater resources can be tracked and their complete profiling including nutrient and bacterial count can be noted down before being used for cultivation so as to include the pre-treatment costs in the final economics of the produced biodiesel.
3. Co-products reduce the economic and environmental burdens of microalgal biodiesel but life cycle impact assessment studies to understand the type of co-products which when produced provide maximum benefit in attaining sustainability, can be done.
4. Use of paddle-wheels in raceway ponds is where maximum allocation of capital is done. In order to reduce the cost burdens (National biodiesel board [NBB], 2009), alternative, less energy intensive technologies for culture mixing can be explored, notwithstanding the water pumping step which also exerts a substantial energy burden.
5. Combined heat and power treatment of the gaseous substances released from the hydrothermal system is used to generate electricity to power the entire cultivation system, but quantification and optimization of the process can be done to get the exact figures for future reference and research.
6. Several resource and environmental challenges exist for scaling up of microalgal culture. To overcome this, complete detailing about the microalgal strain and the cultivation system can be done, as knowledge about the microalgal biology and biochemistry helps us understand the possible response of the microalga to a designed cultivation system with a temperature control mechanism in it as microalgae are extremely susceptible to temperature variations in open cultivation systems.
7. With monoalgal culture being a difficult target to achieve in open raceway pond systems, cultivation of algal consortium can be practiced with an effort to maintain a functional specificity of accumulating lipids rather than a species specificity.
8. With chances of microalgal spills due to cultivation of non-native microalgae in new ecosystems (Gressel et al., 2014), mutagenesis and transgenics can be explored

to delete genes that are unnecessary in culture but obligatory in nature.

9. With different harvesting techniques being experimented with for finding out a faster and efficient technique with minimum energy expenses, bioflocculation and autoflocculation have been found to be most attractive options (Vandamme et al., 2013). So research on the chemicals inside the microalgae leading to the respective phenomenon can be carried out to improvise the process and eliminate any negativity attached to the harvesting technologies.
10. Outside blown in dust, being a major impediment to harvesting costs and a reason for light shading during microalgal cultivation, can be made to settle at the bottom of the pond through some innovative flocculating mechanisms so as to improve productivity in the cultivation systems.
11. Photosynthesis being the starting point for energy capture and dissipation, the complex interplay between spectral range, light capture efficiency and CO_2 fixation can be considered as a crucial area of research.
12. Additionally, development of models of regulatory network in microalgae to assist in better gene and metabolic regulation for optimization of the storage of chemical energy in a particular form, for understanding the signaling mechanism in algal cells in more complex algal populations and for development of predator and pathogen resistance, can allow better biological control in large scale systems.
13. Microalgal metabolism and growth rate being inversely proportional to their cell diameters, the surface-to-volume ratio of the microalgae can be considered to be an important parameter of research while searching for high biomass yielding microalgal strains.

The scope of future research in the field of microalgal biodiesel production, does not limit itself to the few points mentioned here, but goes deep into an elaboration of the points highlighted. With some research projects in progress and few more planned for the future, an analysis of the entire scenario suggests that, today at this moment, the fundamentals are the problem. Lack of fundamental knowledge on the factors governing the variations in the entire algal biodiesel production process result in vague and inconclusive impact assessment reports.

The question of whether microalgae will be a significant contributor to biodiesel production before 2030, generally depends upon the pace of innovations. Few people with a pessimistic view, are with the belief that with the current pace of microalgal biodiesel research scaling up to large quantities by 2030 will be a difficult target to achieve. However, more worrying is the fact that the pace of innovations might be slow enough to making it an uneconomic strategy to invest in at the commercial scale as compared to other opportunities.

If companies fail to innovate, they die. But if they fail to rapidly develop cash-flowing solutions, they cannot attract capital, and they die that way too. Now, cooperative research projects, with companies collaborating with institutions to develop technologies, are an old idea. But planning a roadmap of innovations by using public funds for research and development leading to company formation is a new idea that can be proposed and pursued. The near-term stance for pervasive use of microalgal biodiesel appears dreary, but biodiesel for vocation solicitations such as in aviation may be possible in the medium term.

CONCLUSION

It's honestly extremely turbulent at this moment with a large number of innovations going on at too many fronts, just to make stable forecasts about when microalgal biodiesel will become an affordable reality. It requires scientists to take too many high-risk decisions for a faster pace of innovations. However, it is very clear that counting microalgal biodiesel out, any time before 2030, is a complete no–no for the researchers. What is important to remember here is that, microalgal biodiesel is based on a system of systems, not a single technology. Hence, with patience and perseverance, that which looks daunting today will be a successfully achieved target, couple of years after.

AUTHOR CONTRIBUTIONS

RP read the literature and drafted the manuscript. NM supervised and corrected the manuscript. Both authors have approved the submitted version.

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REVIEW
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Feasibility of Utilizing Wastewaters for Large-Scale Microalgal Cultivation and Biofuel Productions Using Hydrothermal Liquefaction Technique: A Comprehensive Review

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The two major bottlenecks faced during microalgal biofuel production are, (a) higher medium cost for algal cultivation, and (b) cost-intensive and time consuming oil extraction techniques. In an effort to address these issues in the large scale set-ups, this comprehensive review article has been systematically designed and drafted to critically analyze the recent scientific reports that demonstrate the feasibility of microalgae cultivation using wastewaters in outdoor raceway ponds in the first part of the manuscript. The second part describes the possibility of bio-crude oil production directly from wet algal biomass, bypassing the energy intensive and time consuming processes like dewatering, drying and solvents utilization for biodiesel production. It is already known that microalgal drying can alone account for ~30% of the total production costs of algal biomass to biodiesel. Therefore, this article focuses on bio-crude oil production using the hydrothermal liquefaction (HTL) process that converts the wet microalgal biomass directly to bio-crude in a rapid time period. The main product of the process, i.e., bio-crude oil comprises of C16-C20 hydrocarbons with a reported yield of 50–65 (wt%). Besides elucidating the unique advantages of the HTL technique for the large scale biomass processing, this review article also highlights the major challenges of HTL process such as update, and purification of HTL derived bio-crude oil with special emphasis on deoxygenation, and denitrogenation problems. This state of art review article is a pragmatic analysis of several published reports related to algal crude-oil production using HTL technique and a guide towards a new approach through collaboration of industrial wastewater bioremediation with rapid one-step bio-crude oil production from chlorophycean microalgae.

Keywords: bio-crude, biofuel, hydrothermal liquefaction, microalgae, raceway ponds, wastewaters

INTRODUCTION

Renewable energy is typically defined as the energy derived from natural resources and is naturally replenished continuously. Renewable energy resources include solar, wind, rain, tidal waves, geothermal heat, and bioenergy. Renewable energy resources are found over a wide range of geographical regions throughout the world as compared to the fossil fuel resources that are available only in very few countries. A report of Renewable Energy Policy Network for the 21st century (REN Report, 2014; REN Report, 2015), the global investments in renewable technology sectors have been calculated to be US$214 billion. The countries like United States, China, Norway, and Denmark have invested a lot in solar, hydro, wind, and biofuels production sectors (REN Report, 2014; Mathiesen et al., 2015). It is therefore essential to discuss the importance of bioenergy, a kind of renewable energy, derived from various biological sources to generate heat or to produce the liquid transportation fuels often coined as biofuels. As per an analysis of the scenario in the recent years, biofuels are among the most widely used renewable energies that provide approximately 9–10% of the global primary energy supply (IEA Report, 2013). NS news agency (NS Energy Report, 2019) has reported that 2,616 thousand bbl day^{-1} of biofuels were produced in the world during 2019. The United States followed by Brazil have conquered the biofuels market with a collective share of approximately 87% of the world's production. The United States is the primary biofuel producer in the world with a total production of 1,190 thousand bbl day^{-1}. The country has harnessed ~46% of the world's biofuel production in the year of 2018. It is also to be mentioned that United States is the world's top biodiesel producer with a total share of 19%, equivalent to 136.2 thousand bbl day^{-1} in 2018 (https://www.nsenergybusiness.com). These countries and few more, have diverted their attention towards biofuel production from green chlorophycean "microalgae" (Galadimaa and Muraza, 2018). The use of microalgae for biofuel production has numerous advantages over the other biodiesel production sources, and hence, these microalgae are often coined as the "Green gold" for biofuel production (Bagchi et al., 2018).

The major advantages of using microalgae for biofuel production are enumerated below:

Biofuel derived from the grains and oil seeds have a large land and carbon imprint, instigating the food vs. fuel argument (Mandal and Mallick, 2009). Microalgal biomass with a much faster growth rate has a usual capability to bio-mitigate CO_2 while trapping sunlight with an efficiency of 10–50 times higher than the common terrestrial plants (Li et al., 2008), Microalgae are capable of producing higher amounts of oil (58,700–136,900 L ha^{-1} $year^{-1}$) per unit area of land as compared to other oil-producing crops (Chisti, 2007). Comprehending such high oil yielding potentials of microalgae with effective CO_2 bio-fixation up to 12–15% in air mixture, the locally isolated green microalga *Scenedesmus obliquus* was shown to be a potent alternative as a renewable source for biomass production with a maximum biomass yield of 7.01 g L^{-1} under mixotrophic cultivations in photobioreactors under controlled culture environment (Bagchi and Mallick, 2016). As microalgae are aquatic, the microalgae can be cultivated in freshwater, sea-water, brackish-water, or even various wastewaters (de Godos et al., 2009). Microalgae are used for the algal biorefinery studies by sequentially extracting many important compounds (Patnaik and Mallick, 2015; Patnaik et al., 2019). After the extraction of bio-oil from microalgae, the algal biochar can be used as an enhancer of soil fertility along with preventing soil degradation through efficient carbon sequesteration in the soil.

However, despite these advantages use of microalgae for biofuel production is yet to be cost-competitive with fossil-based fuel due to the upstream and downstream challenges such as high cost of nutrients, energy-intensive harvesting, drying, lipid extraction, and transesterification techniques. One of the methods to counter these problems is large scale cultivation for enhanced biomass production (Patnaik and Mallick, 2015; Severo et al., 2019). The large-scale mass cultivations of microalgae in raceway ponds are well established by several researchers. The large-scale practice in raceways had started in the early years of 1950s by cultivating the green microalga *Chlorella* (Brennan and Owende, 2010) and was in full operational phase in the late 1960 using the "high rate algal ponds (HRAP)" with Oswald's large-raceway-pond designs (Oswald and Golueke, 1960). Microalgae mass cultivation in raceway ponds has now been considered as the most promising means for large scale biomass production in terms of less capital investment and low running cost compared to the engineered photobioreactor (PBR) systems. Basically, photobioreactors are useful to maintain the monoalgal culture but the overall operational cost is extremely high as compared to the open large-scale raceway ponds (Bagchi and Mallick, 2016; Bagchi et al., 2019). One of our recent studies has already shown that an annual biomass productivity upto a high value of 13.12 tons ha^{-1} $year^{-1}$ can be achieved if cultivated for ten cultivation cycles per annum. The study was conducted in four numbers of 40,000 L capacity raceway ponds by Bagchi et al. (2019). In another study by us at the same geographical location, it was observed that *Scenedesmus accuminatus* organism produced a biofuel yield of 2.14 tons ha^{-1} $year^{-1}$ in open raceway pond batch cultivation (Koley et al., 2019). However, contrary to this Zhang et al. (2018) reported a comparatively lower biofuel yield of 0.79 tons ha^{-1} $year^{-1}$ algal biofilm raceway ponds.

Nowadays, waste disposal is a worldwide problem. In the current scenario, waste discharges from various industries and city sewages are the primary sources of water pollution. Conventional wastewater treatment systems do not seem to be the definitive solution to pollution and eutrophication problems. Secondary sewage treatment plants are specifically designed to control the number of organic compounds in wastewaters. However, pollutants, mainly nitrogen, phosphorus, sulfur are only slightly affected by this type of treatment. Wastewater treatment by microalgae using the wastewater or waste disposal as the growth medium for large-scale algal cultivations is possibly the best way to solve these tailbacks effectively (Mallick et al., 2016).

Another serious challenge for the upscaling of biodiesel production is the exploitation of various low-energy intensive harvesting and drying techniques and the development of cost-

effective lipid extraction methods. Removal of water from the wet algal biomass is necessary for prolonged storage of the feedstock and carry out further downstream processes like lipid extraction followed by biodiesel production. Generally, wet microalgae contain ~90% moisture. The drying technologies are typically utilizing high extensive heat energy, which puts a significant obstacle to the microalgal biodiesel market assessment on a profitable basis (Lardon et al., 2009; Patil et al., 2011; Bagchi et al., 2015). It is also a well-known fact that the key constraint for downstream process of microalgal biodiesel production is the enormous expenditures associated with the extraction of lipids followed by the transesterification process. There are a lot of researches being carried out for developing the lipid extraction processes from microalgae. The Folch method (Folch et al., 1957) and the Bligh and Dyer (Bligh and Dyer, 1959) technique are the most acclaimed and commonly practiced total lipid extraction protocols for microalgal biodiesel production also adopted for large-scale extraction processes (Kumar et al., 2015). These techniques are performed by using a considerable volume of solvents as chloroform: methanol: 2:1. The modified method of the above for the extraction of all lipids classes was suggested by Matyash et al. (2008) in which Methyl-tert-butyl ether (MTBE) was utilized as a solvent. The method proved to be successful in the extraction of almost all lipids classes to portray entirely accurate lipidomic profiles.

From the above discussions, it is now well understood that despite numerous independent research works on microalgal cultivation, wastewater remediation and HTL, a workable strategy combining all the three factors for reducing the economic gap between fossil-based and biomass-based fuels is not available to the extent of our knowledge. Therefore, we in this review report intend to categorically discuss the important points reported in various research works related to algal cultivation using wastewaters and propose a strategy for "waste to wealth" generation combining microalgal growth and lipid accumulation with wastewater bioremediation followed by HTL technique for deriving bio-crude oil directly from wet algal biomass, thereby recommending a synergistic approach for sustainable biofuel production.

WASTEWATER UTILIZATION FOR MICROALGAL CULTIVATION

Laboratory Based Studies

It is prominent that microalgae can bioremediate wastewater by the removals of ammonium, nitrate, nitrite, and phosphate from a variety of wastewater sources (Mallick, 2002). Various Researchers reported that the microalgae could grow in different kinds of wastewaters, and the wastewater resources are proved to be the best potential source of cost-effective biofuel production (Woertz et al., 2009). However, the wastewater utilization to enhance algal growth, thereby low-cost lipid production, and the exploration of microalgae's pollutant removal efficiency is still a minimal approach in terms of outdoor large-scale algal culture exploitations.

The chlorophycean microalga *Scenedesmus obliquus* has shown an elevated biomass and lipid yield by utilizing the mixture of poultry litter and municipal secondary settling tank discharges in the amount of 15 g L^{-1} (Mandal and Mallick, 2011). The swine manure wastewater was successfully utilized for the cultivation of 97 microalgae obtained from algae-bank and 50 other algal strains isolated from the local waterbodies in Minnesota, United States of America. The maximum biomass yield was achieved up to 2.03 g L^{-1} for the locally isolated microalgal strain UMN 271 (Zhou et al., 2012). One report observed that the mixed microalgal consortium was cultivated in two phases comprising initial growth phase (biomass enhancement; 8 days) under mixotrophic mode using domestic sewage wastewater followed by temperature stressed starvation phase. The biomass yield was recorded high enough in this production process (Venkata Subhash et al., 2014). Another report also demonstrated that the microalga *Chlorococcum* sp. was grown in sea-water based saline medium supplemented with waste glycerol available from the biodiesel industries with a maximum biomass yield was 0.85 g L^{-1} (Beevi and Sukumaran, 2015) (**Table 1**). In continuation, the biomass yield were found significantly higher as 6.0 g L^{-1}, for the mixed algal consortium cultivated with the dairy manure as a rich nutrient source (**Table 1**) (Chowdhury and Freire, 2015). The green microalga *Chlorella vulgaris* was grown under ammonia-rich wastewater (Markou, 2015). The utilization of wastewaters was also quite useful for algae cultivation as per the research work carried out in our lab. The microalga *Chlamydomonas debaryana* IITRIND3 was successfully cultivated in different wastewaters from domestic, sewage, paper mills, and dairy wastewaters, respectively. The maximum biomass yield was depicted as 3.66 g L^{-1} in dairy wastewater whereas 3.56 g L^{-1} in domestic wastewater, respectively (Arora et al., 2016). Biomass yield found in this process by utilizing the wastewaters was quite productive, and the yield values are significantly higher than many other reports published till date. In another study done in this laboratory, the crude glycerol (CG) was used as low cost by-product obtained from the biodiesel production process for the cultivation of the microalga Chlorella minutissima (MCC27), with the maximum biomass yield 3.13 g L^{-1}, respectively (**Table 1**) (Katiyar et al., 2018). It was also demonstrated that the microalga *Chlorella* sp. was successfully cultivated in the aerated seafood processing wastewater for higher biomass accumulation, lipid production as well as the major nutrients' removal from the wastewater. The study also has shown that the total nitrogen (TN) and total phosphorous (TN) contents in the wastewater were constantly decreased during the end of the cultivation period of the microalgae. The total nitrogen concentration was reduced to a deficient level of 4.11 mg L^{-1}, which was only 3.4% of the initial concentration. Further calculations have also indicated that ~93 and ~50% of the eliminated nitrogen and phosphorous were assimilated by the alga during the end of the course of the investigation, showing that the tiny organisms "microalgae" are effectually the potential sources to utilize for removing the nitrogen and phosphorous from the wastewater bodies (Gao et al., 2018) (**Table 1**). Elystia et al. (2020) has reported that the green

TABLE 1 | Tabulations of various reports on elevated biomass yield by using cost-effective cultivations as the utilization of waste disposal and wastewaters.

Name of the microalga	Operational description	Maximum biomass yield (g L^{-1})	References
Scenedesmus obliquus	Poultry litter + municipal secondary settling tank wastewater discharges	2.0	Mandal and Mallick (2011)
Locally isolated microalga	Digested swine manure wastewater	2.03	Zhou et al. (2012)
Mixed microalgae culture	Mixotrophic mode using sewage wastewater followed by temperature stressed starvation phase	2.49	Venkata Subhash et al. (2014)
Chlorococcum sp. RAP13	Sea water-based medium, supplemented with biodiesel industry waste glycerol	0.85	Beevi and Sukumaran (2015)
Mixture of algae	Dairy manure as a nutrient source	6.0	Chowdhury and Freire (2015)
C. vulgaris	Ammonia-rich wastewater by using poultry litter	1.5	Markou (2015)
Chlamydomonas debaryana IITRIND3	Algal cultivation in different wastewaters as domestic, sewage, paper mills, and dairy wastewaters	3.66	Arora et al. (2016)
Chlorella minutissima	Crude glycerol (CG) used as low cost by-product	3.13	Katiyar et al. (2018)
Chlorella sp.	Seafood processing wastewater	1.55	Gao et al. (2018)
Scenedesmus pecsensis	Rice mill effluent wastewater	5.29	Keerthana et al. (2020)

microalga *Chlorella pyrenoidosa* was successfully cultivated using palm oil mill effluent (POME) wastewater as a growth medium and the maximum specific growth rate was 0.306 $days^{-1}$ with the highest number of cells was 3.530×10^7 cells ml^{-1}. However, in this experiment, the researchers have not specified the exact biomass yield or biomass productivity. The fresh water chlorophycean microalga *Scenedesmus pecsensis* was proved to be a potential agent of wastewater bioremediation by 68.2% phosphate and 49.3% nitrogen removal. The alga was cultivated using rice mill effluent wastewater as a low-cost medium. The biomass yield was also found to be quite higher as 5.3 g L^{-1} (Keerthana et al., 2020).

Utilization of Wastewaters for Large-Scale Microalgal Cultivation Systems

Basic Concept of Raceway Ponds

Scientists have urged on the essentiality of large-scale microalgae cultivation for commercial level biofuel production (Moazami et al., 2011; Moazami et al., 2012). There are two main types for large-scale microalgae cultivation, closed systems (photobioreactors) and open ones. A possible low-cost culture system strategy for bio-oil production on a commercial scale is the use of raceways ponds or circular tanks (Moheimani and Borowitzka, 2007; Ashokkumar and Rengasamy, 2012). Compared to the photobioreactors, raceway ponds are generally preferable for large-scale algal biomass production due to the significantly less capital investment and lower maintenance cost, utilization of wasteland or barren lands, and easy operation techniques (Chisti, 2008). It is also an essential factor that the microalgal cultivation inside the raceway ponds requires the optimum stirring for continuous or semi-continuous mixing to recirculate the microalgal culture (Chisti, 2016; Koley et al., 2019). However, there are many limitations of raceway pond culture of microalgae. Open raceways are more prone to contamination by other organisms such as bacteria, fungi, other microalgal starins, diatoms. Achieving elevated productivity and maintenance of mono-algal strain are the real shortcomings of cultivation in open raceway ponds (Bagchi et al., 2018). Therefore, it is very necessary to grow the desired algal starin in the raceways covered with polyhose made with thick transparant polythenes. This is also highly essential to protect the algae from the schorching sunrays particularly in the tropical regions where the temperature are above 45°C during the summer season. Several other essential parameters like pH, DO, light intensity, temperature, aerator speedflow must be monitor time to time for the optimum productivity in large-scale raceway ponds (Bagchi et al., 2019; Koley et al., 2019).

Summarization of Study Reports of Microalgal Cultivation With Wastewaters

The major disadvantages of using the conventional facultative algal ponds are the maintenance of monocultures, the requirement of various chemical flocculation techniques that are generally very costly processes for microalgal harvesting, and may not deliver regular and effective nutrients' removal (Abdel-Raouf et al., 2012). In contrary to this, the use of shallow, paddle-wheel driven and high rated algal ponds can generate much more higher amount of algal biomass up to 30–40 tons ha^{-1} $year^{-1}$ with a provision to explore the bioflocculation or self-flocculation techniques that may afford the cost-effective microalgal harvesting (Slade and Bauen, 2013). However, some little works are carried out on the utilization of various domestic, municipal, or industrial wastewaters for the large- or pilot-scale exploitations of microalgae cultivations in raceway ponds. Therefore, it is imperative to recapitulate those findings and discuss them in a precise manner in this review article. Park and Craggs, in the year 2010, had cultivated the microalga *Pediastrum* sp. in a 31.8 m^2, 8,000 L volume pilot-scale raceway pond with domestic wastewater treatment. The raceway pond was paddle-wheel operated with 1 m wide, galvanized steel paddle-wheel circulated inside the raceway pond water to provide the surface velocity of 0.15 m/s. In this study, the areal biomass productivity was recorded 25 g m^{-2} day^{-1} (**Table 2**). Another study was conducted with a consortium prepared with 15 native microalgal strains, successfully cultivated in a 3,800 L capacity

TABLE 2 | Review on biomass productivity of various microalgal species with the utilization of wastewaters as growth medium under raceway pond cultivation.

Test organism	Cultivation description	Biomass productivity (areal/annual)	References
Pediastrum sp.	Raceway pond microalgal cultivation with domestic wastewater treatment, raceway pond dimensions: surface area: 31.8 m^2, depth: 0.3 m, 8,000 L of raceway pond working volume	Areal productivity: 25 g m^{-2} day^{-1}	Park and Craggs (2010)
A consortium of 15 native microalgae	Raceway ponds had of total 3,800 L capacity, wastewater containing 85–90% carpet industry effluents with 10–15% municipal sewage	Annual productivity: 9.2–17.8 tons ha^{-1} $year^{-1}$	Chinnasamy et al. (2010)
Mixed microalgal consortium	Raceways were constructed in the wastewater treatment plant site. 8,000 L; Area- 21 m^2	Areal productivity: 13.5 g m^{-2} day^{-1}	Lee et al. (2014)
Chlorella vulgaris	Volume - 14.62 billion L, 30 cm culture depth; alga cultivation utilizing wastewaters	Areal Productivity: 15 g m^{-2} day^{-1}	Rogers et al. (2014)
A consortium of microalgal isolates collected from wastewaters	Growth medium: wastewaters from dairy farms; Dimensions: 2.5 × 0.7 × 0.7 m, working volume: 600 L	Annual productivity: 153.54 tons ha^{-1} $year^{-1}$	Hena et al. (2015)
Chlorella pyrenoidosa	Growth medium: domestic wastewater; raceway dimension: 1.5 × 0.6 × 0.4 m, raceway working volume: 360 L	Areal productivity: 36 g m^{-2} day^{-1}	Dahmani et al. (2016)
Mixed microalgal consortium including *Chlorella* sp., *Scenedesmus* sp., and *Stigeoclonium* sp. (CSS)	Growth medium: municipal wastewater; 0.4 ton working capacity high rated raceway pond, optimized culture depth: 20 cm	Areal productivity: 6.16 g m^{-2} day^{-1}	Kim et al. (2018)
Parachlorella sp. JD076	Semi-continuous operation in municipal wastewater under small-scale raceway pond cultivations	Areal Productivity: 22 g m^{-2} day^{-1}	Yun et al. (2018)
Desmodesmus subspicatus	Wastewater mediated algal growth in 2,000 L raceway ponds (2,000 L × 4 raceways)	Areal Productivity: 28 g m^{-2} day^{-1}	Schneider et al. (2018)
Mixed microalgal consortium	High rated algal raceway lagoon (length - 30 m and width of the single channel - 2.5 m), community wastewater utilized.	Areal Productivity: 31.7 g m^{-2} day^{-1}	Buchanan et al. (2018)
Chlorella spp.	Oblong shallow raceway pond having total area was 3.6 m^2, culture depth was set as 40 cm and total height was 50 cm	Areal Productivity: 2.5 g m^{-2} day^{-1}	Romagnoli et al. (2020)

outdoor raceway pond. This was done by utilizing the industrial wastewater containing 85–90% carpet industry effluents with 10–15% municipal sewage wastewater. The culture was supplemented with 6% CO_2 sparging, and the overall annual biomass productivity was calculated as 9.2–17.8 tons ha^{-1} $year^{-1}$ (Chinnasamy et al., 2010). Continuation to this, the 8,000 L capacity raceway ponds were used for the cultivation of the mixed microalgal consortium as *Scenedesmus*, *Chlorella*, *Pediastrum*, *Nitzschia*, *Cosmarium*, and other filamentous microalgae. The ponds were fabricated in the wastewater treatment plants' location, and the effluents of the plants as wastewaters were utilized followed; the average areal biomass was found to be 13.5 g m^{-2} day^{-1} (Lee et al., 2014). It is here worth mentioning that apart from the biomass and lipid productivities reported in this study, the other important part can be incorporated to note that the microalgal biomass harvesting process were made much easier and cheaper in those raceways with the use of some mesh like substrates attached with the microalgae, which were simply removed from the microalgal cultures and the treated wastewaters can be discharged more easily. The green microalga *Chlorella vulgaris* was also grown in the huge-sized, actual large-scale raceway ponds with a full capacity of 14.62 billion L with the supply of various wastewaters available from different sources. The culture depth was set as 30 cm for all studies (**Table 2**). In a contrary to these study reports published, Hena et al. (2015) showed that the annual biomass and lipid productivities could be achieved up to >150 and >25 tons ha^{-1} $year^{-1}$, respectively for the microalgal consortium, isolated from wastewaters and also grown in wastewaters, collected from dairy farms, under the semi-large scale raceway pond cultivation with the working volume of 600 L of one pond (**Table 2**). The supplementation of 10% CO_2 was provided for the elevated growth rate of the microalgae. The raceway pond's dimension was set as 2.5 × 0.7 × 0.7 m, and the mixing speed of the paddle-wheel was fixed at 20 rpm throughout the experimentations. It is indeed highly curious to critically observe this research work's results as it demonstrated an exceedingly high annual biomass and lipid productivities claimed that are incomparable to many studies reports published till date for microalgal biomass and lipid production under large- or semi-large-scale raceway pond cultivations.

Nonetheless to the previous, there are some other reports which have signified the feasibility of utilizing the wastewaters for microalgal cultivation and simultaneous bioremediation of the wastewaters by the algal bodies. In Algeria, the green microalga *Chlorella pyrenoidosa* was successfully cultivated in the raceway ponds constructed in the desert area at the domestic wastewater treatment plant site. The length, breadth, and depth of the raceway were 1.5, 0.6, and 0.4 m, respectively, with the culture working volume of 360 L. The cultures were circulated inside the raceway pond using the paddle-wheel powered by a 70 W electric gear motor. In this experiment, the maximum areal biomass productivity was recorded as >35 g m^{-2} day^{-1} (Dahmani et al., 2016). The ability of the microalga for bioremediation, various parameters like chemical oxygen demand (COD), NH_4 –N, and TP were measured in the course of the cultivation periods, and their average removal efficiencies were reported as 78, 95, and 81%, respectively (Dahmani et al., 2016). Ammonium, and total

phosphate are the primary source of nitrogen and phosphorous in wastewater, and controlling its toxic effects are the foremost challenge in wastewater treatment (Yu et al., 2019). In this direction to bioremediate ammonium, nitrate, nitrate, and total phosphate in wastewaters, the locally isolated microalga Chlorella sp. was cultivated in a fabricated outdoor wetland under the mixotrophic cultivation techniques using the piggery wastewaters. Various significant parameters for this wetland cultivation were investigated, such as the aeration rate, nutrient removal by the alga from wastewaters, biomass yield, and the fatty acid methyl esters (FAMEs) compositions. The maximum biomass productivity was recorded as 79.2 mg L^{-1} d^{-1} and the total nitrogen (TN), phosphorus (TP) removal efficiencies were found to be 80.9 and 99.2% (Lee and Chen, 2016), which was much higher than the overall chemical oxygen demand (COD) value depicted as 74.5%. The best cultivation temperature was found as 25°C.

Schneider et al. (2018) recorded a maximum biomass production of 1.12 g L^{-1} which is equivalent to 28 g m^{-2} day^{-1} from the microalga *Desmodesmus subspicatus* grown with wastewaters in 8,000 L volume raceway ponds. The interesting fact was that the wastewater sample was collected from one university's toilets after one-time treatment with the up-flow anaerobic sludge blankets (UASB), which are the reactors. Another study report also claimed a significantly elevated areal biomass productivity of >30.0 g m^{-2} day^{-1} (Buchanan et al., 2018) for the consortium of mixed algal bloom cultivated in the high rated large-scale raceway lagoons of 30.0 m in length. These facultative raceway ponds were fed with general community wastewater and septic tank effluents (**Table 2**). In 2019, Eladel et al. (2019) recorded a maximum biomass productivity of 0.073 g L^{-1} day^{-1} from *Chlorella sorokiniana* isolated from local municipal wastewater. Moreover, algal growth for 10 days in municipal wastewater depicted a nutrient removal efficiency of 74.20, 83.31, and 78.00% for NO_3^-, NH_3, and total phosphate, respectively. *Scenedesmus obliquus* was proved to be a potent organism in pilot-scale artificial wastewater processing with 96% removal of ammonia content (Liu et al., 2019). Another recent study reported that about 94 and 66% of NH_4^+ and PO_4^{3-} were removed from wastewater medium using microalgae immobilized on agar (Hu et al., 2020). In continuation, the oleagenous microalga *Chlorella* spp. was successfully cultivated using the wastewater obtained from digestate from biogas plants in a raceway pond having length (L):width (W) equal to 2:1, total area was 3.6 m^2. The culture depth was set as 40 cm and total height was 50 cm. The areal biomass productivity was calculated as 12.5 g m^{-2} day^{-1} with a growth yield of 0.25 g L^{-1} obtained in just 08 days of cultivation period (Romagnoli et al., 2020). From the various earlier studies discussed above in detailed, it is well comprehended that the microalgae are not only capable to thrive under open pond cultivations using wastewaters as their growth medium, but also these tiny microorganisms are the best potential candidates for the harmful nutrient removals from the primary treated wastewaters.

It is highly imperative to investigate the feasibility of culturing some oleaginous as well as halophilic or halotolerant and acidophilic microalgal strains to bioremediate wastewaters generated from heavy industries like mining, iron/steel, coal, tanning. The microalgae may offer a favourable and unconventional alternate to traditional and conventional technologies in the treatment of heavy metals like arsenic, copper, cadmium, chromium, and lead which are generally present in the industrial wastewater samples originated from heavy industries (Uberoi, 2003). These heavy metal ions can also cause diabetes, cancer, anemia, osteomalacia, and many neurotic or nephrotic syndromes (Lefebvre et al., 2006). However, the execution is not as easy as it sounds. Researchers reported that the heavy industrial wastewaters were well characterized by their high alkalinity, resulting in a pH value of ~8.0 due to these heavy chemicals used in the technological processes. They have also recorded that the total dissolved solids (TDS) concentrations of the industrial wastewaters are up to the elevated level of 37.0 g L^{-1}. In contrast, the suspended solid concentrations were measured as 5.3 g L^{-1} (Leta et al., 2004). Hence, pragmatically comprehend and perform these experiments are major critical tasks by the researchers for evaluations and commercialization aspects (Kongjao et al., 2008).

Economic Feasibility of Using Wastewater for Microalgal Cultivation

Nowadays, mass- or large-scale cultivations are essentially needed for the algal biofuel industries, and still, several tailbacks are limiting the establishment of commercial level algal bio-oil plants (Grobbelaar, 2012). Microalgal biofuel has gained a tremendous impetus as an alternative to the conventional fossil fuels but the economic feasibility is still a big hindrance for its commercial acceptability throughout the world. One of the several innate challenges is its high cultivation cost. The most acclaimed strategies for the large-scale microalgae productions are the algal growth in raceway ponds that are so termed because of their raceway like shape (Prussi et al., 2014). However, several researchers have addressed that the biggest bottleneck for the microalgal cultivations in the open raceway ponds is the high costs of the chemical-grade growth medium (Kumar et al., 2015; Bhattacharya et al., 2016; Karthikeyan et al., 2016). Nowadays, the microalgal cultivation utilizing the wastewaters in raceway ponds seems to be the preeminent solution in this regard. Wastewater is a cost-effective solution and have already proved as an alternative to the cost incurring algal growth medium (Wu et al., 2014). Since the last decade, microalgae cultivations using various mixotrophic techniques have been practiced and an increased interest in implementing them as part of wastewater treatment coupled with low-cost biofuel generation using the algal slurry. The algae have the potential to utilize the inorganic and organic carbon sources in wastewater bodies vis-à-vis can utilize the inorganic nitrogen and phosphorous particles in wastewaters. From the above discussion, it is clear that various studies have reported that the microalgae can bioremediate more than 90% of the initial nitrogen (ammonia, nitrate, nitrite) and phosphorous (phosphate) from wastewaters. The cost of the produced bio-oil can also reduce substantially using the wastewater where algae can able to thrive even it is highly polluted with nutients and other particles (Mohsenpour et al., 2021).

Moreover, if we look from the industrial and commercial point of view, nowadays it is undoubtedly the most indispensable to

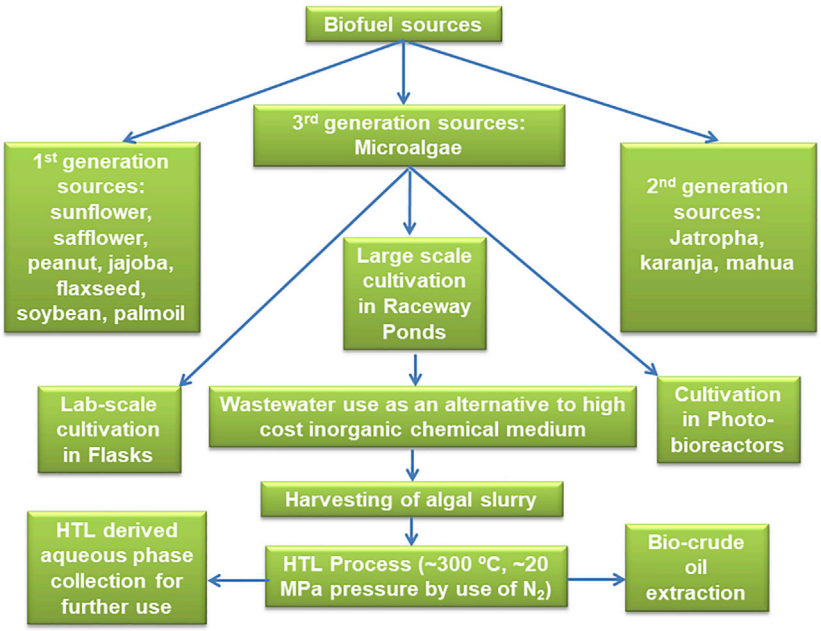

FIGURE 1 | Schematic illustration of microalgal biodiesel production techniques focuses on large-scale cultivation strategies in raceway ponds and direct biomass processing to oil using hydrothermal liquefaction.

follow some more efficient techniques by which the wastewater grown wet algal biomass can directly be converted into bio-crude oil without adopting or involving the numbers of cost-intensive and time-consuming processes essential for biodiesel production such as dewatering, drying, lipid extraction with solvents, followed by the transesterifications. On a serious note, it can be commented that those lengthy conventional techniques are the real stumbling blocks for biodiesel production in the commercial scales. It is also a clear fact that microalgal biodiesel production is practically an energy and cost-intensive approach due to these lavish and time-consuming harvesting, drying, and solvent-mediated lipid production techniques (Mathimani and Mallick, 2018) (**Figure 1**). These two steps harvesting and drying incur a substantial economic bottleneck, for higher energy consumption and lengthy time duration. Scientists have strongly suggested to use alternative thermochemical techniques to produce the bio-crude oil directly from the wet microalgal slurries using thermochemical conversion process (Aliyu et al., 2021).

APPROACHES TOWARDS THE EFFECTIVE HARNESSING OF BIO-CRUDE OIL USING HYDROTHERMAL LIQUEFACTION TECHNIQUE

Prominence of Hydrothermal Liquefaction Process

Since the last two decades, various methods were practiced to efficiently cultivate microalgae under large scale biomass generations and numerous implementations and/or modifications of several techniques associated with the different down-streaming processes like harvesting, drying, lipid extractions, transesterifications, and biodiesel production. However, there are minimal new approaches to make the algal biofuels commercially viable and ready to market available with a quick processing and cost-effective manner. Because of these facts, a recent trend has been followed by utilizing various thermochemical conversion techniques that are comparatively economically worthwhile (Chen et al., 2015). Thermochemical conversions of algal biomass to biofuel in the form of liquid or gas is generally useful with the applications of various techniques such as pyrolysis, direct combustion, torrefaction, gasification, and liquefaction by the involvement of different catalysts with the elevated temperatures (Kumar et al., 2017; Kumar et al., 2018).

The most common process for the thermochemical conversion is pyrolysis, which is involved in the thermochemical decompositions of organic matters into energetically useful and condensed liquids, solid residues, and a mixture of gases in the absence of oxygen and the absence or presence of catalysts (Morgan et al., 2017). However, the liquefaction technique is possibly the best suited for the direct conversion of algal biomass to bio-oil. It offers some energetic gains than the other alternative methods, such as pyrolysis, by using wet algal biomass and relatively effectual products' separations. Remarkably, the bio-crude properties obtained through all of these thermochemical conversion techniques are essentially dependent on the algal biomass feedstock's quality in carbon and hydrogen and must be low in nitrogen, sulfur, oxygen, and ash contents (Cole et al., 2016).

HTL is the technique to convert wet algal feedstocks (~90% moisture content wet basis) directly into bio-crude oil at the

elevated temperature and pressure of ~200–600°C and 10–25 MPa, respectively in the presence/absence of some catalyst with a typical processing time of 10–100 min, depending on the technological efficiencies with their practical implementations (Biller and Ross, 2011; Zhou et al., 2013; Couto et al., 2018). It is indeed an interesting fact that the bio-crude oil extracted with the HTL applications is generally higher than the overall lipid content of the microalgae because the proteins and carbohydrates of the algae may also be converted into oil under the elevated pressure and extreme higher temperature required for HTL technique (Cheng et al., 2018). Therefore, a wide range of algae biomass can be converted into crude oils, and it may be commented that the HTL technology is best suited for the outdoor, raceway ponds' cultivated microalgal biomass processing just after the harvesting. Furthermore, HTL also resolves the issues of energy balance for biofuel production process as water along with the catalysts serves as the reaction medium for this technology (Zhou et al., 2013) evades the requirements of the drying processes which alone generally needs 30% of the total production costs of biomass to biodiesel (Becker, 1994). The most advantageous part of the HTL process is that the aqueous wastewater which is self-seperated and is generated after obtaining the bio-crude oil, can be collected to reuse as the growth medium for the microalgal cultivations (Peterson et al., 2008; Cheng et al., 2017). Nonetheless, it was quite frequently observed that the microalgal lipids might be hydrolyzed and converted into the free fatty acids at a temperature below 250°C, required for the proper operation of HTL process. Further increase in the reaction times and temperatures, the algal cell walls generally break, and the carbohydrates and proteins may undergo deamination, decarboxylation, or re-polymerization (Neveux et al., 2014).

The foremost requirement in the HTL technique is that the microalgal feedstock biomass should not contain a high level of nitrogen, which is not recommended to produce the qualitative bio-crude oil. The elevated nitrogen content in the biomass is not technically feasible for purifying the bio-crude oil using catalysts (Mehrabadi et al., 2015). The bio-crude oil, rich in nitrogen, oxygen, or sulfur, may necessitate ample up-grading with hydrogen before starting a normal purifying process; thereby, the cost-effectiveness and energy inputs for the bio-oil production process will be much higher. However, it is a fact that the microalgal biomass usually contains 3–6% of internal nitrogen, which is stored for use in future times of cellular proliferation. Therefore, it is essential to modulate and lowering the internal nitrogen content of algal cells at the ending of the cultivation periods by the proper management and manipulations of the culture conditions in open raceway ponds before harvesting the wet biomass directly for the bio-crude oil production using HTL process (Duan and Savage, 2011). This major problem was successfully resolved by a recent technique demonstrated by the research work of Cole et al. (2016) in which some suitable organic non-polar solvents were mixed with the algal biomass slurry just before the operational separation of HTL process.

In hydrothermal conversion technique, the biomass of the algae is changed by the extreme hot and compressed water into comparatively shorter carbon chains that have a higher saturation, and thereby the energy values are also relatively higher (Brennan and Owende, 2010). The process's main product contains the heavy bio-crude oil comprised of C16–C18 hydrocarbons, and the crude oil yield from this process is approximately 30–50% of the dry weight (dw) with a heating value in the range of 30–40 kJ/g. The major by-products from this HTL process are the gaseous mixtures, which contain carbon di-oxide, hydrogen, methane, nitrogen, ethane, and acetylene with some residual solids less than 10%wt, and an aqueous phase with 20–30 (wt%) yield (Mehrabadi et al., 2015). The main advantage of considering the HTL technique for large-scale cost-effective biofuel production from algae is that the aqueous phase obtained in the HTL process generally contains a higher amount of essential and major nutrients. This could be recycled again for the microalgal cultivations by taking the aqueous phase mixtures as wastewater sample.

Analysis of Various Studies of Catalytic and Non-Catalytic Hydrothermal Liquefaction Reactions

HTL of the wet algal biomass, generated from the large-scale raceway pond cultivations of microalgae by utilizing the industrial or domestic wastewaters are seemed to be the best promising approach towards the cost-effective production of the renewable and sustainable biofuel, replacements to the conventional fossil fuels. In concern of the large-scale exploration of the wet microalgal biomass processing, HTL is one of the most superior technologies for converting biomass to bio-oil, bypassing the energy and cost-intensive processes like dewatering, drying, and solvent-mediated lipid extractions (**Figure 2**). Several recent works are done for crude bio-oil production from microalgae using different catalysts or without any catalysts under hydrothermal explorations. In 2011, Biller and Ross utilized the HTL technique for the two chlorophycean microalga *Chlorella vulgaris* and *Nannochloropsis occulata* at a temperature and holding time of 350°C and 60 min, respectively. In another study, the bio-crude oil was produced from the alga *Nannochloropsis* sp., with the reactions in water at a temperature of 350°C and holding time of 1 h and in the addition of various heterogeneous catalysts such as Pd/C, Pt/C, Ru/C, Ni/SiO2-Al2O3, CoMo/γ–Al2O3, and Zeolite. The maximum bio-oil production and the heating value were depicted as 57.0 (wt%) and 38.0 MJ kg^{-1}, respectively (Duan and Savage, 2011). The bio-crude oil yield reported in this research process is incredibly higher than many other research works on bio-oil yield by using the HTL technique, published to date. Contrary to using the high-cost catalysts for reactions in HTL technique, researchers proved that the overall oil yield was achieved up to ~45 (wt%) without any catalysts that can act as stimuli. The mixed microalgal consortium was heated at 350°C for 60 min under HTL reactions without any catalyst addition, and the maximum bio-crude oil yield and heating value (% of energy recovery) were shown as 44.5 (wt%) and 39.0 MJ kg^{-1} (Roberts et al., 2013). The bio-oil yield primarily depends on the HTL process temperature, catalyst used, the solvent used, reaction time. Researchers have found exciting findings that the carbohydrates in the algal biomass can rearrange to aromatic

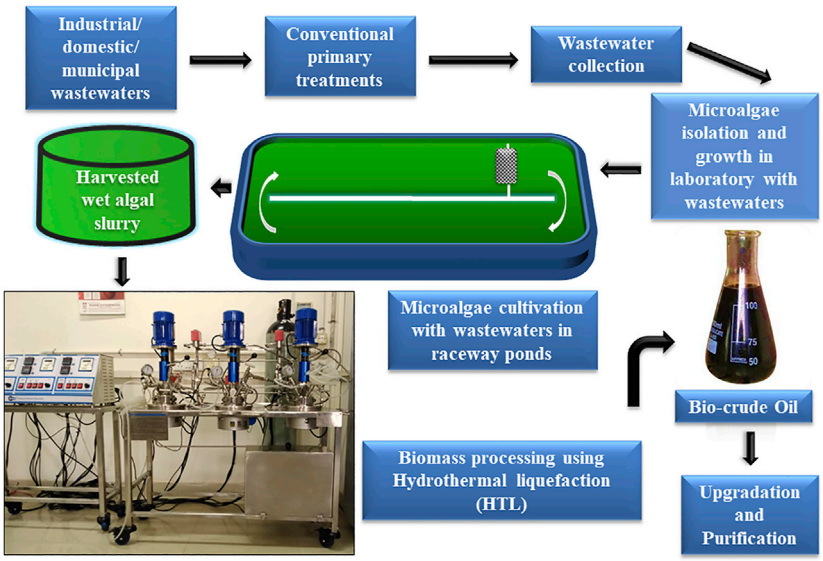

FIGURE 2 | Diagrammatic representation of microalgal cultivation in large-scale raceway ponds with wastewaters coupled with bio-crude oil production using wet algal biomass hydrothermal processing.

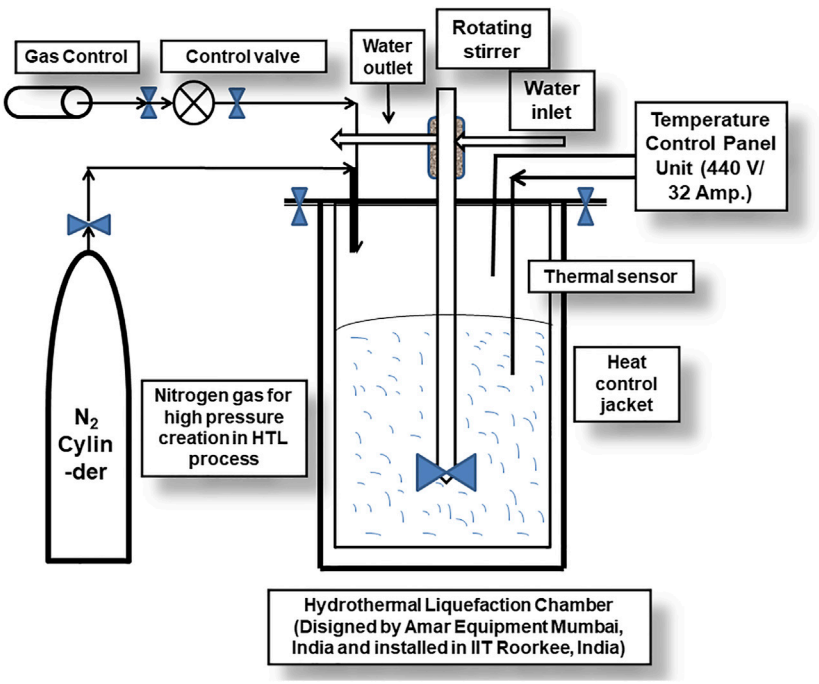

FIGURE 3 | Schematic diagram of a hydrothermal equipment (batch reactor designed by Amar Equipments Mumbai and installed in IIT Roorkee) highlighting its different parts/componants.

compounds, and polymers are converted to monomer units. In contrast, the proteins components are restored to pyrrole, and some other amide compounds during the time course of HTL reaction (Raheem et al., 2018). **Figure 3** illustrates a schematic representation of typical HTL reactor installed in IIT Roorkee.

Some synergistic approaches of the large-scale raceway pond cultivation coupled with direct bio-oil production from the wet algal biomass was successfully performed for the unbranched and filamentous, chlorophycean microalga *Oedogonium* sp. under the large-scale cultivation in a

TABLE 3 | Reviews of various recent experimental reports on catalytic/non-catalytic hydrothermal liquefaction process for bio-crude oil production.

Microalgal species	The temperature of HTL process (°C)	Holding time (min)	Catalyst used	Bio-crude yield (wt%)	Maximum heating value (MJ kg^{-1})	References
Chlorella vulgaris	350	60	No catalyst	38.0	54.2	Biller and Ross (2011)
			1 M Na_2CO_3	28.0	44.2	
			1 M HCOOH	28.0	31.7	
Nannochloropsis occulata			No catalyst	36.0	66.1	
			1 M Na_2CO_3	26.0	50.0	
			1 M HCOOH	28.0	41.1	
Nannochloropsis sp.	350	60	Pd/C; Pt/C; Ru/C; Ni/SiO_2-Al_2O_3; CoMo/γ-Al_2O_3/Zeolite	57.0	38.0	Duan and Savage (2011)
Mixed microalgal consortium	350	60	No catalyst	44.5	39.0	Roberts et al. (2013)
Nannochloropsis oceanica	300	30	No catalyst	40.1	36.3	Cheng et al. (2014)
Chlorella pyrenoidosa	300	60 13 MPa pressure	NaY, USY, HY	64–68	Not reported	Yang et al. (2016)
Oedogonium sp.	350	30	Ni_2P/SiO_2	22–23	22.0	Cole et al. (2016)
Chlorella sp.	300	30	9 MPa, 10% biomass loading, no catalyst	32.5	~34	Reddy et al. (2016)
Galdieria sulphuraria CCMEE 5587.1	350	60	Not specified	30.8	Not reported	Cheng et al. (2017)
Nannochloropsis salina CCMP 1776	310			59.1		
Aurantiochytrium sp. KRS101	400	10	Not specified	51.2	~33.0	Vo et al. (2016)
Cyanidioschyzon merolae	300	30	12 MPa, 10% biomass loading, Catalyst 0.5 M KOH/NaOH	22.7	33.7	Muppaneni et al. (2017)
Microalgal consortium	300	15	No catalyst	44.4	38.1	Couto et al. (2018)
Scenedesmus obliquus	300	60	No catalyst	35.7	35–40	Koley et al. (2018)
			CH_3COOH	45.1		
Chlorella sp.	~350	60	CuO/Al-SBA-15	65.7	Not reported	Jing et al. (2018)
Nannochloropsis sp.	450	60	Ni-Ru/CeO_2 + H_2	57.1	~40	Xu et al. (2018)
Scenedesmus quadricauda	300	30	No catalyst	18.0	Not reported	Kiran Kumar et al. (2018)
Chlorella pyrenoidosa	150–300	Each temp. resting at 10 min	2.73 g of Deionized water	33.3	34.5	Obeid et al. (2019)
Mixed algal culture	280	60	Not specified	26	~35	Carpio et al. (2021)

recirculating aquaculture system consisting of six 10,000 L parabolic raceway ponds with the dimensions of 7.1 m length, 2.2 m width, and 71.0 cm in depth. The wet algal biomass was taken for direct HTL technique at the temperature and pressure of 350°C and 180 bar, respectively. The catalyst was chosen as Ni2P/SiO2, and the maximum crude oil yield was found to be 22–23 (wt%), whereas the heating value (% energy recovery) was 22.0 MJ kg^{-1} (Cole et al., 2016). Though it seems that the bio-crude oil yield was relatively low in this process, the most significant part of the research work was the overall time requirement, i.e., only 3 min, which is relevant in cost-effective bio-oil production (**Table 3**). In accordance, one recent work has demonstrated that the maximum bio-oil yield was 44.4 (wt%) and the heating value (% of energy recovery) was 38.1 MJ kg^{-1}, respectively, at a reaction temperature of 300°C without the addition of any catalysts in the conversion for the hydrothermal process. This study report has also depicted that the overall conversion time for the HTL process was found as 15 min only (Couto et al., 2018). The research work performed by Koley et al. (2018) have shown that the biomass of the green microalga *Scenedesmus obliquus* was hydrothermally processed to obtain the bio-oil varying the temperature and pressure ranges. However, the best suitable temperature was found to be 300°C and pressure of 200 bars with a conversion time of 1 h. The study revealed that the harvested microalgal biomass contained high oxygen and carbon presence of 36.1 and 48.1%, respectively. The crude oil content was enhanced as 45.1 (wt%) with the addition of catalyst as CH_3COOH compared to bio-oil content of only 35.7 (wt%) for no addition of any catalysts. The bio-crude oil content was gained up to the maximum value of 65.7 (wt%), which is significantly higher under the HTL process application for microalgal bio-oil production, reported in other studies to date. In that conversion technique, the most suitable reaction temperature and processing time were recorded as 350°C and 1 h, respectively, with the catalyst as CuO/Al-SBS-15, utilized to fasten the conversion process the HTL chamber (Jing et al., 2018) (**Table 3**). In continuation, Xu et al. (2018) has also experimented with the HTL technique for the wet algal slurry using various catalysts at a temperature of 350°C for 20 min. In that course of studies, various combinations of catalysts were tested for the efficient bio-crude oil yield viz., no catalysts (none), 10% Ni 0.1Ru/CeO2, and 10% Ni/CeO2. The HTL process was

further upgraded to a temperature of 450°C for 1 h with a maximum HTL pressure of 225 bars, and the bio-crude oil yield was found to be 57.14 (wt%) with the addition of Ni-Ru/CeO2 + H2 catalysts. The maximum heating value/% energy recovery has also recorded a value of about 40 MJ kg^{-1}. However, there are also reports available for the lower bio-crude oil production using this HTL process. It was optimized that the maximum crude oil production was only 18.0 (wt%) under the reaction temperature of ~300°C operated at a very low pressure of 60 bars (6 MPa) with no use of catalysts. Interestingly, the microalga *S. quadricauda* was cultivated in the outdoor large-scale open raceway ponds with some other species of *B. braunii* and *C. vulgaris*. The GC-MS analysis of the produced bio-oil confirmed the presence of various organic and fatty acid esters, some nitrogenous and oxygenous compounds, alkanes, and hydrocarbons (Kiran Kumar et al., 2018) (**Table 3**). In a very recent study, it was found that the bio-oil yield was 26.0 (wt%) utilizing HTL technique for demineralized wastewater algal biomass. The wastewater was collected from an wastewater treatment plant in a swine farm at the University of Illinois Urbana-Champaign. The optimized reaction temperature was recorded as 280°C for 1 h reaction time. Moreover, the GC-MS study revealed that the bio-oil was rich in hydrocarbons and found comparable with the fuel properties of various international levels (Carpio et al., 2021).

MAJOR ADVANTAGES AND DRAWBACKS OF HYDROTHERMAL LIQUEFACTION PROCESS

HTL process has certain disadvantages despite being the most effective, suitable, and least time-consuming thermochemical conversion process for the large-scale, raceway pond grown with the wastewater mediated cultivation of microalgal slurries be converted directly into bio-oil for ready to market purpose servings. Compared with the conventional biodiesel manufacturing technologies available, the major advantages of the HTL process are found to be a lot. This HTL technique has eliminated the essentiality of considering only elevated lipid yielding microalgae followed by cell disruption, dewatering, drying, and solvent recovery for lipid extraction process (Cheng et al., 2017). The most exciting part of the use of HTL technique is that the large-scale biomass can be processed and converted into the bio-crude oil in a concise time period of only 30–60 min only. This is very beneficial from the industrial point of view to make the algal oil commercially viable. But the major cons of HTL are the necessity of high energy inputs (~300–500°C) with the elevated input pressure (~15–20 MPa). As the products after the HTL process have constituted a very high content of nitrogen and oxygen, the product seems to be quite unstable. There are some further upgrading of the HTL products therefore decidedly essential. The upgradation involves converting oxygen to CO_2 and nitrogen to ammonia (Faeth et al., 2013; Mehrabadi et al., 2015).

Since its inception, algal biomass is utilized for biodiesel production from lipids using transesterification. However, researchers also tried to harness the biodiesel from lipids during its first phase. It simultaneously generated the bio-crude oil from the residual biomass using HTL technique during its latter stage utilizing the carbohydrates, remaining lipids, and proteins from the same microalgal biomass. The competency of using the defatted biomass after lipid harnessing has a significant impact on the total energy equilibrium for algal biomass to the biofuel production process (Xu et al., 2011). In order to exploit the biomass residues remaining after the lipid harnessing, bio-crude oil can efficiently be generated from the defatted algal biomass residues including carbohydrates and proteins. However, minimal approaches are made in this direction (Cheng et al., 2014), which are actively produced biodiesel in its former stage and co-generated the bio-crude oil in its later stage using HTL technique. Moreover, it is also interesting that a part of the energy recovery of HTL process may also be possible by using the outflow of the HTL chamber to heat the inflow with a convincing positive net energy balance. The aqueous phase that contains the ammonia and specific nutrients can be recycled and may be used as an agricultural fertilizer like the biochar.

The biggest stumbling block for the HTL technology is that it still not proceeding on a commercial scale for cost-effective production of bio-crude oil, so the advantages somewhat seem to be more theoretical (Yang et al., 2014; Smith and Ross, 2016). It is also noticed that the bio-crude oil is composed of various fatty acids, amides, and aliphatic molecules, whereas; a part of the bio-oil contains more nitrogen and oxygen heteroatom aromatic components. For large-scale biomass processing in HTL, these heteroatom nitrogen and high molecular weight containing large molecules in the bio-crude oil are the major concerns for upgrading bio-crude oil (Elliott et al., 2015). These high N-containing and high molecular weight components might be generated from the elevated protein and carbohydrate comprising microalgal biomass to bio-oil conversion process. Nevertheless, researchers have recommended that the pre-treatment processes must remove the carbohydrates for HTL technique rather than the removing of protein components. This is essential because the carbohydrate components can produce some highly aromatic heterocyclic compounds that are very difficult to upgrade in the whole HTL process (Cheng et al., 2017). The above discussions regarding the pros and cons of HTL process evoks that there is a need of design commercial scale hydrothermal equipment which can process a large-scale microalgal biomass in a very short period to bio-oil. Recently, Johannsen et al. (2021) have nicely designed a large-scale HTL batch reactor for processing of biomass. The core part of the HTL plant is equipped with 58 type-K thermocouples. 32 of these are located in the trim heater measuring the temperature of the individual heat clamps. The rest are located along the 147 m pipe system, approximately 6 m apart, ensuring a detailed overview of the temperature profile. The main unit is also outfitted with a trim heater, reactor, cooler, thermocouples, and heat exchangers. This large-scale HTL plant made up with the polycarbonate coffer and inner protective steel and it has the active suction from all areas (Johannsen et al., 2021). This kind of experimentations are highly

needed for pilot plant set up with HTL technique with which the large-scale algal slurry can be processed to bio-oil in a timely manner.

UTILIZATION OF HYDROTHERMAL LIQUEFACTION AQUEOUS PHASE AND USE OF BIOCHAR

Hydrothermal processing is gaining immense importance for biomass processing, starting from lignocellulose feedstocks to the tiny algae for crude oil production (Gai et al., 2014; Guo et al., 2015; Zheng et al., 2017). However, the HTL process applications for the wet algal biomass grown under the wastewater mediated large-scale microalgae cultivation have not yet been fully explored to date. It is also interesting that the hydrothermal aqueous phase can be reutilized as a wastewater source for the outdoor cultivation of microalgae. This aqueous phase which is derived as a huge quantity at the end of the HTL process, is generated due to the elevated moisture content (~90–95% wet basis) of the wet algal biomass (Lee and Chen, 2016; Leng et al., 2018). The aqueous phase is generally comprised of high levels of organic carbon and nitrogen compounds as well as various toxic components viz. some heavy metals and oxygen or nitrogen heterocyclic counterparts (ring structure cyclic compound) like pyrrole and Pyrrolidine. However, a rare study report is for the disposal of this huge quantity of aqueous phase for the re-utilization of it coupling with various wastewaters as the additional growth-promoting nutrient-rich medium for microalgae (Jena et al., 2011; Hognon et al., 2015).

On the other hand, there is an extreme prerequisite of huge quantities of the wastewaters rich in nitrogen and phosphorous for the large-scale commercial level cultivation of microalgae. Nevertheless, only utilizing the aqueous phase derived through the HTL process for the mass cultivation of microalgae may not be able to meet the complete requirement in this case. But the synergistic approach to utilize the aqueous phase of HTL conversion process with the industrial and/or domestic wastewaters as the growth medium for the mass cultivation of microalgae could be the best possible approach towards the cost-effective renewable biofuel production in a commercial scale. There are many reports are available for the reutilization of this aqueous phase for the evaluation of microalgal cultivations (Pham et al., 2013). One study report has already demonstrated the successful outdoor cultivation of microalga *Chlamydomonas reinhardtii* with the hydrothermal mediated aqueous phase wastewater (Becker et al., 2014). However, several researchers performing different studies have confirmed that the aqueous phase generated after the HTL reactions generally was contained various nitrogen, high phosphate ions that are quite beneficial for the algal growth but the existence of the heavy metal ions, phenolic and some furans compounds such as toluene, 2-Methylbenzofuran, and various toxic nitrogenous compounds such as amino-phenol, pyridine, piperidinone were also noticed (Huang and Yuan, 2016; Toufiq Reza et al., 2016). These certain chemical compounds are also inhibitory for the microalgal growth; hence it has a limitation and the proper research should be carried out in this area in the near future (Gollakota et al., 2018). While discussing the recent study reports on HTL process used for algal bio-crude production, it can also be suggested that the processed bio-crude may be used like petro-crude in the petroleum refineries. However, this could be done only after its proper denitrogenation and deoxygenation. It may also be commented that the HTL derived aqueous phase re-utilization with cost-effective hydrothermal processing of algal biomass to biofuel is a very recent and limited approach and thereby, challenges still exist in this field. This can be overcome with future research studies by the technocrats and scientists' efficient combined and mutual works in this direction. A recent study have focused to use the pulse electric field as the pretreatment of microalgae for the HTL process. This pretreatment method can reduce the final nitrogen content in the biocrude. In another way the steam catalytic cracking technique may reduce the oxygenate level in the bio-crude oil and can enhance the hydrocarbon content (Aliyu et al., 2021).

Biochar, one of the most important co-products of algal biorefinery approach are obtained from the hydrothermally processed algal slurry. One recently published article has focused on the co-carbonization of algae with some different feedstocks to generate nitrogen-doped highly microporous biochar specifically named as hydrochar (Aliyu et al., 2021). With these techniques the last product of HTL after the aqueous phase extraction, i.e. biochar will be safe for agricultural fields with a proper balance of nitrogen, carbon and oxygen content.

CONCLUSIONS AND FUTURE PERSPECTIVES

Biomass-based energy is the main form of renewable energy today. As per the International Energy Agency and many other national and international organizations, if commitments for global climate-change are to be met with, then, bioenergy has tremendous potential to effectively provide a solution for a low carbon global energy system in the future, specially through decarbonisation of aviation, shipping and road transport sectors. However, currently exploration and exploitation of biomass-based resources for bioenergy production is tragically much below the required quantity needed to be deployed. Under such circumstances, ramping up of sustainable biofuel generation through accelerated usage of renewable resources is pertinent, particularly in the transportation sector where fuel consumption is estimated to triple by 2030. But biofuel is a difficult and slightly complicated topic, specially when it comes to addressing the sustainability index in the low-carbon global society in the future. This review article therefore re-examines the current state of biofuel research worldwide and suggests solutions to overcome the challenges related to lower biomass and oil yield, longer biomass treatment procedures and multi-step oil extraction methods during algal biofuel production. It also provides a workable roadmap through utilisation of biomass-based renewable resources, more specifically the green chlorophycean microalgae grown in industrial untreated wastewater for a one-step bio-oil production

using HTL technique. Additionally, it also addresses the problem of water scarcity through re-usage of the processed water after cultivation and/or HTL. Furthermore, such a strategy suggesting the combination of three factors, namely, microalgal cultivation, wastewater bioremediation and HTL technique is not available/very scantily available to the extent of our knowledge. This review article is therefore expected to be extremely beneficial to the readers for cost-effective, environmental friendly algal fuel production on a larger scale for commercial application.

DECLARATION OF AUTHOR AGREEMENT

All the authors listed have approved the manuscript and agreed to authorship and submission of the manuscript for peer review.

AUTHOR CONTRIBUTIONS

SB: Conceptualization, Writing - Original Draft RPa: Review and Editing, Language improvement RPr: Visualization, Supervision, Manuscript Correction.

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