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Growth engineering of *Synechococcus elongatus* PCC 7942 for mixotrophy under natural light conditions for improved feedstock production

Aditya Sarnaik [a], Reena Pandit [a*], Arvind Lali [a,b]

[a]*DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai-400019, Maharashtra, India.*

[b]*Department of Chemical Engineering, Institute of Chemical Technology, Mumbai- 400019, Maharashtra, India.*

E-mail: drreenapandit@gmail.com Contact Number: +91-9821211769

Abstract

Synechococcus elongatus PCC 7942 has been widely explored as cyanobacterial cell factory through genetic modifications for production of various value-added compounds. However, successful industrial scale-ups have not been reported for the system predominantly due to its obligate photoautotrophic metabolism and use of artificial light in photobioreactors. Hence, engineering the organism to perform mixotrophy under natural light could serve as an effective solution. Thus, we applied a genetically engineered strain of *Synechococcus elongatus* PCC 7942 expressing heterologous hexose transporter gene (*galP*) to perform mixotrophy under natural light in a temperature controlled environmental chamber (EC). We systematically studied the comparative performances of these transformants using autotrophy and mixotrophy, which showed 3.4 times increase in biomass productivity of mixotrophically grown transformants over autotrophs in EC. Chlorophyll-a yield was found to have decreased in mixotrophic conditions, possibly indicating reduced dependency on light for energy metabolism. Although pigment yield decreases under mixotrophy, titer was found to have improved due to increased biomass

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productivity. Carotenoid analysis showed that zeaxanthin is the major carotenoid produced by the species which is essential for photoprotection. Our work thus demonstrates that mixotrophy under temperature controlled natural light can serve as the viable solution to improve biomass productivity of *Synechococcus elongatus* PCC 7942 and for commercial production of natural or engineered value added compounds from the system.

Keywords: *galP;* mixotrophy*;* natural diurnal light; *Synechococcus*; zeaxanthin

Abbreviations: **Auto**, Autotrophy; **EC**, Environmental chamber/ laboratory (State of the art glass house facility at DBT-ICT Centre for Energy Biosciences); **IC**, Incubator; **Tr**, *Synechococcus* transformant; **WT**, Wild Type

1. Introduction

Currently, intensive global research has been aimed towards genetic engineering of micro-organisms for production of hydrocarbons and value-added compounds where photosynthetic cyanobacteria are attracting considerable interest as platform cell factories.[3, 4, 6, 8, 32, 33, 41, 42] The most explored species amongst these are *Synechococcus elongatus* PCC 7942*,* *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7002, mainly due to availability of their genome databases and established genomic modification tools.[54] They have been explored extensively for their potential as cell factories to produce industrially important compounds.[21] However, their slower growth rate compared to other well characterized bacterial cell factories like *E. coli* poses one of the major obstacles to achieve the desired titer of the engineered compounds. The underlying cause of this could be their strict autotrophic mode of growth that encounters three important limitations; the inability of their photosystems to harvest

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majority of the incoming light energy, the limited generation of ATP and NADPH through photosystems, and the poor efficiency of RuBisCo to fix CO_2 .[54]

As cyanobacterial growth rate is driven by light energy, with increasing light intensity, rate of photosynthesis improves, enhancing biomass substantially.[31] However, at higher light intensities that exceed photosynthetic capacity of cyanobacteria, excess photo-excitation may occur, ultimately causing photo-inhibition and photo-toxicity.[27] Cyanobacteria combat this through various means such as reducing antenna size, scattering of excess irradiation through non-photochemical quenching, state transitions, and increasing the rate of synthesis of antioxidants like carotenoids.[18, 22, 35, 44, 48] Autotrophy under natural light conditions may be a compromise between increasing the biomass productivity and cell maintenance for protecting against photo-oxidative damage. To overcome this, researchers have tried to alter cyanobacterial antenna proteins (PBS) through genetic mutations to reduce photoinhibition and improve biomass productivity.[7, 17, 19, 27, 30, 35] Although the laboratory scale experimentations have been successful in demonstrating the efficiency of these mutants, their performance is not consistent during scale up.[36,37] Moreover, none of them could effectively exhibit the natural sunlight sustenance of these mutants.

Sunlight is a large source of renewable light energy that drives an energy metabolism of photosynthetic organisms like cyanobacteria. Hence, studying its effect on cyanobacteria would help in optimizing growth parameters during scale-up. However, as sunlight intensity shows diurnalas well as seasonal variations, autotrophic sustenance and performance can be challenging for the cells. Under light stress and high intensity conditions, rate of generation of reactive oxygen species may exceed beyond the quenching capacity of cellular antioxidants, thereby causing oxidative damage to the cellular components, especially targeting synthesis of D1

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protein; ultimately causing irreparable damage toPS II activity during photosynthesis.[14, 59] Thus, exercising mixotrophy under natural light and controlled temperature conditions would serve as an effective alternative to increase biomass productivity and improve titers of the products from them.

Synechococcus elongatus PCC 7942 (hereafter referred as *Synechococcus*) has been widely exploited as a cyanobacterial factory through genetic modification for production of fatty acids, alcohol, antioxidants, isobutyraldehyde, etc.[4, 32, 33, 41] Modifying these cells as demonstrated by McEwen *et al* to uptake externally supplied glucose by expressing glucose transporter protein and to perform mixotrophy is one of the promising ways to improve their biomass and so the product titers. Hence, we integrated hexose/H^+ symporter (*galP*) into the *Synechococcus* genome and performed comparative analysis of the transformants (Tr) with the wild type (WT) strains under different light conditions using autotrophy and mixotrophy.[26] The strategy can be efficiently used for industrial production of various value added products from *Synechococcus*.[25] This investigation focuses on vital aspects of mixotrophic cultivation of *Synechococcus* under natural light conditions and evaluation of their pigment profiles.

2. Materials and methods

2.1. Culture conditions

E.coli Top10F cells (*ThermoFisher Scientific*) were grown at 37 ^{0}C in Luria Bertani (LB) broth (*HiMedia*) and LB supplemented with 100µg/ml spectinomycin in case of transformants.

The unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 was grown in BG-11 medium under incubator as well as environmental chamber conditions, using atmospheric CO_2. In incubator (IC), artificial cool white light with an intensity of 55 ± 0.5µmol/m^2/s was

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used for continuous illumination and the temperature was maintained at 28 ± 1^0C. Light and temperature values were manually recorded every 2 hrs. (Light and temperature conditions in IC for all the experiments are plotted in Supplementary Information, *SI: 1B*). Environmental chamber (EC) is a state of the art facility, set at DBT-ICT Centre for Energy Biosciences, Mumbai, India. The facility is a glass house which facilitatessunlight penetration and thus allow to undertake experiment under diurnal and seasonal variations of natural light (sunlight). The temperature in EC was maintained at 28 ± 2 ^{0}C using air conditioners and ventilators. Light and temperature values were manually recorded in every 2 hours. Cultures grown in EC were exposed to natural light that could raise upto 1200 ± 100µmol/m^2/s at noon. (Light and temperature conditions in EC for all the experiments were similar and are plotted in Supplementary Information, *SI: 1A*)

For all the experiments, cultures were grown in 100ml BG-11 medium without or with (only during acclimatization and/or induction of the transformants) 30µg/ml spectinomycin on shakers at the speed of 120rpm, in 500ml Erlenmeyer flasks.[6, 26] Cell growth was monitored by measuring optical density and dry cell weight. Culture absorbance was measured at 750nm (Abs_{750}).[6] For determining dry cell weight, cells were dewatered and harvested by centrifugation at 10,000 rpm for 15 min. The biomass was washed thrice with water for desalting and dried at 60-65 ^{0}C for 18 hrs.[47]

All the cultures were examined for the contamination by plating 50µl of the culture at the regular time interval on Luria-Bertani (LB) agar plates and incubating at three different conditions, 37 ^{0}C incubator, IC and EC for 24-48 h where no growth was observed in any of the plates.

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2.2. Plasmid construction and transformation

The cloning protocol for construction of recombinant plasmid *pGalP48* has been adopted from McEwen *et al,* 2013. The *galP* gene was amplified from *E. coli* W3110 genomic DNA using the primers GalPM_F: CTAACAATTGATGCCTGACGCTAAAAAACAGGGGCG, GalPB_R: GGATAGATCTTTAATCGTGAGCGCCTATTTCG, digested with *MfeI* and *BglII* respectively, and then ligated in plasmid pAM2991 (*AddGene #40248*), digested with *EcoRI* and *BamHI.*[26]

Transformation of wild type *Synechococcus* was carried out, where a liquid culture of *Synechococcus* was grown to the Abs_{750} of 0.7. Cells were centrifuged and suspended in 10ml of 10mM NaCl solution and re-centrifuged. Pellet was then suspended in 300µl BG-11 medium with 1µg of recombinant plasmid. The tube was wrapped in aluminum foil and incubated at 28 ^{0}C overnight with gentle agitation. Transformants were directly selected on BG-11 plates supplemented with 30µg/ml of spectinomycin. Transformants were grown in liquid batch cultures supplemented with 40µg/ml of spectinomycin after four passages on antibiotic containing plates. Gene integration was confirmed using colony PCR.[6]

5'NSI_F (CGGCTTCAGCAATTCGG) primerwas used along with GalPB_R as confirmation primers to check precise gene integration at the neutral site (NS I) in *Synechococcus* genome.

2.3. Experimental design

2.3.1. Growth assay with wild type (WT)

Growth studies were performed with WT *Synechococcus* by exposing it to EC and IC conditions under autotrophy mode. The experiment was performed in biological triplicates for 6

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days. Parameters such as photosynthetic pigment yields and biomass productivity were measured to evaluate the effect of different light conditions on *Synechococcus* growth.

2.3.2. Growth assay with transformant (Tr)

The transformants were exposed to different growth conditions and were induced with 1mM IPTG. Two different aspects were assessed during these growth assays. First was mixotrophic performance of the transformants over wild type, wherein, induced Tr and WT were grown in BG-11 (autotrophic) and BG-11 supplemented with 0.5% and 1% w/v glucose (mixotrophic), and were assessed for their performances (*Fig 1*).[26] The assay was performed in biological triplicates for 6 days.

Secondly, the effect of acclimatization of Tr to different light conditions was also investigated to evaluate its effect on their mixotrophic growth. Cultures were acclimatized to IC or EC light conditions for 3 days and then exposed to different conditions in biological triplicates. One set of Tr was acclimatized in IC and was induced with 1mM IPTG during the exponential growth phase. These Tr were then grown in three different light conditions i.e. natural diurnal conditions in EC, continuous light conditions where cultures were maintained in EC for 8hrs when the light intensities are more than that in IC (9:00AM to 5:00PM) following which the cultures were transferred to IC for 16hrs (hereafter referred as 8:16) and the high-low light transition was repeated throughout the experiment to analyze the effect of different photoperiods on cell growth, and the third set was continuously maintained in IC as a 'control' (*Fig 1*,*Supplementary information SI: 1A and 1B*). Similarly, another set of Tr was acclimatized to natural diurnal light in EC and induced with 1mM IPTG during exponential growth phase. This set of EC based Tr was then exposed to two different conditions; natural diurnal condition

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in EC and continuous light condition (8:16) as IC based Tr. *Fig 1* summarizes details of different growth conditions of WT and Tr in each experiment.

2.4. Nutrient consumption studies

2.4.1. Glucose consumption

Amount of residual glucose and absolute glucose uptake rates (mmol of glucose/ g dry cell weight/ h) were measured to trace the functionality of the cloned transporter protein. 1ml culture was centrifuged at 10,000 rpm for 10 min and the supernatant was tested for residual glucose using GOD-POD (*Accurex*) kit; a substrate specific and sensitive enzymatic technique.[5,53]

2.4.2. Nitrate consumption

Nitrate estimation was performed according to standard methods for examination of water and wastewater as prescribed by American Public Health Association (APHA) manual.[2] 1ml culture was centrifuged at 10,000 rpm for 10 min, the supernatant was diluted 1:100 in deionized water and the absorbance was read at 220nm. The concentration of residual nitrate in the medium (µg/ml) was calculated using equation of the standard curve.[2]

2.5 *Pigment analysis*

2.5.1. Pigment measurement

Cyanobacterial pigment content was determined using spectrophotometric method. 1ml of the culture was centrifuged and cell pellet was extracted in 1ml absolute methanol for 1hr with

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sonication in dark. Chlorophyll a (Chl-a) and total carotenoid concentrations were determined using following equations:[39]

Chl-a (μg/ml) = 16.5169 (Abs_{665}) - 8.0962 (Abs_{652})

Total carotenoids (μg/ml) = 4 x Abs_{480}

2.5.2. Carotenoid analysis

Filtered pigment extracts were used for HPLC analysis of carotenoids with Agilent C_{18} RP column. Mobile phase A was methanol: acetonitrile: water (21:16.5:62.5) with 10mM ammonium acetate and B was Methanol: Acetonitrile: Ethyl acetate (50:20:30). The solvent B gradient program (time [min], %B, flow rate [ml min^{-1}]) employed was as follows: 0, 20, 0.750; 10, 70, 1.0; 40, 100, 1.0 and 60, 100, 1.0; taking absorbance at 480 nm.[11] Peaks were compared with the standard zeaxanthin (*Sigma-Aldrich*) and β-carotene (*Sigma-Aldrich*). Equations of the standard curves were used to calculate concentration of these two carotenoids from the methanol extracts of the cultures. (*Supplementary information, SI 2*)

2.6. Flux balance analysis

Photon uptake rates of autotrophic WT and mixotrophicTr grown with 1% glucose supplementation were determined using *i*JN678 genome-scale metabolic network model (http://bigg.ucsd.edu/models/iJN678).[31] Although the model is designed for *Synechocystis sp* PCC 6803 that can naturally uptake glucose; we have used it for *Synechococcus*, modified to uptake extracellular glucose under mixotrophy. We used *linprog* in MATLAB R2016b to solve the linear optimization problem. Here, the experimentally calculated variables i.e. growth rates, glucose and nitrate uptake rates were utilized to predict the values of CO_2 uptake rates (mmol of

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CO_2/ g dry cell weight/ h) by the cultures. These CO_2 uptake rates were then used to determine the photon uptake rates (mmol of photons/ g dry cell weight/ h) by *Synechococcus* grown under autotrophy and mixotrophy in EC conditions.

3. Results

3.1. Engineering Synechococcus for cloning galP

galP gene from *E. coli* W3110 was cloned into pAM2991 under the control of P_{trc} promoter to generate recombinant plasmid (*pGalP48*). The plasmid was successfully transformed first into *E. coli* Top10F to increase its copy number. Following cyanobacterial transformation, *Synechococcus* transformants with *pGalP48* were successfully obtained after four passages under selective antibiotic pressure. Clones generated through homologous recombination were confirmed for the gene integration using colony PCR. *Supplementary information, SI 4* shows the agarose gel picture of 5'NS I_*galP* amplicon amplified from *Synechococcus* Tr using confirmation primers showing DNA bands corresponding to 3.5 kbps from colony PCR.

3.2. Growth profiles

3.2.1. Effect of natural light on Synechococcus:

Synechococcus WT were studied for difference in their responses to different light intensities in EC and IC under autotrophy (*Supplementary information, SI: 1A and 1B*). As shown in *Fig 2A,* there was 50% decrease in Chl-a content for cells grown under EC as compared to IC with concomitant 50% increase in their biomass productivity. Different light conditions also influenced the carotenoid yield. The weight ratio of carotenoid: Chl-a (mg of

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carotenoid/g of biomass: mg of Chl-a/g of biomass) was found to have increased from 0.58 ± 0.002 in IC to 0.97 ± 0.1 in EC and it was evident through different colors of the cultures (*Fig 2B*). This signifies that EC as compared to IC favors the growth of *Synechococcus* thereby increasing the biomass productivity.

3.2.2. Mixotrophic performance of Synechococcus Tr

Synechococcus Tr were primarily evaluated for functioning of the hexose transporter by comparing their glucose uptake with those of WT. Glucose consumption profiles of *Synechococcus* Tr showed glucose uptake under mixotrophy while WT could not (*Table 1B,Fig 3C*). This indicates successful transformation and functioning of the cloned hexose transporter protein. Mixotrophy imparted positive effect on the growth of Tr where the productivity with 1% glucose supplementation was found to be 3.4 times higher than that with autotrophic Tr and WT (*Table 1A,Fig 3A and 3B*).

Further, the nitrate consumption profiles indicated that WT and Tr cultures consume nitrate at comparable rates however the absolute nitrate uptake rate (mmol of nitrate/ g dry cell weight/ h) varies inversely with glucose supplementation i.e. degree of mixotrophy (*Table 1A,Fig 3D*). Thus, nitrate uptake was found to be 31% reduced in case of Tr supplemented with 1% glucose as compared to autotrophically grown Tr (*Table 1A*).

3.3. Effect of light conditions on mixotrophy

Studying the effect of different light acclimatization and growth conditions (as depicted in *Fig 1*) was essential to optimize biomass productivity of the Tr. *Table 2* shows growth rates of all the cultures acclimatized in IC and EC. Least growth rate was observed with the 'control' cultures acclimatized and maintained under continuous low light in IC under autotrophic and

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mixotrophic conditions. *Fig 4A* shows that the cultures acclimatized in EC give higher biomass productivity over that under IC. Moreover, EC acclimatized cultures grown mixotrophically under natural diurnal conditions shows significantly higher biomass productivity than those under continuous light (8:16) where alternating periods of artificial lights have been employed.

3.4. Pigment quantitation and profiling

Chlorophyll and carotenoids have significant role in photosynthesis and photoprotection in cyanobacteria. Typically, *Synechococcus* displays two major carotenoids, zeaxanthin and β-carotene.[10] The pigment profiling studies under current investigation showed difference in carotenoid and Chl-a contents with respect to the conditions. IC acclimatized cultures showed Chl-a yield of 7.3 ± 0.2 and 6.9 ± 0.2 mg/g under autotrophy, and 6.4 ± 0. 2 and 4.8 ± 0.1mg/g with mixotrophy when cultivated under natural diurnal and continuous (8:16) light respectively. However, EC induced cultures showed Chl-a yield of 8 ± 0.1 and 8.2 ± 0.2 mg/g under autotrophy and 4.1 ± 0.2 and 5 ± 0.1 mg/g with mixotrophy when cultivated under natural diurnal and continuous (8:16) light respectively. Both results indicated the Chl-a yield (mg/g) was significantly higher under autotrophy than mixotrophy. In contrast, Chl-a titers (mg/L) have found to have reduced under autotrophy compared to those with mixotrophy. As higher biomass productivities and Chl-a yields were obtained with cultures acclimatized and induced in EC, further pigment evaluations have been restricted to EC acclimatized cultures (*Fig 4B*).

Carotenoid analysis also exhibited similar pattern where the yields were significantly high under autotrophy than mixotrophy, however the titers were higher under mixotrophy than autotrophy; owing to higher biomass productivity of mixotrophs. Carotenoid analysis of EC acclimatized cultures showed that the zeaxanthin yields were 2 ± 0.1 and 1.9 ± 0.1 mg/g under

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autotrophy while 0.8 ± 0.02 and 0.84 ± 0.04 mg/g with mixotrophs cultivated under natural diurnal and continuous light conditions respectively. On the other hand, β-carotene yields were 1.31 ± 0.02 and 1.32 ± 0.02 mg/g under autotrophy whereas 0.6 ± 0.03 and 0.75 ± 0.03 mg/g under mixotrophy cultivated under natural diurnal and continuous light conditions respectively *(Fig 4C)*.

As chlorophyll and carotenoid concentrations are indicators of cell's response to light stress, it was necessary to correlate both these parameters as their weight ratios (mg of carotenoid/ g of biomass: mg of Chl-a/ g of biomass).[9] In all EC acclimatized samples, the weight ratio of zeaxanthin: Chl-a was significantly higher in autotrophic cultures than mixotrophic cultures. However, there was no prominent difference in the weight ratios of β-carotene: Chl-a amongst any of the samples *(Fig 4D)*.

3.5 Flux balance analysis

Flux balance analysis (FBA) using *i*JN678 model was performed using average experimental growth rates, and glucose and nitrate uptake rates of autotrophically grown WT and mixotrophically grown Tr. On the basis of phenotypic phase planes, PhPP (*Supplementary information, SI 3*) and model predictions; CO_2 and photon uptake rates of autotrophic cultures were found to be 0.4 mmol of CO_2/ g dry cell weight/ h and 10 mmol of photons/ g dry cell weight/ h respectively. However, the uptake rates were found relatively less i.e. 0.3 mmol of CO_2/ g dry cell weight/ h and 5 mmol of photons/ g dry cell weight/ h respectively in case of mixotrophic cultures (*Table 3*). (*Supplementary information*, *SI 3A* and *3BPhPP*).

4. Discussion:

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Synechococcus owing to its natural competency and high transformation efficiency has been studied as an efficient cyanobacterial host system for the production of commercially valuable hydrocarbons like ethanol, isobutyraldehyde, 2,3-butanediol, fatty acids, fatty alcohols through genetic modifications.[3, 4, 6, 8, 32, 33, 41, 42] Despite such promises, industrial scale developments have not been reported for *Synechococcus*. This could be attributed to the obligate photoautotrophic mode of energy metabolism of *Synechococcus*. Strict autotrophy has several limitations like inefficient CO_2 fixation by RuBisCo and limited generation of ATP and NADPH.[54] Hence, providing the cells with additional energy source like organic carbon and reducing their sole dependency on light could serve the purpose of increasing the biomass productivity. Thus, our study was primarily focused on improving the productivity of *Synechococcus* through mixotrophy by using the cells engineered for expressing glucose transporter protein to uptake externally supplied organic sugar and use inorganic carbon i.e. CO_2, simultaneously. Both, light and organic carbon, have independent as well as interdependent influence on the cell growth and biomass productivity. Under mixotrophy, supply of organic carbon, CO_2 and light conditions drive the energy metabolism of the cell.[50, 54] Hence, it was imperative to evaluate the influence of light on Tr at every stage of experimentation including induction phase.

McEwen *et al.* has successfully transformed *Synechococcus* with *galP* gene, thus enabling cells to consume externally supplemented organic C under simulated diurnal conditions. Under current investigation we have generated these transformants to study their metabolic changes over autotrophs and also to increase their biomass productivity under natural diurnal conditions (max. 1200µmol/m^2/s).[26]

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Majority of the reports on *Synechococcus* employ artificial light to simulate natural light conditions for their growth, but as we move to industrial scale, studying the effect of actual natural light and its diurnal variations on cell growth becomes crucial. Growth of *Synechococcus* WT under sunlight was thus assessed, through its comparative growth under artificial continuous, low light. Results indicated that the biomass productivity of WT cultures grown in EC was 50% higher than that under IC. As the temperatures in EC and IC were similar (28 ^{0}C) (*SI 1*), difference in the productivities was due to the different light conditions in EC and IC (*Fig 2*). Moreover, chlorophyll tends to be photo-oxidized at high irradiance and carotenoids can prevent it efficiently through dissipation of excess energy of excited chlorophyll and the elimination of reactive oxygen species.[9, 45] Consequently, relationship between carotenoids and Chl-a may be used as a potential indicator of photo-oxidative damage that could be caused under high irradiance like in EC.[9] Thus, we correlated yield of these carotenoids to that of Chl-a. Pigment analysis revealed that the Chl-a yield was reduced, whereas carotenoid: Chl-a ratio was increased in cultures grown in EC as compared to IC (*Fig 2B*). This could be attributed to the fact that cyanobacterial photosynthesis occurs in narrow spectrum of the visible light and the light intensity in IC is comparatively low, thus cells grown in IC display more photosynthetic reaction centers i.e. Chl-a to harvest maximum light available in the incubator. On the other hand, light intensity in EC goes beyond the photosynthetic capacity of the species where the cells have reduced Chl-a yield, thereby high amount of excitation energy might be lost under fluctuating irradiance.[21, 45] This modulation capacity has thus helped *Synechococcus* cells to acclimatize to light stress in EC; shielding the cells from harvesting excessive light photons and prevent photo-damage and photo-oxidation.[20]Thus, *Synechococcus* growth studies were then performed in EC.

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Mixotrophy was considered to further improve the biomass productivity significantly. However, it cannot be exercised in *Synechococcus* as the cells cannot natively uptake externally supplied simple sugars like glucose, xylose, etc.[26] Therefore, we sought to engineer *Synechococcus* strain that was able to utilize externally supplemented glucose. Colony PCR and glucose uptake profiles showed that successful homologous recombination and expression has been achieved (*Fig 3C, Supplementary Information SI 4*).

Growth studies performed with these Tr using mixotrophy in EC exhibited promising increase in the biomass productivity over WT. Under mixotrophy, Tr grown with 1% glucose supplementation gave highest productivity that was 3.4 times higher than autotrophically grown Tr (*Fig 3A and 3B*). Glucose uptake profile showed only Tr could effectively consume glucose while WT could not (*Fig 3C*). This signifies efficient functioning of the cloned transporter protein in *Synechococcus*. Results were also supported by the nitrate consumption profiles.

Nitrate uptake in cyanobacteria appears to be regulated by the system that integrates photosynthetic N and C assimilatory metabolism.[13] Nitrate uptake rate increases proportionally with increasing light intensity and this parallels photosynthetic activity.[15] Almost 30% of the electrons generated through photosynthetic water oxidation are utilized for nitrate reduction to ammonium, making nitrate assimilation a genuine photosynthetic process.[43] Thus, in our study *Synechococcus* nitrate uptake was also analyzed and correlated to their autotrophic and mixotrophic growth regimes.[15] As the number of photosynthetic reaction centers reduce under mixotrophy, rate of nitrate assimilation will consequently decrease. Thus, we observed that the absolute nitrate uptake rate (mmol of nitrate/ g dry cell weight/ h) was higher under autotrophy than under mixotrophy (*Fig 3D*). Glucose supplementation in the medium showed improved

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nitrate uptake by WT (*Fig 3D*). This might be attributed to different N:C ratio in the medium that could be perceived by the cells for nitrate assimilation, however it needs further investigation.

Our work further involved the study of effect of light and/or photoperiod on the growth of Tr. Despite undertaking combinations of EC/IC acclimatization conditions and diurnal/continuous (8:16) photoperiods, *Synechococcus* Tr showed higher productivities with mixotrophs acclimatized and induced in EC (*Fig 4A*). Results ascertained that culture acclimatization conditions play a significant role in natural light tolerance of the cells. Further comparison of biomass productivities under diurnal and continuous light (8:16) revealed that alternating period of low artificial light does not result in any substantial variation in the growth rate of Tr (*Table 2*). Natural diurnal conditions in EC were thus found to be the most conducive for acclimatization and cell growth using mixotrophy giving improved biomass productivity.

Results were also supported by flux balance analysis (FBA) of the cultures on the basis of experimentally evaluated data. Simulation results from FBA models showed that CO_2 and photon uptake rates of autotrophic cultures were higher than those of mixotrophs. But not all photons harvested by photosynthetic pigments are productively used for ATP and NADPH generation.[46] Excess light could be non-photochemically quenched via heat dissipation or state transition in cyanobacteria.[29, 46] However under high light stress, survival of the cell is crucially dependent on preventing over-reduction of photosystems during electron transport due to excess photo-excitation, D1 protein turnover, and efficient repair of photo-inhibited PS II.[38, 51] Hence, under natural diurnal high light conditions, cell needs to compromise between cell maintenance for survival and increasing the biomass productivity, when grown autotrophically. Also in case of cyanobacteria, respiratory electron transport pathways intersect with photosynthetic electron flow in the same thylakoid membrane, as they share common pool of plastoquinone and Cyt *b6f*

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complex.[48, 52] Thus, rate of photosynthetic electron flow could affect respiratory electron transport and vice-versa. As mixotrophic cultures have externally supplied glucose as alternative source of C and energy, and due to synergistic effect of photosynthesis and respiratory glucose oxidation, even with relatively lower CO_2 and photon uptake rates, they display higher growth rates than autotrophs under natural diurnal conditions.[58]

Photosynthetic pigment comparisons between autotrophs and mixotrophs acclimatized in EC indicated that their titers (mg/L) are inversely proportional to their yields (mg/g of biomass) (*Fig 4B and 4C*). Now, in batch cultures of *Synechococcus*, with increasing cell densities there is continual decrease in the effective light intensity, as a result of which the cells shade each other.[28,32] This effect was observed more with mixotrophic cultures than autotrophic, as mixotrophs had comparatively higher cell densities. Pigment titers were significantly higher under mixotrophy than those of autotrophic cultures, owing to their higher biomass productivities.

The pigment profiles indicated that mixotrophic cultures had lower yields of Chl-a as compared to photoautotrophic cultures (*Fig 4B*). Apart from self-shading, mixotrophic metabolism could itself be the cause of reduced yield of Chl-a. Here glucose, in addition to light, operates the energy metabolism of *Synechococcus*. Hence under mixotrophy, cells can compromise on number of photosynthetic reaction centers, as decreasing the chlorophyll content is one of the ways to relieve photoinhibition and improve productivity.[56] This supports our hypothesis that under mixotrophy photo-reliance of *Synechococcus* Tr reduces and biomass productivity increases as compared to those of autotrophy under natural light conditions. Conversely, mixotrophs showed higher titer of Chl-a which can be attributed to their higher biomass productivities. In *Synechococcus*, carotenoid biosynthesis is up-regulated in response to

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light stress.[49, 59] However due to self-shading, yield of carotenoids was lower under mixotrophy and the titers were higher attributing to improved biomass than autotrophs (*Fig 4C*).

Current work also involved profiling of the carotenoids from the EC acclimatized cultures exposed to diurnal and continuous (8:16) light conditions, grown under autotrophy and mixotrophy, as their productivities were comparatively higher than those acclimatized under IC. Essentially *Synechococcus* does not possess xanthophyll cycle which is an important photoprotecting mechanism in eukaryotic photoautotrophs.[24] Hence, the organism needs to stay solely dependent on zeaxanthin synthesized slowly from β-carotene.[46] Secondly, it lacks other carotenoid pathway enzyme genes like those for the synthesis of lutein, astaxanthin and canthaxanthin.[23] Both these conditions ultimately lead to accumulation of β-carotene as well as zeaxanthin in the cells. Carotenoid profiles showed that zeaxanthin content was higher under all growth regimes than β-carotene (*Fig 4D*). Weight ratios of zeaxanthin: Chl-a were also higher than β-carotene: Chl-a (*Fig 4D*). In addition, zeaxnthin: Chl-a ratios increased under autotrophy and reduced under mixotrophy while β-carotene: Chl-a ratios remained almost unchanged (*Fig 5D*). This showed that zeaxanthin plays a role in photo-protection under natural light and these results also uphold antioxidant potential of zeaxanthin over β-carotene in *Synechococcus.*[16]

In conclusion, *Synechococcus,* genetically modified to express heterologous hexose transporter protein, gives improved biomass productivity when grown mixotrophically in EC under natural diurnal light and controlled temperature conditions. Results suggested that acclimatization of cultures under natural light conditions plays significant role in improving biomass productivity of *Synechococcus*. Comparative pigment profiles showed, under mixotrophy as glucose serves to be an alternative C and energy source, *Synechococcus* displays reduced light dependency by reducing the number of Chl-a molecules. Specific nitrate uptake

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rate under mixotrophy is significantly reduced as compared to autotrophy, owing to reduced photosynthetic reaction centers. Glucose uptake from the medium, change in nitrate uptake rate, altering antenna pigment density and thereby modulating light dependency of cell makes mixotrophy under natural light a promising mode of operation for industrial scale up of *Synechococcus* and production of natural or engineered value added compounds from them.

5. Acknowledgement

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There is no conflict of interest declared for any of the authors of this manuscript.

6. References

1. Adamska I, Lohscheider JN, Strittmatter M, Ku H. Vertical Distribution of Epibenthic Freshwater Cyanobacterial *Synechococcus spp*. Strains Depends on Their Ability for Photoprotection. 2011; 6(5). doi:10.1371/journal.pone.0020134.
2. American Public Health Association, Water Environment Federation, American Water Works Association. *Stand Methods Exam Water Wastewater Part 4500*. 1999:733.

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Accepted Article

3. Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature*. 2008; 451(7174):86-89. doi:10.1038/nature06450.

4. Atsumi S, Higashide W, Liao JC. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Letters Nat Biotechnol*. 2009; 27(12):1177-1180. doi:10.1038/nbt.1586.

5. Buch A, Archana G, Kumar GN. Heterologous expression of phosphoenolpyruvate carboxylase enhances the phosphate solubilizing ability of fluorescent pseudomonads by altering the glucose catabolism to improve biomass yield. *Bioresour Technol*. 2010; 101(2):679-687. doi:10.1016/j.biortech.2009.08.075.

6. Clerico, M. E., Ditty, J. L., Golden SS. Identification of clock genes: Specialized Techniques for Site-Directed Mutagenesis in Cyanobacteria. In: *Circadian Rhythms: Methods and Protocols*. ; 2001:153-172.

7. De Mooij T, Janssen M, Cerezo-Chinarro O, et al. Antenna size reduction as a strategy to increase biomass productivity: a great potential not yet realized. *J Appl Phycol*. 2014:1063-1077. doi:10.1007/s10811-014-0427-y.

8. Deng M De, Coleman JR. Ethanol synthesis by genetic engineering in cyanobacteria. *Appl Environ Microbiol*. 1999;65(2):523-528.

9. Gonçalves JFDC, Marenco RA, Vieira G. Concentration of photosynthetic pigments and chlorophyll fluorescence of mahogany and tonka bean under two light environments. *Rev Bras Fisiol Veg*. 2001;13(2):149-157. doi:10.1590/S0103-31312001000200004.

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Accepted Article

10. Götz T, Windhövel U, Böger P, Sandmann G. Protection of Photosynthesis against Ultraviolet-B Radiation by Carotenoids in Transformants of the Cyanobacterium *Synechococcus* PCC 7942. *Plan Physiol*, 1999; 120(June):599-604.

11. Graham JE, Bryant DA. The biosynthetic pathway for synechoxanthin, an aromatic carotenoid synthesized by the euryhaline, unicellular cyanobacterium *Synechococcus sp*. strain PCC 7002. *J Bacteriol*. 2008;190(24):7966-7974. doi:10.1128/JB.00985-08.

12. Hartmann A, Albert A, Ganzera M. Effects of elevated ultraviolet radiation on primary metabolites in selected alpine algae and cyanobacteria. *J Photochem Photobiol B*. 2015;149:149-155. doi:10.1016/j.jphotobiol.2015.05.016.

13. Herrero A, Muro-pastor AM, Flores E. Nitrogen Control in Cyanobacteria Nitrogen Control in Cyanobacteria. 2001;183(2):411-425. doi:10.1128/JB.183.2.411.

14. Hsieh P, Pedersen JZ, Bruno L. Photoinhibition of Cyanobacteria and its Application in Cultural Heritage Conservation. 2014:533-543. doi:10.1111/php.12208.

15. Hu Q, Westerhoff P, Vermaas W, Hu Q, Westerhoff P. Removal of Nitrate from Groundwater by Cyanobacteria : Quantitative Assessment of Factors Influencing Nitrate Uptake. 2000; 66(1):133-139. doi:10.1128/AEM.66.1.133-139.2000.

16. Jahns P, Holzwarth AR. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochim Biophys Acta - Bioenerg*. 2012;1817(1):182-193. doi:10.1016/j.bbabio.2011.04.012.

17. Kana R, Kotabová E, Barbora Š, Papageorgiou GC. The slow S to M fluorescence rise in cyanobacteria is due to a state 2 to state 1 transition. *Biochimica et Biophysica Acta*. 2012:1-11. doi:10.1016/j.bbabio.2012.02.024.

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Accepted Article

18. Kirst H, Formighieri C, Melis A. Maximizing photosynthetic efficiency and culture productivity in cyanobacteria upon minimizing the phycobilisome light-harvesting antenna size. *Biochim Biophys Acta - Bioenerg*. 2014; 1837(10):1653-1664. doi:10.1016/j.bbabio.2014.07.009.

19. Kwon JH, Bernát G, Wagner H, Rögner M, Rexroth S. Reduced light-harvesting antenna: Consequences on cyanobacterial metabolism and photosynthetic productivity. *Algal Res*. 2013; 2(3):188-195. doi:10.1016/j.algal.2013.04.008.

20. Lakatos M, Bilger W, Büdel B. Carotenoid composition of terrestrial cyanobacteria: response to natural light conditions in open rock habitats in Venezuela. *Eur J Phycol*. 2001; 36(4):367-375. doi:10.1080/09670260110001735518.

21. Lau NS, Matsui M, Abdullah AAA. Cyanobacteria: Photoautotrophic Microbial Factories for the Sustainable Synthesis of Industrial Products. *Biomed Res Int*. 2015; 2015. doi:10.1155/2015/754934.

22. Lea-Smith DJ, Bombelli P, Dennis JS, Scott SA, Smith AG, Howe CJ. Phycobilisome-Deficient Strains of *Synechocystis sp.* PCC 6803 Have Reduced Size and Require Carbon-Limiting Conditions to Exhibit Enhanced Productivity. *Plant Physiol*. 2014; 165(2):705-714. doi:10.1104/pp.114.237206.

23. Liang C, Zhao F, Wei W, Wen Z, Qin S. Carotenoid biosynthesis in cyanobacteria: Structural and evolutionary scenarios based on comparative genomics. *Int J Biol Sci*. 2006; 2(4):197-207. doi:10.7150/ijbs.2.197.

24. Lohscheider JN, Strittmatter M, Küpper H, Adamska I. Vertical Distribution of Epibenthic Freshwater Cyanobacterial *Synechococcus* spp. Strains Depends on Their Ability for Photoprotection. *PLoS one*, 2011; 6(5). doi:10.1371/journal.pone.0020134

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Accepted Article

25. McEwen JT, Kanno M, Atsumi S. 2, 3 Butanediol production in an obligate photoautotrophic cyanobacterium in dark conditions via diverse sugar consumption. *Metab Eng*. 2016;36:28-36. doi:10.1016/j.ymben.2016.03.004.

26. McEwen JT, Machado IMP, Connor MR, Atsumi S. Engineering *Synechococcus elongatus* PCC 7942 for continuous growth under diurnal conditions. *Appl Environ Microbiol*. 2013; 79(5):1668-1675. doi:10.1128/AEM.03326-12.

27. Montgomery BL. The Regulation of Light Sensing and Light-Harvesting Impacts the Use of Cyanobacteria as Biotechnology Platforms. *Front Bioeng Biotechnol*. 2014; 2(July):22. doi:10.3389/fbioe.2014.00022.

28. Mori T, Binder B and Johnson CH. Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proc Natl Acad Sci,* 1996; 93(September):10183-10188.

29. Mullineaux CW. Electron transport and light-harvesting switches in cyanobacteria. *Front Plan Sci* 2014; 5(January):1-6. doi:10.3389/fpls.2014.00007.

30. Nakajima Y, Ueda R. Improvement of microalgal photosynthetic productivity by reducing the content of light harvesting pigment. *J Appl Phycol*. 1999; 11(2):195-201. doi:10.1023/A:1008015224029.

31. Nogales J, Gudmundsson S, Knight EM, Palsson BO, Thiele I. Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. *Proc Natl Acad Sci U S A*. 2012; 109(7):2678-2683. doi:10.1073/pnas.1117907109.

32. Nozzi NE, Oliver JWK, Atsumi S. Cyanobacteria as a Platform for Biofuel Production. *Front Bioeng Biotechnol*. 2013; (September):1-6. doi:10.3389/fbioe.2013.00007.

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Accepted Article

33. Oliver JWK, Machado IMP, Yoneda H, Atsumi S. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. *Proc Natl Acad Sci U S A*. 2013; 110(4):1249-1254. doi:10.1073/pnas.1213024110.

34. Ooms MD, Dinh CT, Sargent EH, Sinton D. Photon management for augmented photosynthesis. *Nat Publ Gr*. 2016;7:1-13.doi:10.1038/n

35. Ort DR, Zhu X, Melis A. Optimizing Antenna Size to Maximize Photosynthetic Efficiency. *Plant Physiol*. 2011; 155(1):79-85. doi:10.1104/pp.110.165886.

36. Page LE, Liberton M, Pakrasi HB. Reduction of photoautotrophic productivity in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by phycobilisome antenna truncation. *Appl Environ Microbiol*. 2012; 78(17):6349-6351. doi:10.1128/AEM.00499-12.

37. Page LE, Liberton M, Sato H, Pa HB. Phycobilisome Antenna Deletion in a Cyanobacterium does Not Improve Photosynthetic Energy Conversion Efficiency or Productivity in a Bench-Scale Photobioreactor System. 2013:744-747.

38. Pollari M, Rantama S, Huokko T, Kårlund-marttila A, Virjamo V, Tyystjärvi E, and Tyystjärvi T. Effects of Deficiency and Overdose of Group 2 Sigma Factors in Triple Inactivation Strains of *Synechocystis* sp Strain PCC 6803. *J. Bacteriol*, 2011; 193(1):265-273. doi:10.1128/JB.01045-10.

39. Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A, Legrand J. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. *Bioresour Technol*. 2011; 102(1):150-158. doi:10.1016/j.biortech.2010.06.153.

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Accepted Article

40. Ranade S, Zhang Y, Kaplan M, Majeed W, He Q. Metabolic Engineering and Comparative Performance Studies of *Synechocystis* sp. PCC 6803 Strains for Effective Utilization of Xylose. *Front Microbiol*. 2015; 6(December):1484. doi:10.3389/fmicb.2015.01484.

41. Ruffing AM. Metabolic Engineering of Hydrocarbon Biosynthesis for Biofuel Production. *Liq Gaseous Solid Biofuels - Convers Tech*. 2013:263-298. doi:10.5772/52050.

42. Ruffing AM, Trahan CA, Jones HDT. Genetic Engineering of Cyanobacteria as Biodiesel Feedstock. 2013;(January).

43. Sakamoto T, Bryant D a. Nitrate transport and not photoinhibition limits growth of the freshwater Cyanobacterium *Synechococcus species* PCC 6301 at low temperature. *Plant Physiol*. 1999;119(2):785-794. doi:10.1104/pp.119.2.785.

44. Schäfer L, Sandmann M, Woitsch S, Sandmann G. Coordinate up-regulation of carotenoid biosynthesis as a response to light stress in *Synechococcus* PCC 7942. *Plant, Cell Environ*. 2006; 29(7):1349-1356. doi:10.1111/j.1365-3040.2006.01515.x.

45. Schagerl M, Müller B. Acclimation of chlorophyll a and carotenoid levels to different irradiances in four freshwater cyanobacteria. *J Plant Physiol*. 2006;163(7):709-716. doi:10.1016/j.jplph.2005.09.015.

46. Schuurmans RM, Alphen P Van, Schuurmans JM, Matthijs HCP and Helingwerf KJ. Comparison of the Photosynthetic Yield of Cyanobacteria and Green Algae : Different Methods Give Different Answers. 2015:1-17. doi:10.1371/journal.pone.0139061.

47. Show K, Lee D, Chang J. Algal biomass dehydration. *Bioresour Technol*. 2013; 135:720-729. doi:10.1016/j.biortech.2012.08.021.

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48. Sonoike K, Hihara Y, Ikeuchi M. Physiological significance of the regulation of photosystem stoichiometry upon high light acclimation of Synechocystis sp . PCC 6803. *Plant Cell Physiol*. 2001; 42(4):379-384.

49. Steiger S, Schafer L, Sandmann G. High-light-dependent upregulation of carotenoids and their antioxidative properties in the cyanobacterium *Synechocystis* PCC 6803. 1999; 52:14-18.

50. Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R. Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. *Environ Int*. 2013; 51:59-72. doi:10.1016/j.envint.2012.10.007.

51. Tyystjärvi T, Aro E, Jansson C, Pirkko M. Changes of amino acid sequence in PEST-like area and QEEET motif affect degradation rate of D1 polypeptide in photosystem II. 1994:517-526.

52. Vermaas WFJ. Photosynthesis and respiration in cyanobacteria. *Encycl Life Sci*. 2001:1-7.doi:10.1002/9780470015902.a0021539.

53. Wagh J, Bhandari P, Shah S, Archana G, Kumar GN. Overexpression of citrate operon in *Herbaspirillum seropedicae* Z67 enhances organic acid secretion, mineral phosphate solubilization and growth promotion of *Oryza sativa*. *Plant Soil*. 2014; 383(1-2):73-86. doi:10.1007/s11104-014-2161-2.

54. Wan N, Abernathy M, Tang JKH, Tang YJ, You L. Cyanobacterial photo-driven mixotrophic metabolism and its advantages for biosynthesis. *Front Chem Sci Eng*. 2015; 9(3):308-316. doi:10.1007/s11705-015-1521-7.

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55. Wu H, Gao K, Villafañe VE, Helbling EW, Villafan VE, Watanabe T. Effects of Solar UV Radiation on Morphology and Photosynthesis of Filamentous Cyanobacterium *Arthrospira platensis*. 2005; 71(OCTOBER):5004-5013. doi:10.1128/AEM.71.9.5004.

56. Yan R, Zhu D, Zhang Z, Zeng Q, Chu J. Carbon metabolism and energy conversion of *Synechococcus* sp. PCC 7942 under mixotrophic conditions: Comparison with photoautotrophic condition. *J Appl Phycol*. 2012; 24(4):657-668. doi:10.1007/s10811-011-9683-2.

57. Yao L, Qi F, Tan X, Lu X. Improved production of fatty alcohols in cyanobacteria by metabolic engineering. 2014:1-9.

58. Yu H, Jia S, Dai Y. Growth characteristics of the cyanobacterium *Nostoc flagelliforme* in photoautotrophic , mixotrophic and heterotrophic cultivation. *J. Appl Phycol*, 2009:127-133.doi:10.1007/s10811-008-9341-5.

59. Zakar T, Laczko-dobos H, Toth TN, Gombos Z. Carotenoids Assist in Cyanobacterial Photosystem II Assembly and Function. 2016; 7(March):1-7. doi:10.3389/fpls.2016.00295.

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Growth engineering of *Synechococcus elongatus* PCC 7942 for mixotrophy under natural light conditions for improved feedstock production

Aditya Sarnaik[a], Reena Pandit[a*], Arvind Lali[a,b]

[a]*DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai-400019, Maharashtra, India.*

[b]*Department of Chemical Engineering, Institute of Chemical Technology, Mumbai- 400019, Maharashtra, India.*

E-mail: drreenapandit@gmail.com Contact Number: +91-9821211769

Figures

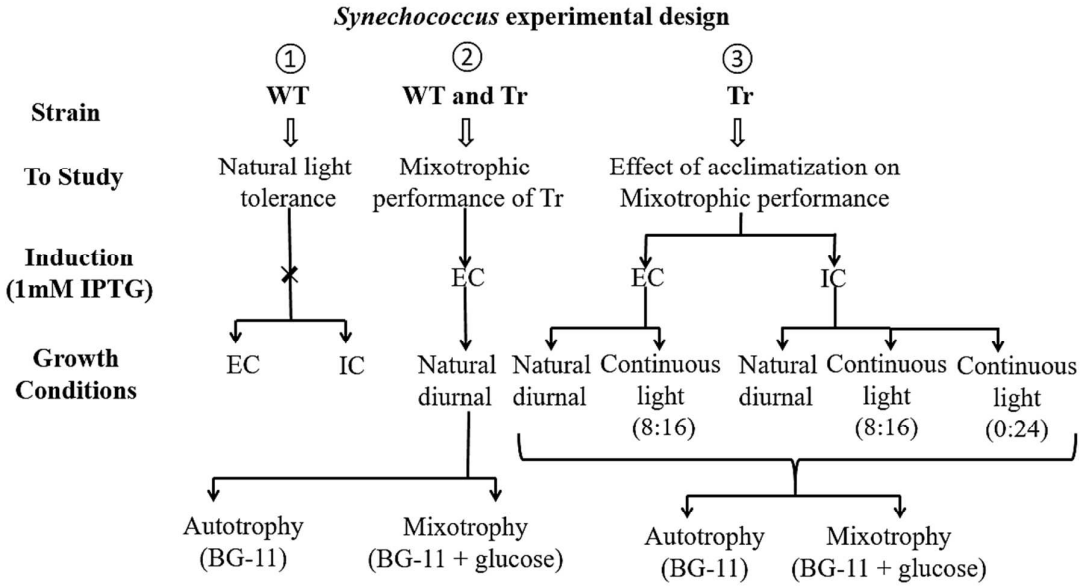

Fig 1: Representation of the overall experiment performed for *Synechococcus* growth study under different light conditions. Experiment 1 was performed to ensure natural light tolerance of the wild type (WT) *Synechococcus* and to study its effect on biomass and photosynthetic pigments. Experiment 2 was performed under natural diurnal conditions (in EC) to analyse the comparative growth performances of WT and *galP* transformants (Tr) under autotrophy and mixotrophy. Experiment 3 was performed with Tr to study the effect of different

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photoperiods on their biomass and pigment production. EC, Environmental chamber (max. light intensity 1200 ± 100µmol/m^2/s); IC, Incubator (light intensity 55 ± 0.5µmol/m^2/s).

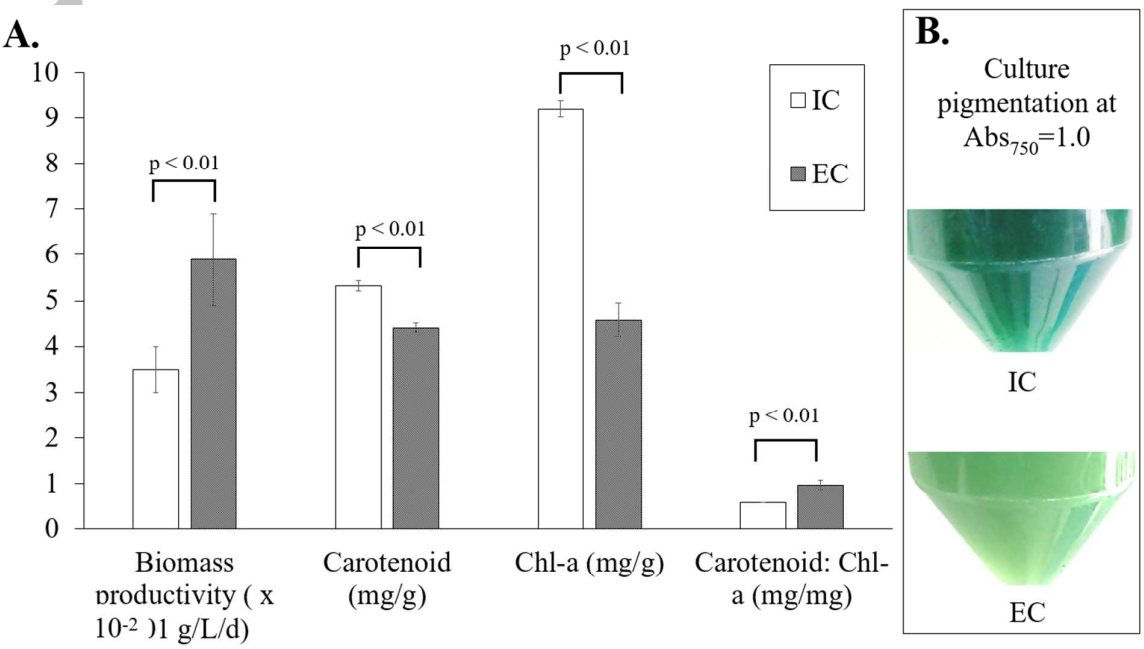

Fig 2: Artificial and natural light performances of *Synechococcus* WT.(A) IC and EC exhibit different light, but similar temperature conditions. Effect of these conditions on growth and photosynthetic pigment yields of *Synechococcus* WT was studied to investigate the most favorable condition supporting higher biomass productivity (α=0.05). (B) This pigment modulation can be prominantly observed by the colour of the cultures in IC and EC (*side panel*). (EC, Environmental chamber (max. light intensity 1200 ± 100µmol/m^2/s); IC, Incubator (light intensity 55 ± 0.5µmol/m^2/s)).

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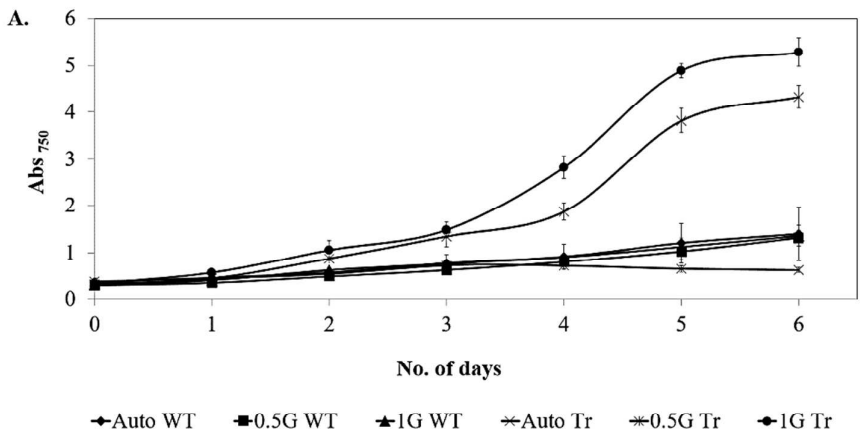

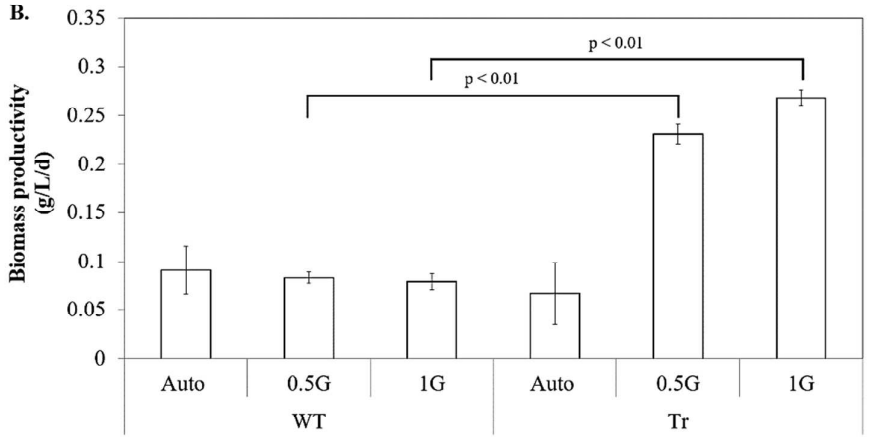

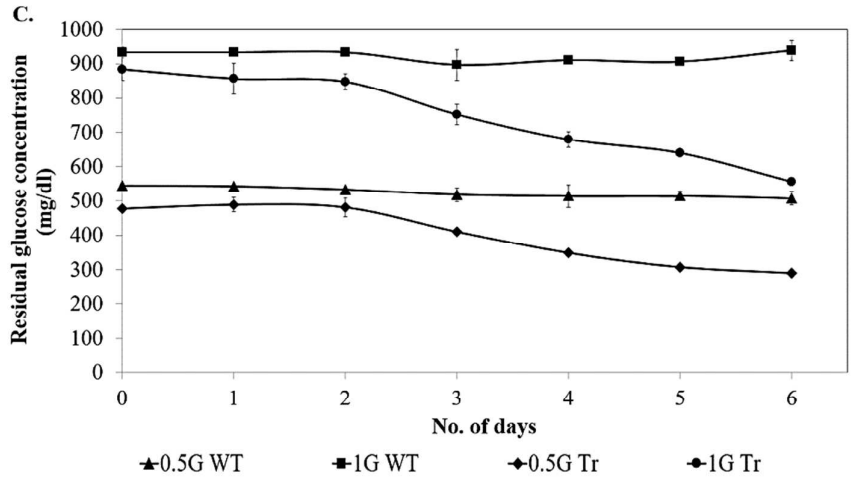

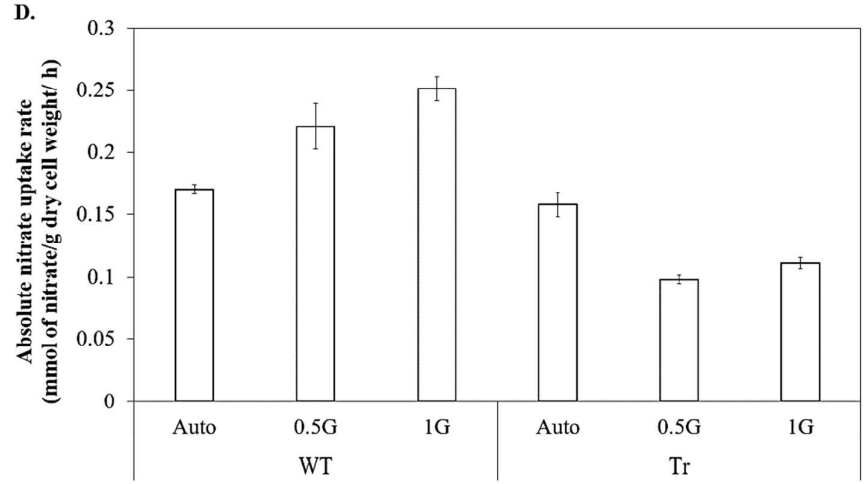

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Fig 3: Growth and nutrient uptake profiles of *Synechococcus* WT and Tr under EC. (A) Comparative growth studies of WT and Tr were performed in EC under autotrophic and mixotrophic conditions (B) Dry cell weights (g) for all the WT and Tr cultures grown in EC under autotrophy and mixotrophy for 6 days were determined and represented as their biomass productivities (g/L/d) ($\alpha = 0.05$). (C) Glucose uptake profiles of WT and Tr grown in BG-11 media supplemented with glucose (0.5% and 1% w/v) were determined daily as concentration of residual glucose (mg/dl) in the medium. (D) Absolute nitrate uptake rate (mmol of nitrate/g dry cell weight/ h) of the WT and Tr cultures. (Auto, Autotrophy; 0.5G, BG-11 + 0.5% glucose; 1G, BG-11 + 1% glucose)

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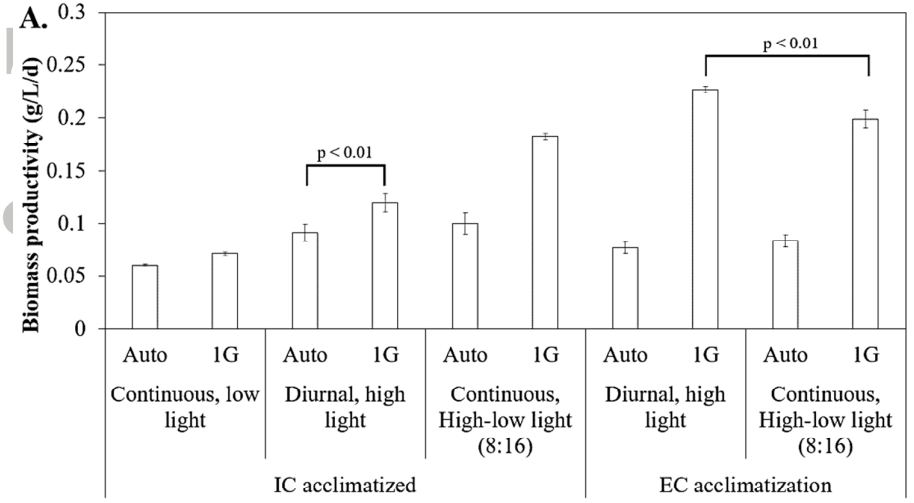

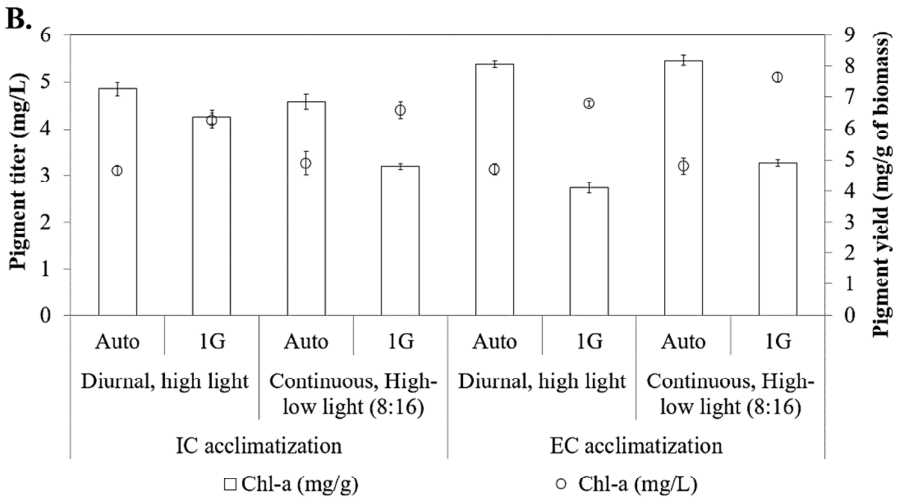

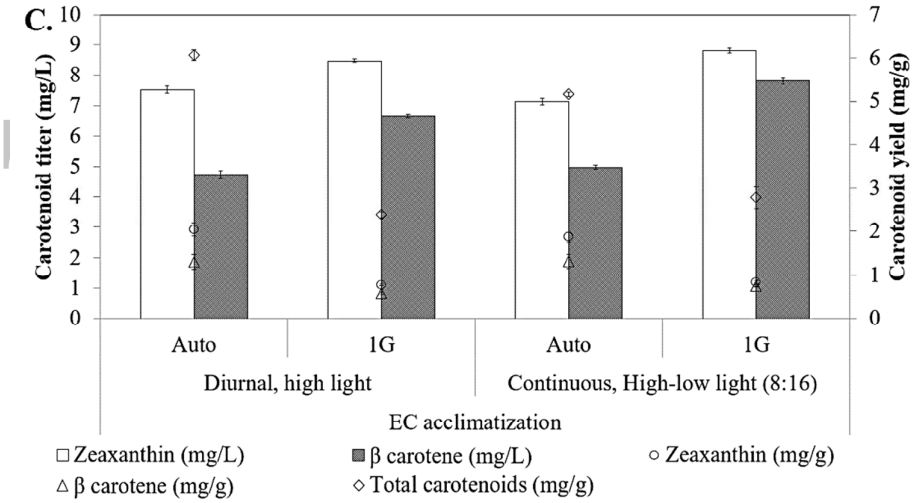

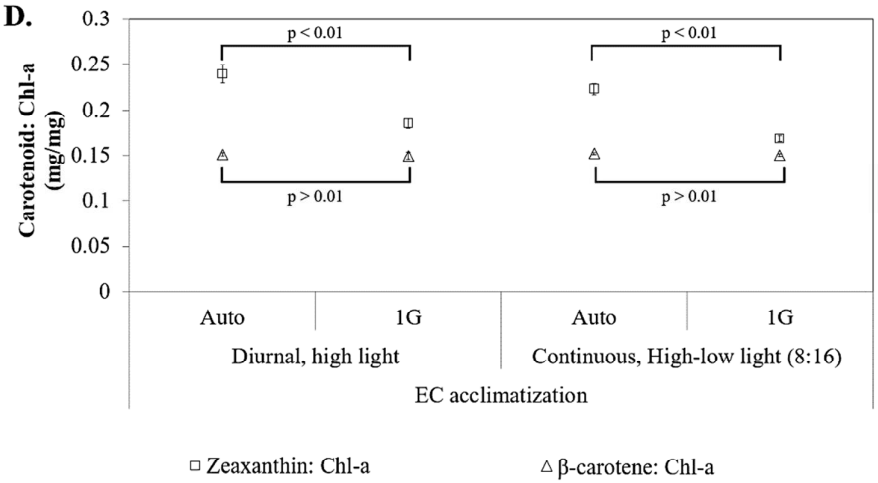

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Fig 4: Effect of different acclimatization conditions and photoperiods on biomass productivity and pigment profiles of *Synechococcus* Tr. (A) Comparative biomass productivities for Tr acclimatized in IC and EC and grown under diurnal and continuous (8:16) light conditions under autotrophic and mixotrophic conditions for 8 days. "Control" culture of Tr was acclimatized and maintained in IC to validate the effect of acclimatization on growth performance of the Tr. ($\alpha = 0.05$) (B) Comparative Chl-a yield (mg/g) and titer (g/L) for IC and EC acclimatized cultures (C) Comparative Carotenoid yield (mg/g) and titer (g/L) for EC acclimatized cultures.(D) Weight ratios (mg of carotenoids: mg of Chl-a per g of cells) for zeaxanthin and β-carotene extracted from EC acclimatized Tr cultures. ($\alpha = 0.05$) (Auto, Autotrophy; 0.5G, BG-11 + 0.5% glucose; 1G, BG-11 + 1% glucose; EC, Environmental chamber; IC, Incubator)

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Aditya Sarnaik[a], Reena Pandit[a*], Arvind Lali[a,b]

[a]*DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai-400019, Maharashtra, India.*

[b]*Department of Chemical Engineering, Institute of Chemical Technology, Mumbai- 400019, Maharashtra, India.*

E-mail: drreenapandit@gmail.com Contact Number: +91-9821211769

Tables

Table 1: Comparative performances of WT and Tr under autotrophic and mixotrophic regimes. (A) Growth rates (h^{-1}), biomass productivities (g/L/d) and (B) absolute glucose uptake rates (mmol of glucose/g dry cell weight/h) of WT and Tr grown in EC.(Auto, Autotrophy; 0.5G, BG-11 + 0.5% glucose; 1G, BG-11 + 1% glucose).

A.

Parameters		WT			Tr		
		Auto	0.5G	1G	Auto	0.5G	1G
EC (No. of days=6)	Growth rate (h^{-1})	0.0112 ± 0.0012	0.0101 ± 0.0010	0.0108 ± 0.0001	0.0063 ± 0.0021	0.0173 ± 0.001	0.0204 ± 0.001
	Biomass productivity (g/L/d)	0.09 ± 0.012	0.08 ± 0.006	0.08 ± 0.008	0.07 ± 0.032	0.23 ± 0.010	0.27 ± 0.007

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B.

Conditions		Absolute glucose uptake rate (mmol of glucose/g dry cell weight/h)
EC	0.5G Tr	0.19 ± 0.012
	1G Tr	0.22 ± 0.015

Table 2: Comparative growth rates (h^{-1}) of Tracclimatized in IC and EC for 2 days and grown under different light conditions for 8 days. (Auto, autotrophy; 1G, BG-11 + 1% glucose).

Growth rates (h^{-1})	IC acclimatization			EC acclimatization	
	Diurnal, high light	Continuous, High-low light (8:16)	Continuous, low light	Diurnal, high light	Continuous, High-low light (8:16)
Auto	0.015 ± 0.001	0.018 ± 0.001	0.010 ± 0.0001	0.018 ± 0.0007	0.017 ± 0.001
1G	0.021 ± 0.001	0.022 ± 0.002	0.013 ± 0.0001	0.026 ± 0.0003	0.025 ± 0.0006

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Table 3: Experimental evaluations and corresponding FBA predictions. Experimentally evaluated values of growth rate (h^{-1}) and nutrient uptake rates (mmol/ g dry cell weight/ h) were used to simulate FBA model. The table has average experimental values of the experimentally determined variables and the corresponding FBA predicted variables, atmospheric CO_2 and photon uptake rates.

Conditions	Average experimental variables			Predicted variables	
	Specific growth rate (h^{-1})	Nitrate uptake rate (mmol of nitrate/ g dry cell weight/ h)	Glucose uptake rate (mmol of glucose/ g dry cell weight/ h)	CO_2 uptake rate (mmol of CO_2/ g dry cell weight/ h)	Photon uptake rate (mmol of photons/ g dry cell weight/ h)
Auto WT (EC)	0.011	0.17	--	0.4	10
1G Tr (EC)	0.020	0.11	0.22	0.3	5

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