Mixotrophic transition induced lipid productivity in *Chlorella pyrenoidosa* under stress conditions for biodiesel production

Hari Prasad Ratnapuram, S.S. Vutukuru, Rajasri Yadavalli *

*Corresponding author.
E-mail address: rajasriy@sreenidhi.edu.in (R. Yadavalli).

**Abstract**

Influence of mixotrophic mode and its transition to various trophic modes under stress conditions was assessed during two stage cultivation of *Chlorella pyrenoidosa*. Significant lipid productivity was triggered under low light intensity, glucose + bicarbonate supplementation and nitrogen starvation. The association between biomass and lipid productivity, fatty acid composition during mixotrophic transition was critically evaluated. Biomass in growth phase (GP) and stress phase (SP) was 6.14 g/l and 5.14 g/l, respectively, in mixotrophic mode. Higher lipid productivity of 284 g/kg and 154.3 g/kg of neutral lipids was achieved in SP in mixotrophic-mixotrophic (MM) and mixotrophic-heterotrophic (MH) modes, respectively. Stress conditions resulted in high unsaturated fatty acid methyl esters in MH mode. In addition, neutral lipid content was 58% in MH and 52% in MM, that can be attributed to carbon source that is supplemented even in stress phase. Exploring such novel strategies can generate sustainable avenues for biodiesel production.

Keywords: Biotechnology, Bioengineering, Environmental science
1. Introduction

Microalgae are being employed as potential generation vehicles to harness various useful products like biofuels, neutraceuticals, animal feeds and biomaterials etc (Singh et al., 2016). They are solar driven photosynthetic cellular factories which synthesize secondary metabolites like lipids under stress conditions that enhance the biodiesel production significantly (Solovchenko, 2012). Research indicates that to endure adverse environmental conditions, microalgae typically store lipids in the form of triacylglycerides (Markou and Nerantzis, 2013; Sharma et al., 2012). Recent studies have demonstrated that the lipid content can be enhanced in microalgae by manoeuvring the cultures and subjecting them to diverse stress conditions (Chandra et al., 2014; Yadavalli et al., 2012). Amongst the various available cultivation methods, two-stage systems are of intense interest. In the first stage, optimum conditions are maintained to maximize biomass production whereas in the second stage, the accumulation of lipids is facilitated under stress conditions (Markou and Nerantzis, 2013; Devi and Mohan, 2012). Literature suggests that the major stress conditions applied to enhance lipid accumulation are temperature, light intensity, pH, nitrogen starvation, phosphate limitation (Yadavalli et al., 2010; Takagi et al., 2006).

As many avenues exist in the microalgae production, to increase efficiency or eliminate extraneous costs, research is warranted to improve the biomass yield and thereby enhancing its lipid productivity to harness biodiesel or other therapeutically/nutrionally valuable products. This can be achieved by adopting one of the strategies viz. optimizing metabolic pathways during algal cultivation in different trophic modes. Metabolic pathways reflect the availability and form of nutrients, particularly carbon, in the media in which the microalgae are cultured.

Previous studies established that in microalgae factors such as nitrogen, light intensity, CO₂ are highly crucial for metabolic activities like photosynthesis, cell division, respiration, intracellular transportation, protein synthesis etc (Lv et al., 2010; Rawat et al., 2011). Microalgae accumulate energy in the form of polysaccharides, and/or neutral lipids under stress conditions. One of the most widely used strategies to improve lipid accumulation is nitrogen starvation which could lead to reduced cell division (Amaro et al., 2011). Consequently, a metabolic shift in the lipid biosynthetic pathways leads to the synthesis of more neutral lipids than membrane lipids (Li et al., 2015a,b; Converti et al., 2009).

Whilst most of the existing literature reflects on autotrophic microalgae culture, emerging studies are exploring the potential of heterotrophic and even mixotrophic growth to optimize the process for biofuel production (Wang et al., 2012; Wan et al., 2011). A perusal of the available information suggests that albeit heterotrophic cultivation can potentially increase the lipid content and mixotrophic mode is desirable to achieve both higher yields of biomass and lipid productivity to
harness biodiesel (Lee, 2001; Chen, 1996). For achieving this objective, a viable strategy like two stage cultivation of microalgae with transition of trophic modes needs to be optimized (Fan et al., 2015; Hena et al., 2015; Rohit and Mohan, 2016).

In this investigation, we evaluated the biomass and lipid productivity of *C. pyrenoidosa* in two stage cultivation viz., growth phase (GP) and stress phase (SP) during transition from mixotrophic mode (Growth phase) to autotrophic, heterotrophic and mixotrophic modes (stress phase) and vice versa [mixotrophic mode-autotrophic mode (MA), mixotrophic mode-heterotrophic mode (MH), mixotrophic mode-mixotrophic mode (MM), autotrophic mode-mixotrophic mode (AM), autotrophic mode-heterotrophic mode (AH)]. In this research, autotrophic mode is taken as positive control wherein only light is provided with no external carbon supplementation. On the other hand, heterotrophic mode served as a negative control in the absence of light and supplemented with external carbon in the form of glucose (since photosynthesis do not occur). In mixotrophic mode both light intensity and external supplementation of carbon are ensured to promote the enhanced lipid growth in algae. Apparently, the presence or absence of light intensity profoundly influences the microalgal growth in MM rather than a relative affect.

A perusal of the available literature indicates that but for the report of (Lohman et al., 2015), information on glucose supplementation and dissolved inorganic carbon as NaHCO3 in growth and stress phases of mixotrophic mode is scanty. However, they did not take the trophic transition effect into consideration for biodiesel production. We hypothesize that maximum lipid productivity will be obtained during transition from mixotrophic to heterotrophic mode under additional supplementation of carbon source, low light intensities and nitrogen starvation conditions in stress phase. Changes in the biomass growth, chlorophyll content, lipid productivities and fatty acid compositions were evaluated and the corresponding results are presented in this paper.

### 2. Materials and methods

#### 2.1. Microalgae and media composition

*Chlorella pyrenoidosa* (NCIM NO: 2738) was obtained from National Centre for Industrial Microorganisms (NCIM), Pune, India. Stock culture of *Chlorella pyrenoidosa* was photoautotrophically cultivated in BG11 media at 28 °C under continuous light illumination in four 100 ml borosil flasks. Each litre of the BG11 medium contained NaNO₃-1.5 g, K₂HPO₄-0.04 g, MgSO₄ 7H₂O-0.075 g, CaCl₂2H₂O-0.036 g, Citric acid-0.006 g, NaCO₃-0.02 g, H₃BO₃-0.00286 g, MnCl₂ 4H₂O-0.00181 g, ZnSO₄ 7H₂O-0.00022 g, Na₂MoO₄ 2H₂O-0.00039 g, CuSO₄ 5H₂O-0.00008 g, Co(NO₃)₂ 6H₂O-0.00005 g,(NH₄)₆Mo₇O₂₄ 4H₂O-0.003 g.
g, Na₂EDTA-0.00001 g. The inoculums were prepared by transferring the cells from stock culture, and incubated aseptically in a 1000 ml flask containing 700 ml of fresh BG11 medium. A continuous illumination of 34 μmol m⁻² s⁻¹ at 28 °C was provided to the culture for four days on an orbital shaker set at 120 rpm.

### 2.2. Experimental methodology

Experiments were designed and operated in two phase mode viz., growth phase (GP) followed by lipid accumulation phase (SP). Conditions maintained for experiments were represented in Table 1. For growth phase, experiments were conducted thrice with 250 ml flasks and 160 ml culture was maintained in each mode. At the end of growth phase, one flask is utilized for biomass and lipid estimations in each of the five modes. Subsequently, stress phase experiments were continued in second flask for biomass and lipid estimations. Flasks were mounted on a temperature controlled shaking incubator at 120 rpm in the presence of a cool white fluorescent light under continuous illumination. In this study, 60 μmol m⁻² s⁻¹ was considered as high light intensity as maintained in growth phase except in heterotrophic mode. Similarly, 30 μmol m⁻² s⁻¹ (low light intensity) was provided in stress phase except in heterotrophic mode.

### 2.3. Biochemical analysis

After growth phase and stress phase experiments, the obtained biomass were centrifuged (Thermo Scientific Sorvall ST16R) at 5000 rpm for 5 min at 28 °C). All the samples were analyzed using UV-Visible spectrophotometer at 620 nm (ELICO SL 210). Algal biomass was estimated for every 24 h. The dry cell weight (DCW) was measured gravimetrically by using Whatman filter paper and subjected to drying in hot air oven at 55 °C. The experiments were carried out in triplicates and the results presented here represent a mean of three solitary operations.

### Table 1. Conditions for growth and stress phase with organic and inorganic carbon source in various modes of cultivation.

| Mode of nutrition | Growth phase conditions (BG11 medium) under 60 μmol m⁻² s⁻¹ | Stress phase conditions (BG11 medium-N) under 30 μmol m⁻² s⁻¹ |
|-------------------|-------------------------------------------------------------|-------------------------------------------------------------|
| MA                | Glucose-3 g/l+NaHCO₃(3 g/L)                                 | Glucose-0 g/l                                               |
| MH                | Glucose-3 g/l+NaHCO₃(3 g/L)                                 | Glucose-6 g/l                                               |
| MM                | Glucose-3 g/l+NaHCO₃(3 g/L)                                 | Glucose-3 g/l + NaHCO₃(3 g/L)                               |
| AM                | Glucose-0 g/l                                               | Glucose-3 g/l + NaHCO₃(3 g/L)                               |
| HM                | Glucose-6 g/l                                               | Glucose-3 g/l + NaHCO₃(3 g/L)                               |

(MA = mixotrophic to autotrophic mode transition; MH = mixotrophic to Heterotrophic mode transition; MM = Mixotrophic to mixotrophic mode transition; AM = autotrophic to mixotrophic mode transition; HM = Heterotrophic to Autotrophic mode transition).
2.3.1. Chlorophyll estimation

Fresh algal cells (10 ml) were centrifuged and the pellet was subjected to acetone and ethanol (1:1) extraction. The extract was centrifuged at 6000 rpm for 5 min and the optical density of the supernatant was measured at 647 nm and 664 nm to deduce the Chlorophyll a and b concentrations based on following equations (Jeffrey and Humphrey, 1975)

\[
\text{Chlorophyll A} = (-1.93 \times \text{Abs}_{647} + 11.93 \times \text{Abs}_{664})
\]

(1)

\[
\text{Chlorophyll B} = (20.36 \times \text{Abs}_{647} - 5.5 \times \text{Abs}_{664})
\]

(2)

Total Chlorophyll = Chlorophyll A + Chlorophyll B

(3)

2.3.2. Carbohydrates, nitrates and phosphates estimation

Carbohydrates were estimated by Phenol–sulphuric acid method (Vigeolas et al., 2012). Briefly, 5 mg of dried biomass was subjected to acid hydrolysis with 2.5 N HCl and then 0.2 ml of sample was collected and the volume was made up to 1 ml. Then 5 ml of concentrated sulphuric acid and 1 ml phenol were added to determine the carbohydrates in the samples by measuring the absorbance at 480 nm.

Nitrates and phosphates were analyzed as per the universally accepted protocols given in standard methods (APHA, 1998). Briefly, 10 ml of culture was collected for both nitrates and phosphates, and these samples were centrifuged to collect supernatant. Nitrates were analyzed by adding 1 ml concentrated hydrochloric acid and then concentration was determined at 220 nm. For phosphates estimation, 5 ml of ammonium molybdate was added to the collected supernatant. Then 4–6 drops of stannous chloride were added and incubated at room temperature for 10 min and later analyzed for concentration at 690 nm.

2.3.3. Lipid extraction

Extraction of total lipids was carried out using (Bligh and Dyer, 1959) employing chloroform: methanol (2:1 v/v) as solvents, while neutral lipids were extracted with n-Hexane adopting solvent extraction procedure (Lee et al., 2010). Prior to extraction, the dried algal biomass was subjected to sonication (2 min; 40 kHz; Lab Tech) in requisite solvent/solvent mixture (chloroform: methanol/n-Hexane) and transferred to thimbles made with Whatman filter paper No. 1 for neutral lipids. On the other hand, the biomass was directly placed in to the reaction vials for total lipids extraction and dried at 70 °C. Lipid percentage per 1 g of biomass was quantified gravimetrically on a dry weight basis by using recommended formula

http://dx.doi.org/10.1016/j.heliyon.2017.e00496
Lipid percentage \(= \left(\frac{x_2 - x_1}{\text{WAL}}\right) \times 100\) \hspace{1cm} (4)

Where

\(X_1 =\) Empty weight of dried vial

\(X_2 =\) Final weight of vial after lipid extraction

\(\text{WAL} = \) Weight of the Algal sample taken for Lipid extraction

2.3.4. Transesterification

The transesterification process was done by refluxing the total and neutral lipids extracted in chloroform/methanol and hexane with methanol in the presence of 2% sulphuric acid as a catalyst. Refluxing was performed at 65–70 °C for 4 h in a round bottom flask and of ethyl acetate and water was added (1:1) and later washed in a retort funnel with distilled water to maintain the neutral pH. After this step, the organic phase containing Fatty Acid Methyl Esters (FAME) present in the reaction mixture was separated from aqueous phase. To remove traces of water, sodium sulphate (anhydrous) was added to the organic layer. Finally, the organic phase was evaporated for recovering the solvent, while leaving the FAME mixture in the tube, which again was dissolved in N-Hexane.

2.3.5. Gas chromatography

Fatty acid methyl esters (FAME) were analyzed from the concentrated samples by Gas Chromatogram Performed by (Agilent 7890 B) equipped with FID through DB− 225 capillary column (30 m × 0.25 mm i.d × 0.25 um film thickness). The injector and detector temperatures were maintained at 300 and 325 °C, respectively, and split ratio of 50:1 using nitrogen as carrier gas (1.5 ml/ min). Initially, the temperature was maintained at 160 for 2 min in the oven which was subsequently raised to 300 °C at a ramping of 5 °C/min and maintained for 20 min. The FAME composition was compared with the Standard FAME mix C4–C24 (18919-1AMP SUPELCO).

3. Results and discussion

Two stage cultivation was employed in the present experiment wherein the growth phase (GP) is intended to enhance the biomass under nitrogen replete condition (Fig. 1). The corresponding results are presented hereunder.

3.1. Biomass growth

Biomass enrichment has been observed in different modes of trophic mode transition. At the end of the growth phase (EGP), the biomass increment in
Mixotrophic, heterotrophic, and autotrophic modes was 6.14 g/l, 3.42 g/l and 1.85 g/l, respectively, over a period of 8 days in GP (Fig. 2). Maximum biomass of *C. pyrenoidosa* was achieved in mixotrophic mode which could be due to external supplementation of carbon (Glucose) and dissolved inorganic carbon (NaHCO₃). The results are in agreement with earlier study which reported that mixotrophic nutrition facilitates higher biomass growth through carbon utilization (Mishra et al., 2014). In contrary, the biomass decreased in MA, HM, MH and MM modes and was found to be 2.85 g/l, 4.35 g/l, 2.37 g/l, 3.82 g/l and 5.14 g/l, respectively, at end of SP. Whilst high yield of biomass was achieved in MM in GP and SP, the decrease in stress phase of other trophic modes could be attributed due to nitrogen depletion. However, the DCW marginally increased in AM mode from 1.94 g/l at the end of growth phase (EGP) to 2.85 g/l at the end of stress phase (ESP), which could be due to supplementation of glucose + NaHCO₃ in stress phase (mixotrophic).

### 3.2. Effect of transition mode on chlorophyll enrichment in *C. pyrenoidosa*

Chlorophyll pigment, a key molecule in photosynthesis is considered as one of the indices for the biomass growth of microalgae since the biosynthesis of starch and
lipids are both dependent on active photosynthetic activity. In this investigation, the effect of transition mode on chlorophyll enrichment in \textit{C. pyrenoidosa} is assessed since the role of photosynthesis is very crucial in metabolic pathways of starch and lipid productivity except in heterotrophic mode. Chlorophyll pigmentation will be normal in autotrophic mode and hence taken as positive control while heterotrophic mode acts as negative control since light is not provided. In MM, predominant chlorophyll pigmentation is observed which could be due to supplementation of both organic and inorganic carbon (glucose + NaHCO$_3$).

In AM, the chlorophyll content in GP yielded 1.38 mg/mg which is considered as the optimum value (positive control), whereas in HM, very less amount of chlorophyll 0.54 mg/mg was observed in the absence of light. High chlorophyll was obtained in GP of MM (1.92 mg/mg). The results suggest that trophic transition mode (AM, MA, HM and MH) has a remarkable role in chlorophyll accumulation in SP (Fig. 3). Increase in chlorophyll was observed in AM and HM from 1.38 mg/mg to 2.08 mg/mg and 0.54 mg/mg to 2.26 mg/mg, respectively. This is due to mixotrophic condition in stress phase, augmented with glucose and NaHCO$_3$ which helps in enhancing the synthesis of enzymes related to the

\textbf{Fig. 2.} Biomass growth rate of \textit{C. pyrenoidosa} in Growth and Stress phases. (MA = mixotrophic to autotrophic mode transition; MH = mixotrophic to Heterotrophic mode transition; MM = Mixotrophic to mixotrophic mode transition; AM = autotrophic to mixotrophic mode transition; HM = Heterotrophic to Autotrophic mode transition; GP = growth phase; SP = stress phase).
photosynthetic activity (Lohman et al., 2015). On the other hand, decrease in chlorophyll content in SP of MH is due to absence of light which was found to be 1.78 and 1.43 mg/mg, respectively, in GP and SP. In MM maximum chlorophyll (1.92 mg/mg) was obtained in GP and 3.72 mg/mg in SP, which is due to external supplementation of CO2 in dissolved form (NaHCO3) and also organic carbon catabolism that also ends with CO2 as a by-product. This condition eventually could have resulted in hiked CO2 levels in the media which tends to the enrichment of pigments in both the phases.

3.3. Nutrient utilization for biomass productivity of C. pyrenoidosa

Nitrates and phosphates are vital nutrients required for the growth of microalgae since they are essential for the growth and division of the cell. However, the physiological advantage lie in the fact that nitrogen and phosphate depletion leads to the synthesis of the storage molecules like starch and lipid in C. pyrenoidosa.

Considerable variation in the composition of vital biochemical constituents under conditions of nutrient limitation can be observed in algae. These variations crucially depend upon the type and degree of nutrient limitation. In general, there exists a linear and positive correlation between the growth rate of algae and the
uptake rate of the most limiting nutrient (Subhash and Mohan, 2015). In the present study, we examined the uptake of nitrates and phosphates of the two key nutrients that play a significant role in the biomass and lipid productivity of *C. pyrenoidosa*.

### 3.4. Nitrates and phosphates

Nitrogen and phosphate are two vital macronutrients required for the growth and metabolism of algal cells (Adams and Bugbee, 2014). Nitrogen is the backbone of all the naturally amino acids that are involved in protein and nucleic acids synthesis, vitamins as it is a fundamental element. Nitrates are easily utilized by microalgae for synthesis of cellular growth proteins which are responsible for increase in biomass suggesting that the concentration of nitrates play vital role in the biomass growth in algal cells. Fig. 4a illustrates that maximum utilization of nitrates (97.5%) was observed in mixotrophic mode followed by heterotrophic mode (96.62%) and autotrophic mode (85.72%). In *C. pyrenoidosa* the maximum biomass productivity observed in MM > HM > AM can be attributed to utilization of the nitrates in the formation of organic nitrogen in their cell tissues (Devi and Mohan, 2012).

Phosphates play a vital role in metabolism like DNA synthesis and energy generation (ATP) which is directly related to cell division and biomass growth (Adams and Bugbee, 2015). The uptake of phosphates too demonstrated a similar pattern as in nitrates with the maximum uptake of 99.75%, 99.6% and 98.6% in heterotrophic, mixotrophic and autotrophic modes of trophic transition, respectively (Fig. 4b). Among the nutrients evaluated, phosphates documented slightly higher uptake (99.75%), compared to nitrates (97.5%) indicating that both the nutrients are required for the growth of *C. pyrenoidosa*.

### 3.5. Role of nitrogen in accumulation of starch and lipid in *pyrenoidosa*

Nitrogen depletion triggers metabolic changes characterized by insufficient carbon source which eventually end up in lower starch levels (Li et al., 2015a,b). Under nitrogen starvation, the limited endogenous nitrogen present in algal cells is rapidly depleted negatively influencing the proteins/enzymes involved in starch biosynthesis and its accumulation (Alonso et al., 2000). To overcome this limitation of less starch content in SP, carbon source was supplemented to enhance starch content which triggers its conversion to lipids.

The starch content in GP was 46 mg/g, 182 mg/g and 203 mg/g in autotrophic, heterotrophic and mixotrophic modes, respectively (Fig. 5a). As the external carbon was supplemented in SP, starch accumulation peaked up to a certain level and subsequently decreased. This could be due to simultaneous synthesis of starch.
and its (Li et al., 2015a,b) utilization. This trend was not observed in MA condition which is autotrophic in stress phase.

Lipid accumulation usually precedes that of starch, while lipid content rapidly increases after depletion in starch (Li et al., 2015a,b). But according to an earlier report (Fan et al., 2011), lipid accumulation in N-starved cells strictly depends on carbon augmentation, and that lipid content progressively increases along with

**Fig. 4.** (a) Uptake of nitrates by *C. pyrenoidosa* in growth phase. (b) Phosphate utilization of *C. pyrenoidosa* in growth phase. (MA = mixotrophic to autotrophic mode transition; MH = mixotrophic to Heterotrophic mode transition; MM = Mixotrophic to mixotrophic mode transition; AM = autotrophic to mixotrophic mode transition; HM = Heterotrophic to Autotrophic mode transition).
glucose concentration. The present experiment also demonstrated a similar trend wherein a parallel increment was observed in both starch and lipid. The total lipid content at ESP was 17.5%, 24.3%, 21.18%, 26.6% and 28.4% and neutral lipid content was 6.14%, 9.4%, 11.02%, 16.43%, and 15.02% in AM, MA, HM, MH, MM.

**Fig. 5.** (a) Carbohydrate accumulation in various trophic transition modes of *C. pyrenoidosa*. (b) Lipid accumulation in *C. pyrenoidosa* at the end of stress phase in various trophic transition modes. (MA = mixotrophic to autotrophic mode transition; MH = mixotrophic to Heterotrophic mode transition; MM = Mixotrophic to mixotrophic mode transition; AM = autotrophic to mixotrophic mode transition; HM = Heterotrophic to Autotrophic mode transition).

glucose concentration. The present experiment also demonstrated a similar trend wherein a parallel increment was observed in both starch and lipid. The total lipid content at ESP was 17.5%, 24.3%, 21.18%, 26.6% and 28.4% and neutral lipid content was 6.14%, 9.4%, 11.02%, 16.43%, and 15.02% in AM, MA, HM, MH.
and MM, respectively (Fig. 5b). This suggests that algal cells in N-depleted conditions utilize carbon sources differently, and that other factors beyond carbon availability determine the upper limits of lipid accumulation. This finding is in agreement with the earlier data (Li et al., 2011) which also reported parallel starch and lipid accumulation patterns in the microalga *Pseudochlorococcum*, suggesting that algal cells utilize starch both as a short-term carbon source and energy storage product. Therefore, it appears that nitrate level regulates both starch and lipid accumulation in algal cultures independent of trophic mode transition. Since more concentration of nitrates and phosphates helps in biomass production and their absence cause stress and induces lipidogenesis, this study is carried out by trophic transition mode. The results of lipid percentages at the end of growth phase were less compared to end of stress phase strongly suggesting that nitrogen starvation helps in lipidogenesis.

3.6. Effect of transition mode, photosynthesis and low light intensity on lipid productivity in *Chlorella pyrenoidosa*

3.6.1. Transition mode

In the present study, we made an attempt to examine the effect of mixotrophic transition mode. Trophic mode transition resulted in biomass production in GP and lipids in SP. The overall lipid productivity in MM was 284 g/kg followed by 266 g/kg in MH and 243 g/kg in MA. This could be attributed to the partitioning of excess carbon from photosynthesis into the lipid synthesis pathway (Suen et al., 1987). Fig. 6a & b suggest that maximum total lipid productivity was obtained in the order of MM > MH > MA modes. The results further illustrate that neutral lipid productivity which is crucial for harnessing the biodiesel was more in MH (154.3 g/kg dry mass) > MM (150.2 g/kg dry mass) > HM (110.2 g/kg dry mass). The experimental results obtained in the present study also substantiate our

![Graphs showing biomass vs total lipid productivity and neutral lipid productivity in various trophic transition modes of C. pyrenoidosa.](http://dx.doi.org/10.1016/j.heliyon.2017.e00496)

**Fig. 6.** (a) Biomass Vs total lipid productivity in various trophic transition modes of *C. pyrenoidosa*. (b) Biomass Vs neutral lipid productivity in various trophic transition modes of *C. Pyrenoidosa*. (MA = mixotrophic to autotrophic mode transition; MH = mixotrophic to Heterotrophic mode transition; MM = Mixotrophic to mixotrophic mode transition; AM = autotrophic to mixotrophic mode transition; HM = Heterotrophic to Autotrophic mode transition).
hypothesis that maximum neutral lipid productivity is obtained in MH and MM. Trophic mode transition significantly influences the biomass and lipid productivity in *C. pyrenoidosa*. MM yielded higher biomass and lipid productivity using glucose + NaHCO₃ as carbon source both in GP and SP. In addition, low light intensities and supplementation of glucose along with NaHCO₃ in stress phase results in maximum neutral lipid productivity. The outcome of our research offers a great scope for enhancing the lipid productivity at low cost per kg biomass of the algae which is essential for harnessing biodiesel. However, large scale production by trophic transition mode (MH, HM and MM) can be operated only in closed systems like photobioreactors due to operational constraints like light and contamination.

### 3.6.2. Photosynthesis

According to Li et al. (2015a,b), in mixotrophic mode, replenishing of CO₂ through photosynthetic activity proved to be critical for biomass synthesis, which provides increased availability of carbon leading to the formation of energy storage products. On the other hand, significant enhancement in lipid production has the potential to serve as a protective mechanism in mixotrophic cells. In GP, large amount of algal biomass is produced, which is partly driven by the ATP and NADPH that are generated during photosynthesis. During N limitation (SP), cell growth and proliferation are impaired leading to the depletion of the pool of NADP⁺, the major electron acceptor for photosynthesis, due to high levels of NADPH build-up. This triggers over-oxidation of PSII reaction centre, causing photo-oxidative damage, generation of reactive oxygen species, and eventual cellular damage by oxidative stress. To avoid this scenario, increased lipid production facilitates NADPH consumption in fatty acid biosynthesis, as a compensatory metabolic pathway by the cell to protect itself from oxidative damage (Hu et al., 2008). These findings substantiate the increased lipid productivity in *Chlorella pyrenoidosa* under N depletion, trophic mode transition.

### 3.6.3. Low light intensity

As discussed supra, the photosynthetic activity plays major role in lipid synthesis. It is well known that heterotrophic mode (SP) yields higher lipid in the absence of light. However, this could directly cease other photosynthetic related activities like energy synthesis. In order to study the effect of photosynthetic activity on lipid synthesis we provided low light intensity (30 μmol m⁻² s⁻¹) in stress phase. Higher light intensity was avoided to prevent the photosynthetic machinery degradation due to intensified cellular damage, which nullifies the role of photosynthesis to fatty acid production. Low light intensities induced a positive result in MM with 28% and 26% of total lipid percentage. The lipid productivity peaked in MM which yielded high biomass and lipid content of *C. pyrenoidosa* which are found to be
5.142 g/l and 1.46 g/l, respectively. Recent research (Li et al., 2015a,b) also demonstrated that although photosynthetic light reactions help in lipid accumulation, lipid biosynthesis does not require a high light intensity in the N starved cells. Slow rate of photosynthesis coupled with NADH accumulation inhibits enzyme citrate synthase and prevents acetyl CoA from entering into the TCA cycle. Eventually, elevated acetyl CoA activates acetyl CoA carboxylase, which irreversibly converts acetyl CoA to malonyl CoA. In microalgae, this is the rate limiting step in fatty acid biosynthesis which leads to enhanced lipid accumulation (Wan et al., 2015).

3.7. Fatty acid methyl esters (FAME) composition in *Chlorella pyrenoidosa*

As high neutral lipid productivity was obtained in MH and MM modes, the triplicates of both samples were analysed for FAME composition (Table 2). The results indicate that the composition and quantity of fatty acids varied in different transition modes (MH and MM). The main factors that influenced this variation in SP were lack of light in MH, low light intensity in MM, nutrient depletion and variation of carbon source in MH (Glucose) and MM (Glucose + NaHCO₃).

Levels of saturation and unsaturation of fatty acids were almost same but there is huge variation in the unsaturated fatty acid composition in MM. The percentage of unsaturated fatty acids is high in heterotrophic than in mixotrophic conditions.

**Table 2.** Composition of FAME in MH and MM modes.

| S.no | Fattyacid          | Formula | Percentage | MH    | MM    |
|------|--------------------|---------|------------|-------|-------|
|      |                    |         |            |       |       |
| 1    | Palmitic           | C16:0   | 31.3 ± 1.2 | 28 ± 1.3 |
| 2    | Stearic            | C18:0   | 11 ± 0.9   | 8 ± 0.7  |
| 3    | Arachidic acid     | C20:0   | –          | 12 ±0.6  |
| 4    | Myristoleic acid   | 14:01   | 14.2 ±1.0  | –      |
| 5    | Palmitoleic acid   | 16:01   | 9 ± 0.8    | 10.6 ± 0.89 |
| 6    | Oleic acid         | 18:01   | 14.4 ± 0.98| 17.4 ± 0.79 |
| 7    | Eicosenoic acid    | 20:1    | –          | 6.3 ± 0.77 |
| 8    | Linolic acid       | 18:02   | 16.3 ± 0.99| 4.4 ± 0.07 |
| 9    | Eicosadienoic acid | 20:02   | –          | 8.2 ± 0.09 |
| 10   | Docosadienoic acid | 22:02   | –          | 2 ± 0.04 |
| 11   | Alpha-Linolenic acid| 18:03 | 2 ± 0.04 | 1.7 ± 0.02 |
| 12   | Gamma Linolenic acid | 18:03 | 1.7 ± 0.04 | – |
| 13   | Arachidonic acid   | 20:04   | –          | 1.2 ± 0.01 |
| Total|                    |         | 99.7       | 99.8   |
Chain elongation and high carbon number fatty acids like eicosadienoic acid C20:02, docosadienoic acid, C22:02 and arachidonic acid C 20:4 were observed in only MM or mixotrophic condition. This is due to the availability of light (Illman et al., 2000) and carbon source as glucose and dissolved CO2 where photosynthetic activity is high. Both heterotrophic and mixotrophic mode shared common fatty acids like linolic acid C18:0, alpha-linolenic acid C18:3. Overall biodiesel related fatty acids were observed more in the heterotrophic mode or MH while high amount of food supplements like omega 3 and 6 fatty acids were synthesized in MM.

4. Conclusion

The results of the present study demonstrated that trophic mode transition significantly influenced the biomass and lipid productivity in C. pyrenoidosa. We supplemented glucose + NaHCO3 as carbon source both in GP and SP in mixotrophic mode which yielded higher biomass and lipid productivity. In addition, there is a perceptible increase in the percent of neutral lipid in total lipids (58% in MH and 52% in MM). Low light intensities and supplementation of glucose along with NaHCO3 in stress phase resulted in maximum neutral lipid productivity in MH mode (154.3 g/kg dry mass) > MM mode (150.2 g/Kg dry mass) which is essential for harnessing biodiesel.

Declarations

Author contribution statement

Hari Prasad Ratnapuram: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

S.S. Vutukuru: Analyzed and interpreted the data; Wrote the paper.

Rajasri Yadavalli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

This work was funded by Department of Biotechnology, Government of India under the research grant # BT/PR13125/PBD/26/448/2015.
Additional information

No additional information is available for this paper.

References

Adams, Curtis, Bugbee, Bruce, 2014. Nitrogen retention and partitioning at the initiation of lipid accumulation in nitrogen-deficient algae. J. Phycol. 50 (2), 356–365.

Amaro, H.M., Guedes, A.C., Malcata, F.X., 2011. Advances and prospectives in using microalgae to produce biodiesel. Appl. Energy 88, 3402–3410.

Alonso, Diego López, Belarbi, El Hassan, Fernández-Sevilla, José M., Rodríguez-Ruiz, Juan, Grima, Emilio Molina, 2000. Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga Phaeodactylum tricornutum. Phytochemistry 54 (5), 461–471.

APHA, 1998. Standard Methods for Examination of Water and Waste Waters, 20th Edn. American Public Health Association/American Water works Association/Water Environment Federation, Washington, D.C.

Bligh, E., Dyer, W., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37.

Chandra, R., Rohit, M.V., Swamy, Y.V., Venkata Mohan, S., 2014. Regulatory function of organic carbon supplementation on biodiesel production during growth and nutrient stress phases of mixotrophic microalgae cultivation. Bioresour. Technol. 165, 279–287.

Chen, F., 1996. High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol. 14, 421–426.

Converti, Attilio, Casazza, Alessandro A., Ortiz, Erika Y., Perego, Patrizia, Del Borghi, Marco, 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. Chem. Eng. Proc.: Process Intensif. 48 (6), 1146–1151.

Devi, Prathima M., Mohan, S. Venkata, 2012. CO₂ supplementation to domestic wastewater enhances microalgal lipid accumulation under mixotrophic microenvironment: effect of sparging period and interval. Bioresour. Technol. 112, 116–123.

Fan, Jianhua, Ning, Kang, Zeng, Xiaowei, Luo, Yuanchan, Wang, Dongmei, Hu, Jianqiang, Li, Jing, et al., 2015. Genomic foundation of starch to lipid switch in oleaginous Chlorella. J. Plant Physiol. 169 (4).
Hena, S., Fatihah, N., Tabassum, S., Ismail, N., 2015. Three stage cultivation process of facultative strain of Chlorella sorokiniana for treating dairy farm effluent and lipid enhancement. Water Res. 80.

Hu, H.H., Li, H.Y., Xu, X.D., 2008. Alternative cold response modes in Chlorella (Chlorophyta, Trebouxiophyceae) from Antarctica. Phycologia 47, 28–34.

Illman, A.M., Scragg, A.H., Shales, S.W., 2000. increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27 (8), 631–635.

Li, Chao, Yu, Yanling, Zhang, Dawei, Liu, Jia, Ren, Nanqi, Feng, Yujie, 2015a. Combined effects of carbon, phosphorus and nitrogen on lipid accumulation of Chlorella vulgaris in mixotrophic culture. J. Chem. Technol. Biotechnol. 23, 254–261.

Li, Tingting, Gargouri, Mahmoud, Feng, Jie, Park, Jeong Jin, Gao, Difeng, Miao, Chao, Dong, Tao, Gang, David R., Chen, Shulin, 2015b. Regulation of starch and lipid accumulation in a microalga Chlorella sorokiniana. Bioreour. Technol 180, 250–257.

Li, Y., Han, D., Sommerfeld, M., Hu, Q., 2011. Photosynthetic carbon partitioning and lipid production in the oleaginous microalga Pseudochlorococcum sp. (Chlorophyceae) under nitrogen-limited conditions. Bioreour. Technol. 102 (1), 123–129.

Lee, Y.-K., 2001. Microalgal mass culture systems and methods: their limitation and potential. J. Appl. Phycol. 13, 307–315.

Lee, J.Y., Yoo, C., Jun, S.Y., Ahan, C.Y., Oh, H.M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioreour. Technol. 101, 575–577.

Lohman, E.J., Gardner, R.D., Pedersen, T., Peyton, B.M., Cooksey, K.E., Gerlach, R., 2015. Optimized inorganic carbon regime for enhanced growth and lipid accumulation in Chlorella vulgaris. Biotechnol. Biofuels 82.

Lv, Ming Jian, Cheng, Li Hua, Xu, Xin Hua, Zhang, Lin, Chen, Huan Lin, 2010. Enhanced lipid production of Chlorella vulgaris by adjustment of cultivation conditions. Bioreour. Technol. 101 (17), 6797–6804.

Markou, Giorgos, Nerantzis, Elias, 2013. Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. Biotechnol. Adv. 31 (8), 1532–1542 Elsevier Inc..

Mishra, Sanjiv K., Suh, William I., Farooq, Wasif, Moon, Myounghoon, Shrivastav, Anupama, Park, Min S., Yang, Ji Won, 2014. Rapid quantification
of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour. Technol. 155, 330–333.

Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F., 2011. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl. Energy 88 (10), 3411–3424.

Rohit, M.V., Mohan, S. Venkata, 2016. Tropho-metabolic transition during Chlorella sp. cultivation on synthesis of biodiesel. Renew. Energy 98, 84–91.

Sharma, Kalpesh K., Schuhmann, Holger, Schenk, Peer M., 2012. High lipid induction in microalgae for biodiesel production. Energies 5 (5), 1532–1553.

Singh, Poonam, Kumari, Sheena, Guldhe, Abhishek, Misra, Rohit, Rawat, Ismail, Bux, Faizal, 2016. Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renew. Sustain. Energy Rev. 55, 1–16.

Solovchenko, A.E., 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russ. J. Plant Physiol. 59 (2), 167–176.

Suen, Y., Hubbard, J.S., Holzer, G., Tornabene, T.G., 1987. Total lipid production of the green-alga Nannochloropsis sp. Qii under different nitrogen regimes. J. Phycol. 23, 289–296.

Subhash, G.V., Mohan, Venkata S., 2015. Sustainable biodiesel production through bioconversion of lignocellulosic wastewater by oleaginous fungi. Biomass Convers. Biorefin. 5 (2), 215–226.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae Dunaliella cells. J. Biosci. Bioeng. 101 (3), 223–226.

Vigeolas, Hélène, Kaymak, Esra, Duby, Francéline, Niessen, Guillaume, Motte, Patrick, Franck, Fabrice, Remacle, Claire, 2012. Isolation and partial characterization of mutants with elevated lipid content in Chlorella sorokiniana and Scenedesmus obliquus. J. Biotechnol. 162 (1), 3–12.

Wan, Minxi, Liu, Peng, Xia, Jinlan, Rosenberg, Julian N., Oyler, George A., Betenbaugh, Michael J., Nie, Zhenyuan, Qiu, Guanzhou, 2011. The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in Chlorella sorokiniana. Appl. Microbiol. Biotechnol. 91 (3), 835–844.

Wang, H., Fu, R., Pei, G., 2012. A study on lipid production of the mixotrophic microalgae Phaeodactylum tricornutum on various carbon sources. Afr. J. Microbiol. Res. 6, 1041–1047.
Yadavalli, Rajasri, Rao, C.S., Reddy, Chandrakanth, Sivasai, K.S.R., Ramgopal-rao, S., 2010. Effect of different culture media on cell concentrations of *Chlorella pyrenoidosa* under photoautotrophic conditions. IJNES 4 (3), 47–51.

Yadavalli, Rajasri, Ramgopal Rao, S., Rao, C.S., 2012. Lipid accumulation studies in *Chlorella pyrenoidosa* using customized photobioreactor- effect of nitrogen source, light intensity and mode of operation. J. Eng. Res. Appl. 2 (3), 2446–2453.