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Evaluation of indigenous microalgal isolate *Chlorella* sp. FC2 IITG as a cell factory for biodiesel production and scale up in outdoor conditions

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Abstract The present study reports evaluation of an indigenous microalgal isolate *Chlorella* sp. FC2 IITG as a potential candidate for biodiesel production. Characterization of the strain was performed under photoautotrophic, heterotrophic, and mixotrophic cultivation conditions. Further, an open-pond cultivation of the strain under outdoor conditions was demonstrated to evaluate growth performance and lipid productivity under fluctuating environmental parameters and in the presence of potential contaminants. The key findings were: (1) the difference in cultivation conditions resulted in significant variation in the biomass productivity (73–114 mg l^{-1} day^{-1}) and total lipid productivity (35.02–50.42 mg l^{-1} day^{-1}) of the strain; (2) nitrate and phosphate starvation were found to be the triggers for lipid accumulation in the cell mass; (3) open-pond cultivation of the strain under outdoor conditions resulted in biomass productivity of 44 mg l^{-1} day^{-1} and total lipid productivity of 10.7 mg l^{-1} day^{-1}; (4) a maximum detectable bacterial contamination of 7 % of the total number of cells was recorded in an open-pond system; and (5) fatty acid profiling revealed abundance of palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2), which are considered to be the key elements for suitable quality biodiesel.

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Introduction

Microalgae has gained renewed interest as a potential cell factory for production of biodiesel as these unicellular photosynthetic organisms possess a faster growth rate, higher photosynthetic efficiency, and the ability to produce more oil per acre than plants [8, 14]. The lipid content of different oleaginous microalgal species is reported to vary from 10 to 77 % of dry cell mass [8] and many strategies have been proposed to further enhance the lipid productivity and yield. However, sustainability and the economic feasibility of algae-based biodiesel calls for significant improvement of the technology that can supplant the conventional fossil fuels. For instance, large-scale algal cultivation in open raceway pond suffers from less growth rate and lipid productivity attributed to other species contamination and fluctuating environmental parameters [25]. To that end, high oil-accumulating robust strains with resistance towards changing climatic conditions and undesired contaminations are a prerequisite for sustainable process development [25]. Bioreactors could be another option that can provide axenic conditions for growth. However, inefficient light penetration and high installation cost limits the use of photobioreactors in large-scale biodiesel production under photoautotrophic conditions [25]. High cell-density microalgal cultivation processes can be achieved under heterotrophic conditions at the expense of increased production cost attributed to the organic carbon source. Neutral lipids synthesized from free fatty acids are the main precursors of interest for biodiesel production. These precursors are typically secondary metabolites, the synthesis of which are

induced under specific stress conditions such as nutrient limitations (nitrogen or phosphate), elevated temperature [9], or high light intensity [25], and hence, are not growth-associated. Therefore, biomass productivity and high lipid content of microalgae are mutually exclusive, which in turn decreases the net lipid yield [16]. Hence, the success of algae-based biodiesel production depends on three key parameters: (a) biomass productivity, (b) lipid yield, and (c) lipid productivity, which governs the techno-economic feasibility of the whole process. To that end, it is indispensable to develop an efficient process with a suitable producer strain aiming at both optimal growth and lipid productivity and its implementation on a large scale.

Chlorella sp. has been extensively characterized under photoautotrophic, heterotrophic, and mixotrophic cultivation conditions [12, 25]. With a change in growth conditions (e.g., type of carbon–nitrogen sources or nutrient starvation or repletion), microalgae modulate its metabolism towards the production of neutral lipids and biomass formation [7, 29]. For instance, the lipid content of *Chlorella protothecoides* was reported to be approximately fourfold higher under heterotrophic growth as compared to photoautotrophic conditions [43]. Further, heterotrophic growth of *Chlorella zofingiensis* resulted in accumulation of high levels of neutral lipids, whereas under photoautotrophic conditions, cells accumulated high levels of glycolipid and phospholipid [25]. Interestingly, it is not only the quantity but also the quality of lipids that varies significantly under different cultivation conditions [25]. Polyunsaturated fatty acids (PUFA) were found to be abundant when the cells were grown under photoautotrophic conditions whereas monounsaturated fatty acids (MUFA) dominated in the heterotrophic cultivation [25]. It is important to note that fractional variations of these PUFA and MUFA may have a significant effect on key fuel properties such as cloud point and cetane number [24], which in turn play a major role in meeting the specific standards of high-quality biodiesel [5]. These studies emphasize the need for characterization of microalgae under various growth conditions in order to understand the physiology of biomass formation and lipid production.

The present study reports an indigenous novel freshwater microalgal isolate *Chlorella* sp. FC2 IITG (hereafter referred as FC2) as a potential platform for biodiesel production. The isolate was evaluated in terms of growth and lipid productivity under photoautotrophic, heterotrophic, and mixotrophic conditions. Further, lipid productivity of the microorganism was obtained by transiently exposing the culture from nutrient (nitrogen and phosphate)-sufficient conditions to nutrient-starved conditions. The experiments highlight the metabolic flexibility of the strain in terms of the relationship between dynamic change in biomass composition, growth rate, nutrient utilizations, and lipid productivity. Finally, the culture was grown in outdoor conditions to evaluate its growth performance and lipid productivity under varying environmental parameters and in the presence of contaminants. The quality of fatty acid methyl esters (FAME) in terms of fatty acid compositions of the biomass under different cultivation conditions was evaluated by gas chromatography (GC). The outcome of the research points towards a scope to develop this microalgal strain as a feasible feed stock for sustainable biodiesel generation.

Materials and methods

Isolation and identification of algal culture

The strain FC2 was isolated from a freshwater pond at Guwahati, Assam, India (91°44′E longitude and 26°10′N latitude) in the month of March 2010. The sample collected was inoculated into BG11 medium comprising (g l^{-1}) $NaNO_3$, 1.5; $K_2HPO_4 \cdot 3H_2O$, 0.004; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.036; Na_2CO_3, 0.02; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; and A5 + Co solution (1 ml l^{-1}) that consists of H_3BO_3, 2.86; $MnCl_2 \cdot H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $CuSO_4 \cdot 5H_2O$, 0.079; $Na_2MoO_4 \cdot 2H_2O$, 0.390; and $Co(NO_3)_2 \cdot 6H_2O$, 0.049 [3] and incubated in an orbital shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 28 °C under 20 µmol photons m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. Axenic algal cultures were obtained through conventional streaking methods and the organisms were maintained in BG11 agar slants. Detail about the isolation method is given in the supplementary material (Table T1, supplementary material S1). While multiple algal isolates were enriched in the BG11 medium, this particular strain was selected for further characterization based on its natural propensity to accumulate neutral lipids as confirmed by Nile red-based staining method and imaging under a confocal microscope (Fig. 1a, b). Further, identification of the organism was carried out using morphometric analysis under a phase contrast microscope (Eclipse E200, Nikon, Japan) and molecular analysis via 18S rDNA sequencing. The cells were disrupted and the 18S rDNA sequence was amplified using specific universal forward primer 5′-GGTGATCCTGCCAGTAGTCATATGCTTG-3′ (ss5) and reverse primer 5′-GATCCTTCCGCAGGTTCACCT ACGGAAACC-3′ (ss3) in a thermal cycler [30]. Amplified PCR products were separated by gel electrophoresis and a gel elution kit (Sigma-Aldrich, St. Louis, MO, USA). Sequencing was performed using ABI PRISM 3700 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) and the similarity to sequences was determined using BLAST [1]. A phylogenetic tree was constructed from the 18S rDNA sequences of the isolated strain and related species using the software ClustalX 2.1 and MEGA 5.0.

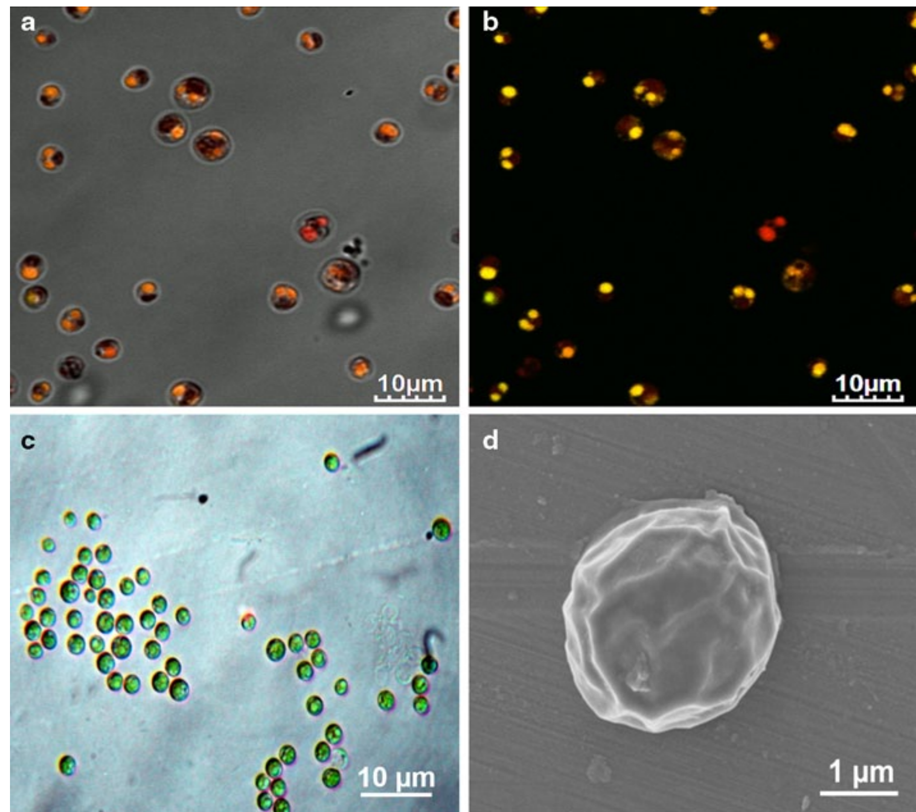

Fig. 1 Morphometric identification of *Chlorella* sp. FC2 IITG. **a** Superimposed image of bright-field cells, auto-fluorescence of cells stained with Nile red, and fluorescence from Nile red-neutral lipid complex. The images were obtained using a confocal microscope. **b** Cells showing auto-fluorescence in *red color* and Nile red-neutral lipid complex fluorescence as *golden yellow color* under a confocal microscope. **c** Cells under phase-contrast microscope and **d** field effect scanning electron microscopic image of the cell obtained at 2.0 kV EHT and 21, 600 X magnification (color figure online)

Characterization of the strain under different cultivation conditions in shake flask and automated bioreactor

All of the characterization experiments were performed using 1 % (v/v) inoculum with absorbance (A_{690}) of 1.0 (~2.0×10^7 no. of cells). Preliminary shake-flask experiments were performed to study the effect of medium pH, temperature, nitrogen, and carbon sources on growth of the organism in BG11 medium. The strain was subjected to grow under different pH levels of the medium (2, 4, 6, 8, and 10), different temperatures (20, 28, 36, and 44 °C), and different nitrogen sources (ammonium chloride, ammonium sulfate, sodium nitrate, sodium nitrite, urea, and glycine) with equimolar concentration of nitrogen (0.018 mM). These experiments were performed under photoautotrophic nutrition in the orbital shaker at 150 rpm, 28 °C, under 20 μmol photons m^{-2} s^{-1} light intensity with a light:dark cycle of 16:8 h. Further, selection of carbon source was carried out under heterotrophic conditions with nine different carbon sources, which includes glucose, sodium acetate, glycerol, sucrose, fructose, maltose, lactose, mannitol, and yeast extract (Himedia, India) at a concentration of 15 g l^{-1}. Further detail regarding shake-flask characterization is given in the supplementary material (S2).

In the next step, detailed characterization of the strain was performed under photoautotrophic (CO_2 1 % v/v), heterotrophic (glucose 15 g l^{-1}), and mixotrophic (maintained at glucose >1.8 g l^{-1} and CO_2 1 % v/v) cultivation conditions (supplementary material S2) in a 3-l automated bioreactor (Bio Console ADI 1025, Applikon Biotechnology, Holland) with a working volume of 1.25 l of BG11 medium.

Effect of nitrate and phosphate starvation on growth and lipid productivity of FC2

Two-stage cultivation was employed to study the effect of nitrate and phosphate starvation on biomass and lipid productivity of FC2. In the first stage, the algal cells were grown under photoautotrophic conditions in the nutrient-sufficient BG11 medium to obtain a biomass concentration of 0.63 g l^{-1} (equivalent to absorbance 3.0). In the second stage, the cells were collected through centrifugation and resuspended in the same volume of nutrient-starved medium. Details about nitrate and phosphate starvation study are given in the supplementary material (S3).

Characterization of the strain in open pond under outdoor conditions

The effect of fluctuating environmental conditions on the performance of growth and lipid productivity of FC2 was evaluated by growing the organism in an open-pond system

(length, 1.39 m; width, 0.99 m; depth, 0.38 m) under outdoor conditions. The experiment was performed using 30 l of seed culture of absorbance 1.0 as inoculum for 300 l of BG11 medium. The compressed air was purged through perforated tubing and the pH of the medium was maintained between 6 and 8 by continuous feeding of CO_2 mixed with air. Contamination of the pond with other microalgae was measured with a microscope through cell counting, whereas the fungal and bacterial contaminants were quantified using serial dilution plating and colony counting in BG11 medium supplemented with 15 g l^{-1} of glucose. The dynamic profile of growth and neutral lipid accumulation was obtained via analysis of the sample collected at 24-h time intervals. The variations in light intensity and the temperature were recorded every hour. The period of cultivation was from March 11, 2013 to March 27, 2013. Further detail is given in the supplementary material (S4).

Analysis of growth, substrates utilization, and biomass composition

Analysis of growth, utilization of substrates, and biomass composition were carried out at every sampling time point. A known volume of sample was centrifuged at 8,000×*g* for 10 min at 4 °C and the supernatant was collected for extracellular substrates analyses. The pellet was utilized for the analysis of biomass compositions.

Cell density was monitored by measuring the absorbance at 690 nm (A_{690}) using a UV–visible spectrophotometer (Cary 50, Varian, Australia). The absorbance values were converted into dry cell weight (DCW) through appropriate calibration equations. For photoautotrophic and heterotrophic conditions, one cell density = 0.21 g dry cells l^{-1} ($R^2 = 0.99$) and for mixotrophic conditions, one cell density = 0.27 g dry cells l^{-1} ($R^2 = 0.98$). Estimation of nitrate in the supernatant was carried out using the salicylic acid method [4] with sodium nitrate as the standard. Phosphate estimation was carried out using the ascorbic acid method with potassium hydrogen phosphate (dibasic) as standard [34]. Glucose estimation in the medium was performed using dinitrosalicylic acid method [31].

Intracellular biomass composition analysis

Estimation of carbohydrate fraction in the biomass was performed by phenol sulfuric acid method with glucose as standard [11]. For protein estimation, cell pellets were subjected to alkaline hydrolysis by boiling with 2 N NaOH at 100 °C for 15 min and then neutralizing to pH 7.0 by adding 1.6 N HCl [36]. The neutralized solution was used for protein estimation using Lowry's method with bovine serum albumin as the standard [26]. The chlorophyll estimation was carried out using the method provided by Pruvost et al. [36]. Total chlorophyll content of the cells was expressed as the sum of chlorophyll *a* and *b*. For Nile red-based neutral lipid analysis, cell pellet with an absorbance 0.7 resuspended in 1.0 ml of 25 % (v/v) dimethyl sulfoxide was used. Nile red was added to the resuspended pellets at a concentration of 4 μg ml^{-1} and incubated at 50 °C in a water bath for 1 min. The fluorescence spectra were obtained in a spectrophotometer (Fluoromax 3, Horiba, USA) with excitation at 480 nm and emission in the region 550–650 nm. The auto-fluorescence of algal cells and the intrinsic fluorescence of Nile red were subtracted from the fluorescence of Nile red neutral lipid complex obtained at 580 nm. Triolein (Supelco, USA) was used as standard for Nile red-based neutral lipid estimation. The dynamic profile of neutral lipid accumulation in the biomass was obtained by Nile red-based assay method and the total FAME along with fatty acid composition were obtained from GC analysis as described below.

FESEM and confocal microscopic analysis

The field-effect scanning electron microscope (FESEM) analysis was performed for morphometric analysis of the cells and the confocal microscopic analysis was carried out to visualize the intracellular neutral lipid bodies in the algal cells. Details regarding FESEM analysis are given in the supplementary material (S5). For confocal microscopic analysis, the algal cells were stained with Nile red. The stained cells were loaded on a slide and viewed with a confocal microscope (LSM Meta 510, Zeiss, Germany) at 63× oil immersion.

Quantification of total lipid and composition analysis using GC-FID

FAME analysis was carried out for the freeze-dried algal cell mass harvested at the end of the batch. A sequential two-step direct transesterification method was employed, which involves alkali catalyst in the first step and acid catalyst in the second step (supplementary material S6). The reaction mixtures after direct transesterification were cooled down to room temperature after completion of the first and second incubation. Deionized water and hexane of equal volume (1.0 ml) were added to the cooled trans-esterified mixture to obtain the FAME in the hexane layer. The hexane layer was washed twice with water to remove any aqueous impurities and used directly for GC analysis after filtration through a 0.2-μm filter. FAME was analyzed directly in GC equipped with a flame ionization detector (GC-FID, Varian 450, Netherlands) and SLB-IL100 column (30 × 0.25 mm i.d., 0.20-μm film thickness). FAME

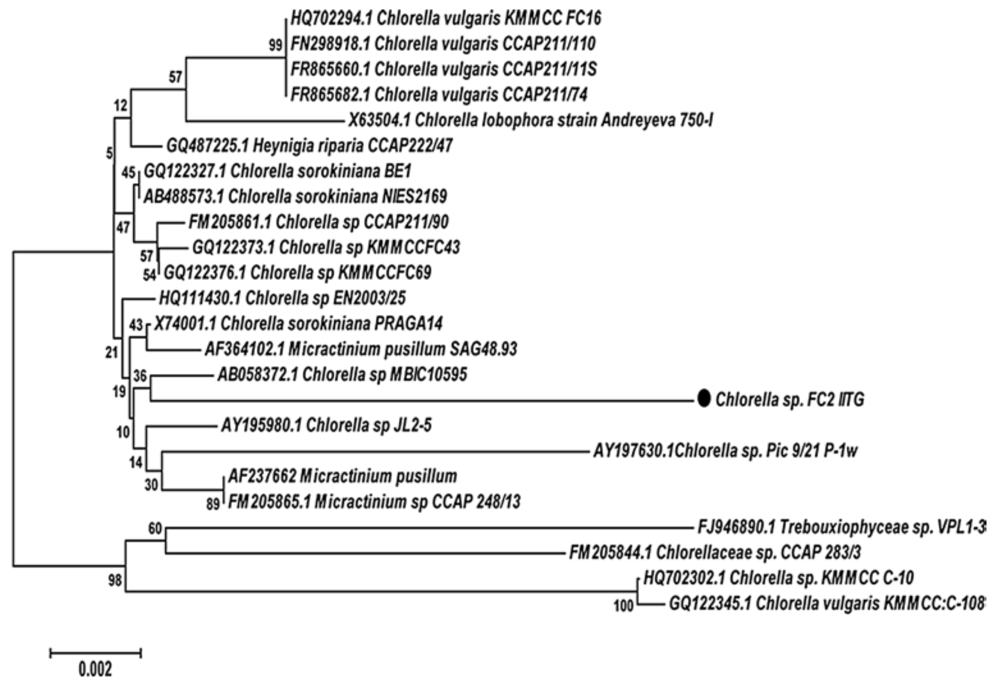

Fig. 2 Molecular analysis of *Chlorella* sp. FC2 IITG. The phylogenetic tree was based on 18S rDNA sequences of the strain and genus within the order Chlorellales. The tree was constructed using the neighbor-joining method with the Jukes–Cantor model. Bootstrap test (1,000 replicates in %) is shown next to the branches and the taxon name starts with the gene accession number. The isolated strain reported in the present study is marked with a *filled circle*

mix C14–C22 (Supelco, USA) was used as the standard for GC-FID and the lipid quantified using this method represents the total lipid of the biomass in % (w/w, DCW). Further details regarding GC analysis are given in the supplementary material (S6).

Statistical analysis

All the experiments were conducted in triplicate and the data are expressed as mean ± standard error. The significant difference in growth, substrate utilization, and biomass compositions at every time point under photoautotrophic, heterotrophic, and mixotrophic cultivation conditions were analyzed through one-way analysis of variance (ANOVA).

Results and discussion

Morphometric and molecular identification of the organism

The colonies on the BG11 agar plate were green in color, spherical convex in shape, and shiny with regular edges. Microscopic observation under a phase-contrast microscope showed that the cells were unicellular, green colored, spherical in shape, and measured about 3–5 µm in diameter (Fig. 1c). FESEM imaging further confirmed the absence of spikes and flagella over the cell surface avoiding confusions with *Micractinium pusillum* (Fig. 1d). The partial 18S rDNA gene (1,609 bp in length) sequence coding for the ribosomal RNA of the strain was sequenced and submitted to GenBank (Accession Number: JX154075). BLAST analysis showed that the strain is the closest relative to *Chlorella* sp. with maximum sequence similarity of 99 %. A phylogenetic tree was constructed based on 18S rDNA sequence of the strain and 24 organisms under the order Chlorellales with 98–99 % similarity (Fig. 2). The figure illustrates that the strain is closely related to *Chlorella* sp. MBIC10595. The isolated strain was designated as *Chlorella* sp. FC2 IITG.

Effect of pH, temperature, and carbon and nitrogen sources on growth of FC2

The strain was able to grow over a wide range of pH (4–10) and its optimal growth was observed in between pH 6 and 8 (Fig. 3a). The ability of the strain to grow at higher alkaline pH may provide us an opportunity for contamination-free cultivation in outdoor conditions, where biological contaminants can be reduced by increasing the pH of the medium while maintaining active concentrations of the desired microbe [33]. Even though the strain could grow over a wide range of temperatures (Fig. 3b), maximum biomass concentration was obtained at 28 °C, which is lower than the other *Chlorella* sp. reported in the literature. For instance, an optimum temperature of 30 °C was reported for a new isolate, *Chlorella sorokiniana* [42], and even a higher optimum temperature (37–40 °C) was reported for other *Chlorella* sp. [10]. While the survival of the organism over a wide pH range points towards metabolic flexibility of the strain in terms of its growth, a lower optimum temperature depicts a unique feature of the strain as a new

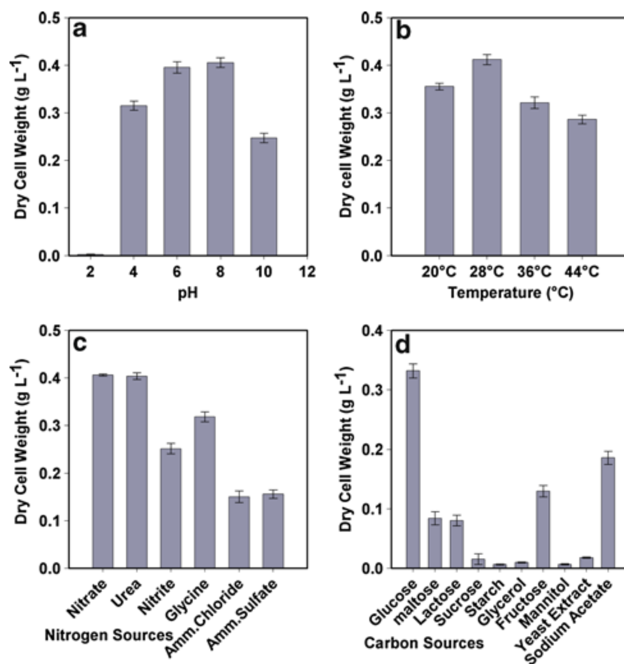

Fig. 3 Effect of various parameters on growth of the strain *Chlorella* sp. FC2 IITG: **a** pH of the medium; **b** temperature; **c** different nitrogen sources, and **d** different carbon sources

subclass [42]. Sodium nitrate and urea were found to be the best nitrogen sources that resulted in highest growth (Fig. 3c). Several cases of the literature support nitrate as the favorable source of nitrogen for the growth of *Chlorella* [39], *Scenedesmus* [2], and *Neochloris oleoabundans* [23]. The rest of the nitrogen sources, glycine, ammonium chloride, ammonium sulfate, and sodium nitrite, were found to be suboptimal for growth. Uptake of ammonia from the medium is coupled with release of H^+ ions into the medium, which may be attributed to lesser growth and early reach of the stationary phase [18]. The strain was further studied under heterotrophic conditions to evaluate its ability to utilize different carbon sources (Fig. 3d). Glucose was found to be the optimal carbon source for growth with the highest biomass titer, followed by sodium acetate and fructose. Maltose and lactose were found to be suboptimal carbon sources for growth. Negligible growth was observed for sucrose, starch, glycerol, mannitol, and yeast extract. Glucose and sodium acetate was reported to support higher growth for *C. zofingiensis* [40] and *C. vulgaris* [15], respectively. Mannitol [19] and glycerol [15] were found to inhibit growth for most of the *Chlorella* species. Further characterization of the organism under different cultivation conditions was carried out at a temperature of 28 °C and pH of 6–8 with nitrate as the nitrogen source. Glucose was used as the carbon source in heterotrophic and mixotrophic conditions.

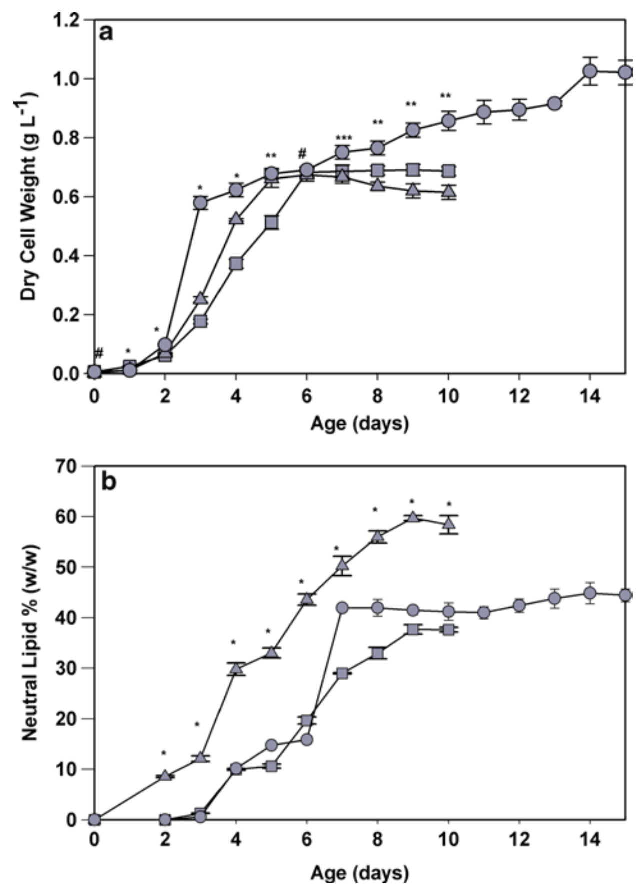

Fig. 4 Dynamic profiles for growth, changes in intracellular neutral lipid composition of the strain *Chlorella* sp. FC2 IITG under photoautotrophic, heterotrophic, and mixotrophic conditions: **a** growth; **b** neutral lipid percentage in the biomass. The strain was grown under photoautotrophic (*closed square*), heterotrophic (*closed upper triangle*), and mixotrophic (*closed circle*) conditions on BG11 medium at 28 °C and 400 rpm in a 3-l automated bioreactor. *Different symbols* indicate significant differences between growth conditions obtained from one way analysis of variance (ANOVA) (*asterisk* represents p value = 0; *double asterisk* represents $0 < p < 0.05$; *triple asterisk* represents $0.05 < p < 0.5$ and *hash symbol* represents $0.5 < p < 1.0$)

Characterization of FC2 under photoautotrophic, heterotrophic, and mixotrophic conditions in an automated bioreactor

Biomass and lipid productivity of the organism FC2 were evaluated under photoautotrophic, heterotrophic, and mixotrophic conditions. Among the three cultivation conditions, the highest biomass titer (1.03 g l^{-1}) was achieved for mixotrophic conditions (Fig. 4a). This biomass titer was ~1.5-fold higher than that achieved for both photoautotrophic and heterotrophic conditions (Table 1). An improved biomass concentration was reported for *C. sorokiniana* [41], *Nannochloropsis oculata*, [41] and *C. vulgaris* [15] when grown under mixotrophic conditions in

Table 1 Kinetic parameters for growth and lipid formation of *Chlorella* sp. FC2 IITG cultivated under photoautotrophic, heterotrophic, and mixotrophic cultivation conditions

Parameters	Cultivation conditions		
	Photoautotrophic	Heterotrophic	Mixotrophic
Specific growth rate μ_{exp} (day^{-1})	0.92 ± 0.007[a]	0.97 ± 0.009[a]	0.97 ± 0.004[a]
Dry cell mass (g l^{-1})	0.69 ± 0.027	0.67 ± 0.014	1.03 ± 0.011
Biomass productivity (mg l^{-1} day^{-1})	114 ± 0.003[b]	112 ± 0.003[b]	73 ± 0.003[b]
Neutral lipid (%, w/w DCW)	37.64 ± 0.79	59.69 ± 0.53	44.83 ± 0.16
Neutral lipid productivity (mg l^{-1} day^{-1})	28.88 ± 0.725[c]	45.80 ± 0.40[c]	35.37 ± 1.68[c]
Total lipid (%, w/w DCW)	45.18 ± 3.11	64.52 ± 0.13	68.75 ± 0.009
Total lipid productivity (mg l^{-1} day^{-1})	35.02 ± 0.003[d]	48.95 ± 0.001[d]	50.42 ± 0.006[d]

Values are mean ± SE ($n = 3$)

[a] Specific growth rate μ_{exp} was calculated as average in the exponential phase of growth (0–5 days of cultivation) for all three cultivation conditions

[b] Data from 6 days of cultivation were used to calculate biomass productivity under photoautotrophic and heterotrophic conditions. Data from 14 days of cultivation were used to calculate biomass productivity under mixotrophic conditions

[c, d] Lipid productivity was calculated based on the data from 9 days of cultivation under photoautotrophic and heterotrophic growth, whereas for mixotrophic conditions, data from 14 days of cultivation were used

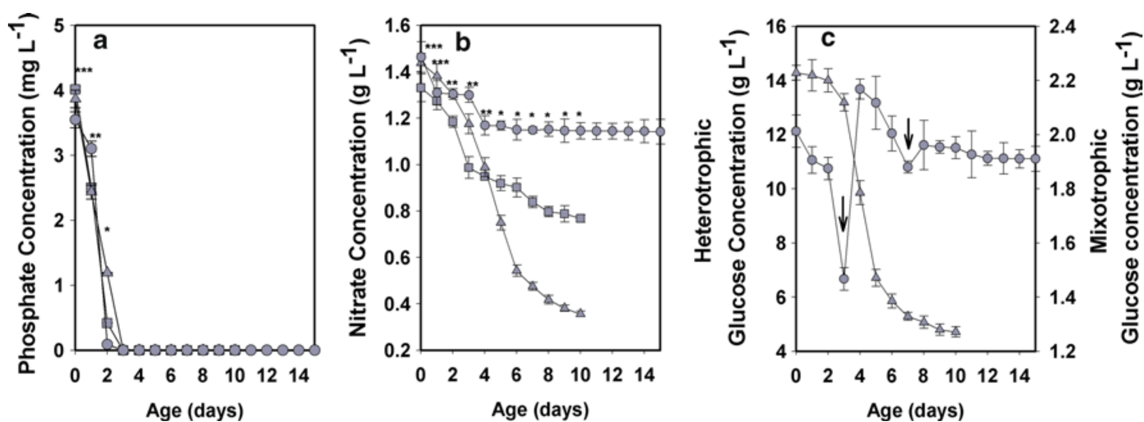

Fig. 5 Substrate-utilization profile of the strain *Chlorella* sp. FC2 IITG grown under photoautotrophic (*closed square*), heterotrophic (*closed upper triangle*), and mixotrophic (*closed circle*) conditions: **a** phosphate utilization; **b** nitrate utilization, and **c** glucose utilization for heterotrophic and mixotrophic conditions. The *arrow* in **c** indicates the time of glucose feeding in mixotrophic conditions. Glucose feeding was performed when the concentration of glucose in the medium was <1.8 g l^{-1}. *Different symbols* indicate significant differences between culture conditions for the strain obtained from statistical analysis (for details refer to the legend of Fig. 4)

comparison to photoautotrophic conditions. Mixotrophic cultivation provides an opportunity to the strain to follow both a heterotrophic route and a light-dependent route of growth in a simultaneous and independent manner [38]. Therefore, growth is not strictly limited by the availability of light or availability of carbon source in the medium, as is the case for photoautotrophic and heterotrophic growth, respectively. However, the biomass productivity of the strain under mixotrophic conditions (73 mg l^{-1} day^{-1}) was found to be less than photoautotrophic (114 mg l^{-1} day^{-1}) and heterotrophic (112 mg l^{-1} day^{-1}) conditions. This was attributed to the longer cultivation period (14 days) required to achieve the maximum biomass concentration in case of mixotrophic conditions as opposed to the other two cultivation conditions (6 days). While the maximum achievable biomass concentration in photoautotrophic conditions was restricted by the combined effect of phosphate exhaustion from the medium (Fig. 5a) and insufficient photon flux attributed to cell shading effect [6], in the case of heterotrophic conditions, the same was limited by both phosphate unavailability and nitrogen source limitation [27] (Fig. 5b). Under mixotrophic conditions, the organism was able to grow beyond the 6th day of cultivation by utilizing glucose as the source of energy even under phosphate exhaustion (Fig. 5a) and photon limitation. This is evident form the glucose-uptake profile showing utilization until the 8th

day of cultivation under mixotrophic conditions with the requirement of two pulse additions (on the 3rd day and 7th day) of glucose in the medium (Fig. 5c). Intermittent addition of glucose for mixotrophic cultivation of *Chlorella* sp. and *Nannochloropsis* sp. was found to enhance the biomass productivity [6]. Given that the comparison of biomass productivity of the present strain with the available literature may be difficult owing to markedly different growth conditions and cultivation systems, a detailed comparison of the biomass productivity of FC2 with other microalgae is given in the supplementary materials (Table T2, Supplementary material).

The difference in cultivation conditions resulted in significant variation in the neutral lipid productivity (28.88–45.80 mg l^{-1} day^{-1}) and total lipid productivity (35.02–50.42 mg l^{-1} day^{-1}) of the strain (Table 1). Neutral lipid accumulation under different cultivation conditions was captured by Nile red-based assay method (Fig. 4b) and total lipid content in terms of FAME was obtained from GC analysis. The highest neutral lipid productivity and neutral lipid content was obtained for heterotrophic culture followed by mixotrophic and photoautotrophic conditions (Table 1). However, the total lipid content and productivity was found to be highest for mixotrophic conditions. Irrespective of the cultivation conditions, the total lipid productivity of our strain under mixotrophic growth was found to be higher than most of the values reported in the literature, with exception of a few microalgal strains (Table T2, Supplementary material). Under heterotrophic conditions, a 2.75-fold increment in neutral lipid content was reported for *C. zofingiensis* [25] in comparison to photoautotrophic growth, which supports the present findings. In contrast, no significant difference in lipid content was reported for four different strains tested under mixotrophic, heterotrophic, and photoautotrophic conditions [6]. Hence, it is possible that the lipid accumulation property of the cells is strain-dependent [6]. The intracellular neutral lipid induction may be attributed to the exhaustion of some of the nutrients during transition from growth phase to the stationary phase of the cultivations. For instance, the utilization profile of the substrates under all three cultivation conditions (Fig. 5a) showed that phosphate was consumed completely within 3 days of cultivation and hence, creating nutritional stress conditions for the cells. Further, in our study, a higher specific growth rate of the strain under heterotrophic conditions leads to faster utilization of nitrate during the exponential growth phase and in turn resulted in lower concentration of nitrate in the medium in comparison to the photoautotrophic and mixotrophic conditions (Fig. 5b). This concentration of nitrate (0.4 g l^{-1}) may be rate-limiting for growth under heterotrophic conditions [27]. A large pool of literature has demonstrated that phosphate and nitrate starvation (or limitation) serve as drivers for lipid accumulation in the microalgal strains [13, 17, 37]. In the present study, a similar trend was observed between nitrate or phosphate utilization and neutral lipid induction. Therefore, we hypothesized that the higher induction of neutral lipid under photoautotrophic and mixotrophic growth may be due to phosphate starvation. In case of heterotrophic growth, it may be due to phosphate starvation and nitrate limitation. In order to prove this hypothesis, the organism was further tested under phosphate and nitrate starvation.

Effect of nitrogen and phosphate starvation on lipid productivity

As a proof for hypothesis that the phosphate and nitrate starvation serve as the drivers for lipid induction in FC2, the lipid productivity of the cells was evaluated under photoautotrophic conditions by transiently exposing the cells from nutrient-sufficient conditions to nutrient (nitrate or phosphate)-starved conditions. The dynamic profile of biomass formation, neutral lipid accumulation, and nitrate and phosphate utilization was obtained for both nitrate (Fig. 6) and phosphate (Fig. 7)-starvation experiments. Induction of neutral lipid was observed only when the cells were exposed to the nutrient-starved conditions and no accumulation of neutral lipid was observed under nutrient-sufficient conditions. A sharp increase in the neutral lipid content from 1.19 to 22.47 % (w/w, DCW) was observed in the initial 24 h of nitrate-starvation phase and reached a maximum neutral lipid content of 54.4 % (w/w, DCW) on the 7th day of starvation (Fig. 6b). In case of phosphate starvation, maximum neutral lipid accumulation of 28.60 % (w/w, DCW) was recorded on the 7th day of cultivation under starved conditions (Fig. 7b). These results point toward the significance of nitrate and phosphate starvation as triggers for neutral lipid accumulation in the cell mass. A significant increase in lipid accumulation was reported for *Nannochloropsis* sp. [37] and *Chlorella* sp. BUM11008 [35] when the cultures were transferred from nitrate-sufficient to nitrate-depleted conditions. Further, an elevated accumulation of intracellular lipid content was reported for *C. zofingiensis* grown under phosphate starvation [13] and for *Monodus subterraneus* grown under phosphate limitation [20]. Neutral lipid productivity of 71.9 and 60.8 mg l^{-1} day^{-1} was obtained for growth (7 days in starvation) of FC2 under phosphate and nitrate starvation, respectively. The total lipid productivity of 85.56 and 63.45 mg l^{-1} day^{-1} was obtained under phosphate-starved and nitrate-starved conditions, respectively. Comparison of lipid productivity under nutritional stress conditions for our strain with the available literature revealed that this organism can serve as a superior platform for lipid overproduction in comparison to the majority of algal strains. However, the lipid productivity of the FC2 was lower in

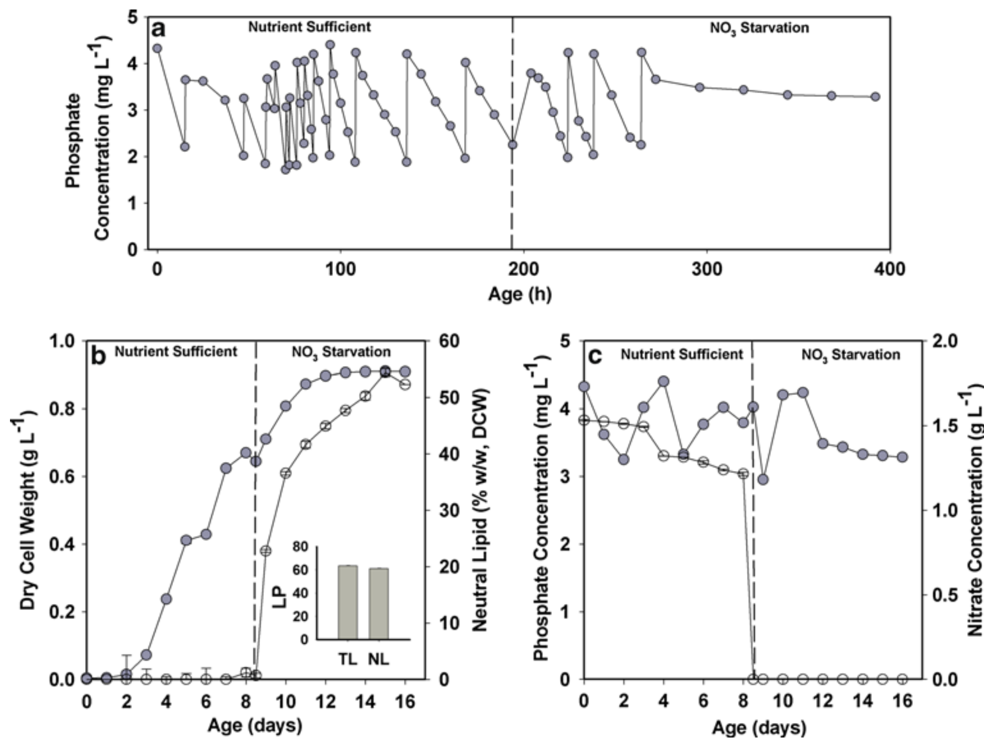

Fig. 6 Dynamic profiles for growth, neutral lipid accumulation, and substrate utilization of the strain *Chlorella* sp. FC2 IITG grown under nutrient-sufficient and nitrate-starvation conditions: **a** phosphate utilization and feeding; **b** growth (*closed circle*) and neutral lipid percentage in the biomass (*open circle*); **c** nitrate (*open circle*) and phosphate utilization (*closed circle*). The figure in the *inset* of **b** shows the total lipid productivity (TL) and neutral lipid productivity (NL) in mg l^{-1} day^{-1} during the starvation phase. The lipid productivity (LP) was calculated as $\frac{(L_f \times X_f)-(L_i \times X_i)}{\Delta t \times 100}$ where, L_i—lipid content in % DCW at the start of the starvation phase; L_f—lipid content in % DCW at the end of the starvation phase; X_i—Initial concentration of biomass under starvation phase; X_f—final concentration of biomass under starvation phase; Δt is duration of starvation phase

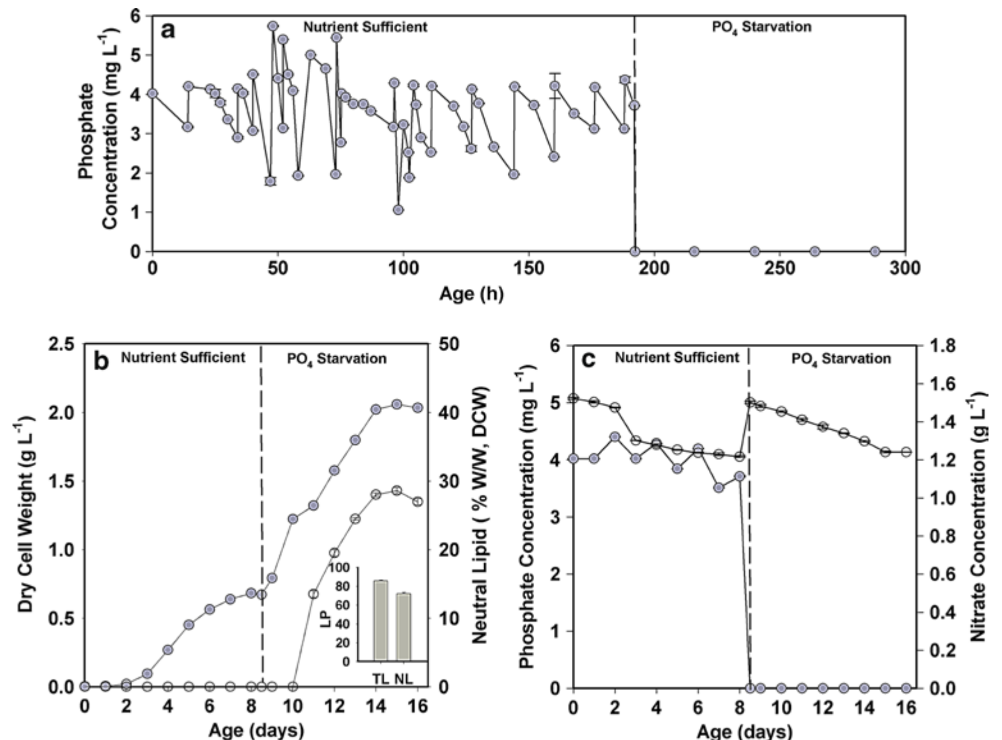

Fig. 7 Dynamic profiles for growth, neutral lipid accumulation, and substrate utilization of the strain *Chlorella* sp. FC2 IITG grown under nutrient-sufficient and phosphate-starvation conditions: **a** phosphate utilization and feeding; **b** growth (*closed circle*) and neutral lipid percentage in the biomass (*open circle*); **c** nitrate (*open circle*) and phosphate utilization (*closed circle*). The figure in the *inset* of **b** shows the total lipid productivity (TL) and neutral lipid productivity (NL) in mg l^{-1} day^{-1} during the starvation phase. For lipid productivity (LP) calculation, refer to the legends of Fig. 6

Table 2 Intracellular composition of macromolecules in the strain *Chlorella* sp. FC2 IITG grown under nutrient-sufficient phase and nutrient-starved phase

Conditions	Sufficient phase (8th day)			Starvation phase (14th day)		
	Carbohydrate (%, DCW)	Protein (%, DCW)	Neutral lipid (%, DCW)	Carbohydrate (%, DCW)	Protein (%, DCW)	Neutral lipid (%, DCW)
Nitrate starvation	45.57 ± 1.2	41.21 ± 1.6	1.19 ± 0.59	18.32 ± 1.3	22.31 ± 1.1	54.41 ± 0.69
Phosphate starvation	47.35 ± 0.8	40.65 ± 1.3	1.00 ± 0.01	32.21 ± 1.4	27.19 ± 0.7	28.60 ± 0.47

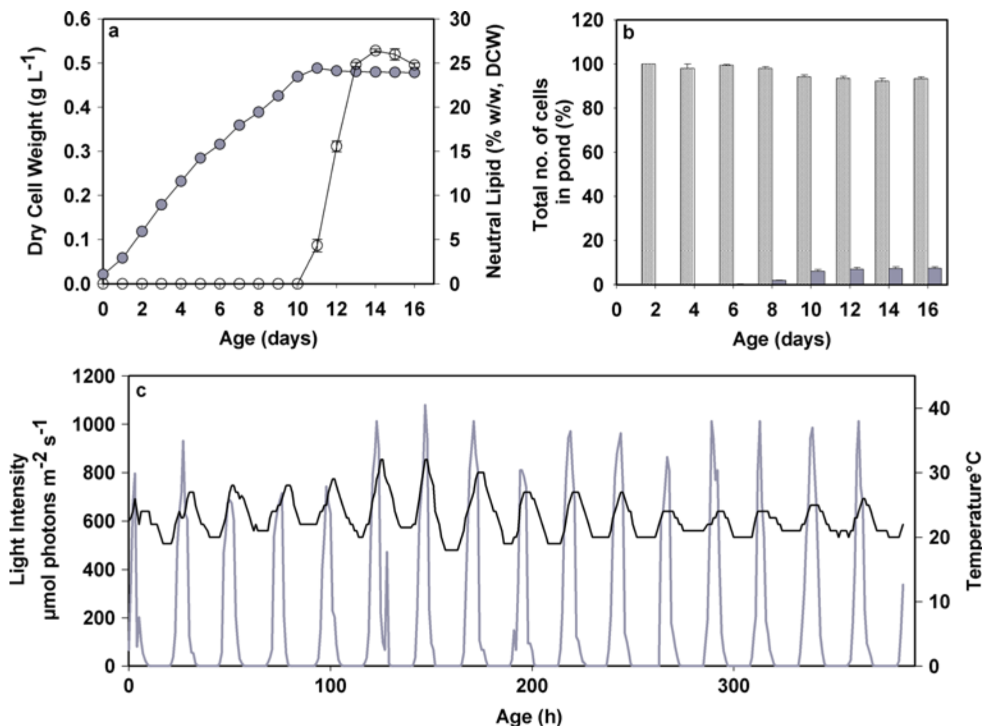

Fig. 8 Dynamic profiles for growth, neutral lipid accumulation of the strain *Chlorella* sp. FC2 IITG grown under outdoor conditions in an open-pond system. **a** Growth (*closed circle*) and neutral lipid content (*open circle*); **b** percentage of contamination during the cultivation period (*grey bars* represent the bacterial contamination and the *dotted bars* represent the percentage of our strain FC2) and **c** dynamic profile of fluctuating environmental parameters light intensity (*continuous grey line*) and temperature (*continuous black line*)

comparison to few other algal strains reported in the literature (Table T3, Supplementary material). It is important to note that these strains were grown under higher CO_2 concentrations, higher light intensity, and a continuous light cycle. Optimization of the cultivation conditions for FC2 will further improve biomass and lipid productivity, which may be comparable with these lipid-overproducing strains.

Under phosphate or nitrate starvation, carbon flux from some of the intracellular components such as protein, carbohydrate, and pigments are redirected towards lipid biosynthesis [32, 37]. These lipids are higher energy-yielding compounds than carbohydrate and hence serve as an efficient energy reserve for the cell [37]. A significant change in the intracellular composition of FC2 was observed while transferred from a nutrient-sufficient conditions to the nutrient-starved conditions (Table 2). For instance, intracellular carbohydrate and protein content was reduced up to 2.5-fold and 1.8-fold, respectively, when the cells were transferred from nutrient-sufficient conditions to nitrate-starved conditions with a concomitant increase in neutral lipid content (Table 2). A similar observation was recorded for phosphate starvation as well. Redirection of carbon flux from carbohydrate and protein fractions of the biomass towards accumulation of neutral lipid was also evident from changes in macromolecular composition of the cells during the transition from exponential phase to the stationary phase of growth under three different cultivation conditions (Fig. F1, Supplementary material). Similar metabolic shift in terms of biomass composition was reported for photoautotrophic growth of *Neochloris oleoabundans* and *Chlorella vulgaris* under nitrogen starvation where a reduction in intracellular protein content was coupled with an increase in neutral lipid accumulation [36]. Further, *Pseudochlorococcum* sp. was found to accumulate starch as the major energy storage compound during nitrogen-sufficient conditions whereas under nitrogen-depleted conditions the strain accumulated neutral lipids with a significant reduction in the carbohydrate content [22]. Increased

Table 3 Fatty acid methyl esters (FAME) profile of *Chlorella* sp. FC2 IITG grown under different cultivation conditions

FAME (%, w/w)	Autotrophic	Heterotrophic	Mixotrophic	Nitrate starvation	Phosphate starvation	Out door
C12:0	0.179 ± 0.003	0.085 ± 0.009	0.251 ± 0.02	0.074 ± 0.011	1.16 ± 0.09	0
C14:0	1.313 ± 0.06	0.875 ± 0.14	1.115 ± 0.01	0.803 ± 0.01	3.99 ± 0.002	0.825 ± 0.030
C14:1	0.079 ± 0.008	0.071 ± 0.002	0.053 ± 0.07	0.105 ± 0.001	1.359 ± 0.01	1.52 ± 0.045
C15:0	0.183 ± 0.002	0.09 ± 0.001	0.13 ± 0.01	0	0	0.12 ± 0.0015
C16:0	33.72 ± 0.09	19.088 ± 0.3	24.46 ± 0.08	32.02 ± 0.09	22.03 ± 0.011	22.88 ± 1.3
C16:1	0.902 ± 0.01	0.778 ± 0.014	0.89 ± 0.02	0.493 ± 0.002	1.055 ± 0.01	4.58 ± 0.25
C16:2	1.223 ± 0.014	0.599 ± 0.003	0.56 ± 0.01	0.376 ± 0.01	0.823 ± 0.01	4.38 ± 0.173
C17:0	0.298 ± 0.05	0.22 ± 0.001	0.19 ± 0.01	0.563 ± 0.007	0	0.373 ± 0.028
C17:1	2.858 ± 0.003	4.362 ± 0.21	5.49 ± 0.001	1.386 ± 0.002	5.11 ± 0.1	11.41 ± 0.33
C18:0	4.006 ± 0.04	3.577 ± 0.18	4.48 ± 0.021	12.04 ± 0.11	2.769 ± 0.11	6.37 ± 0.69
C18:1 *trans*	4.483 ± 0.005	0.506 ± 0.09	3.71 ± 0.12	3.274 ± 0.14	5.217 ± 0.22	3.2 ± 0.048
C18:1 *cis*	21.551 ± 0.04	37.396 ± 1.03	23.25 ± 0.12	24.74 ± 0.24	16.67 ± 0.09	22.9 ± 0.188
C18:1 ω7C	1.109 ± 0.014	1.44 ± 0.012	1.073 ± 0.01	0.473 ± 0.12	0.882 ± 0.03	0.85 ± 0.033
C18:2 *trans*	0.141 ± 0.01	0.064 ± 0.001	0.105 ± 0.002	0.106 ± 0.01	1.409 ± 0.01	0.112 ± 0.002
C18:2 *cis*	17.72 ± 0.01	29.29 ± 0.13	27.72 ± 0.13	15.55 ± 0.03	29.25 ± 0.13	21.09 ± 0.4
C20:0	2.02 ± 0.07	0.088 ± 0.027	0	0.782 ± 0.03	0	1.065 ± 0.015
C18:3	7.59 ± 0.03	0.99 ± 0.011	6.24 ± 0.014	6.62 ± 0.1	7.086 ± 0.15	1.16 ± 0.035
C22:0	0	0.063 ± 0.001	0	0.157 ± 0.05	0.331 ± 0.008	0
SAT FAME[a]	41.72 ± 0.068	24.08 ± 0.811	30.62 ± 0.105	46.44 ± 0.18	30.29 ± 0.13	31.64 ± 0.81
MUFA[b]	30.98 ± 0.055	44.55 ± 0.19	34.46 ± 0.140	30.47 ± 0.16	30.30 ± 0.27	44.43 ± 0.19
PUFA[c]	26.68 ± 0.086	30.95 ± 0.022	34.62 ± 0.013	22.65 ± 0.079	38.56 ± 0.17	23.09 ± 0.43
Others	0.62 ± 0.055	0.41 ± 0.22	0.3 ± 0.115	0.44 ± 0.13	0.85 ± 0.12	0.84 ± 0.19
Total FAME	45.18 ± 3.11	64.52 ± 0.13	68.75 ± 0.093	61.62 ± 0.611	36.26 ± 0.59	35.12 ± 0.517

Total FAME are expressed in %, weight fraction of dry cell weight

[a] Percentage of saturated FAME (% of total FAME)

[b] Percentage of monounsaturated FAME (% of total FAME)

[c] Percentage of polyunsaturated FAME (% of total FAME)

carbon flux towards carbohydrate catabolism and a subsequent rise in acetyl CoA pool with concomitant increase in neutral lipid biosynthesis was observed for FC2 during the transition from phosphate replete to phosphate-starved conditions [32]. A similar increase in carbohydrate metabolism and acetyl CoA pool was also observed under nitrogen-starved conditions for *Micractinium pusillum*, which revealed that carbohydrate forms the major carbon source for triacyl glycerol synthesis rather than photosynthesis, which supports the findings in the present study [21].

Evaluation of growth performance and lipid productivity of FC2 under outdoor conditions

In order to evaluate the biomass and lipid productivity under fluctuating environmental parameters, the organism was grown under outdoor conditions in an open-pond system. The effect of contamination on the growth of the organism was also tested in the open environment. Maximum neutral lipid content of 26.4 % (w/w, DCW) and the biomass concentration of 0.48 g l^{-1} were recorded under outdoor conditions for FC2 (Fig. 8a). The biomass and neutral lipid productivity were found to be 44 and 9.91 mg l^{-1} day^{-1}, respectively. The total lipid content measured by GC showed a maximum of 35.12 % (w/w, DCW) with the lipid productivity of 10.71 mg l^{-1} day^{-1}. A recent study on characterization of *Scenedesmus obliquus* in an open-pond system reported a maximum biomass concentration of 0.53 g l^{-1} and a total lipid content of 19.6 % (w/w) [28]. Studies on performance evaluation of other algal strains under outdoor conditions demonstrated an improved biomass and lipid productivity as compared to our reported strain (Table T4, Supplementary material). However, these strains were grown under aseptic conditions in a closed photobioreactor and in some cases under a nutrient-starved environment. Therefore the strains were able to demonstrate higher biomass and lipid productivity. Interestingly, in the present study, no detectable contamination was recorded for the initial 4 days of cultivation and a maximum contamination of 7 % of total number of cells

was recorded only towards the end of the batch (Fig. 8b). This contamination was caused solely due to bacterial growth and the pond was free from any fungal and other algal contaminants. Further, the culture experienced a fluctuating light intensity from zero (in the night) to a maximum of 1,100 μmol photons m^{-2} s^{-1}. The pond temperature was found to vary from 18 to 32 °C over the entire period of cultivation (Fig. 8c). Thus, the strain was found to be robust under fluctuating environmental parameters and can be grown in open-raceway pond efficiently with minimal contamination.

FAME composition of FC2 under different cultivation conditions

The FAME composition of FC2 was analyzed under various cultivation conditions using gas chromatography (Table 3). Palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) were the three major fractions that constitute the majority of total fatty acid compositions under different cultivation conditions. These fatty acids were also found to be abundant in other *Chlorella* sp. reported in the literature [25, 44]. Linolenic acid (C18:3) was found to be present in the range of 1–7 % weight fraction of total fatty acids, which is less than the permissible limit of 12 mol% as per European standards [8]. It is interesting to note that the ratio of unsaturated (monounsaturated plus polyunsaturated) to saturated fatty acid fractions were significantly distinct and varied from 1.1 to 3.1 depending on the cultivation conditions (Table 3). While the saturated fatty acids contribute to increased oxidation stability and cetane number of biodiesel, the unsaturated fraction is useful for achieving increased cold filter plugging point and hence higher stability at lower temperatures. In the present study, the strain FC2 was able to produce fatty acids with 65–85 % contributions from saturated (C16:0) and unsaturated (C18:2, C18:2) fatty acids, which are considered to be the key elements for suitable quality biodiesel [25]. Hence, FC2 can be a potential candidate for good-quality biodiesel production.

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Ethical standards The experiments in this work comply with the current laws of the country in which they were performed.

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