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# High cell density lipid rich cultivation of a novel microalgal isolate *Chlorella sorokiniana* FC6 IITG in a single-stage fed-batch mode under mixotrophic condition

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## HIGHLIGHTS

- Reports a novel microalgal isolate *Chlorella sorokinana* FC6 IITG.
- Glucose & acetate was found to be growth supporting & lipid inducing, respectively.
- Single-stage fed-batch mixotrophic cultivation for high cell density lipid-rich FC6.
- Maximum productivity (mg $L^{-1}$ $day^{-1}$): biomass-1930 & lipid-550 in fed-batch.
- Biodiesel produced from FC6 meets international standards.

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## ABSTRACT

A single-stage mixotrophic cultivation strategy was developed with a novel microalgal isolate *Chlorella sorokiniana* FC6 IITG for high cell density lipid-rich biomass generation. The strain was evaluated for growth and lipid content under different physico-chemical parameters, nutritional conditions and trophic modes. Finally, a single-stage mixotrophic fed-batch cultivation strategy was demonstrated with intermittent feeding of key nutrients along with dynamic increase in light intensity for high cell density biomass and sodium acetate as elicitor for lipid enrichment. The key findings: (i) glucose and sodium acetate was identified as growth supporting and lipid inducing nutrients, respectively; (ii) mixotrophic batch cultivation resulted in maximum biomass and lipid productivity (mg $L^{-1}$ $day^{-1}$) of 455.5 and 111.85, respectively; (iii) single-stage mixotrophic fed-batch cultivation showed maximum biomass productivity of 1.93 g $L^{-1}$ $day^{-1}$ (biomass titer 15.81 g $L^{-1}$) and lipid productivity of 550 mg $L^{-1}$ $day^{-1}$; (iv) biodiesel properties were in accordance with international standards.



## 1. Introduction

Oleaginous microalgae are considered as one of the most promising feedstock for renewable and sustainable production of biodiesel. This increasing importance of microalgae may be attributed to its key inherent properties such as higher photosynthetic efficiency, higher biomass and lipid productivity in comparison to other plant resources (Chisti, 2007). However, microalgae based biodiesel production technology suffer from several limitations at different stages of the upstream and downstream processes e.g. strain selection, growth of microalgal strains under outdoor conditions, contamination, light penetration through dense culture, harvesting, drying, extraction and transesterification (Lam and Lee, 2012). Therefore, there is a need to design a sustainable process using hyper producing strains with improved biomass and lipid productivity. Recently, efforts have been aimed to identify robust microalgal strains with enhanced biomass productivity, and high net lipid productivity along with suitable fatty acid methyl ester (FAME) content for biodiesel production (Lam and Lee, 2012).

Microalgal biomass production has mainly been achieved by photoautotrophic cultivation in open pond and photobioreactors in the presence of light and $CO_2$ (Brennan and Owende, 2010; Kumar et al., 2011). However, some strains have been reported

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to utilize organic carbon sources like acetate, glycerol and glucose under heterotrophic conditions (Alagesan et al., 2013; Muthuraj et al., 2014). The green alga *Chlamydomonas reinhardtii* can grow photoautotrophically utilizing $CO_2$, heterotrophically utilizing acetate, and mixotrophically utilizing both carbon sources (Heifetz et al., 2000). Mixotrophic microalgae have the ability to utilize both the organic and inorganic carbon sources for cellular growth in the presence of light as the energy source (Chen et al., 2011). Heterotrophic or mixotrophic cultivation in bioreactor provides high degree of growth control and in turn, can ensure higher biomass and lipid productivity in comparison to photoautotrophic cultivation which further reduces harvesting cost (Brennan and Owende, 2010). Various microalgal strains have been identified as potential strains for biodiesel production with ability to accumulate significant amounts of lipids under different cultivation conditions (Xu et al., 2006; Muthuraj et al., 2014). For instance, *Chlorella* sp. FC2 IITG was found to grow under different trophic modes with lipid productivity in the range from35.02 mg $L^{-1}$ $day^{-1}$ to 50.42 mg $L^{-1}$ $day^{-1}$ (Muthuraj et al., 2014). Therefore, alterations in the growth conditions will have significant influence on biomass and lipid productivity which depicts the need for characterization of the potential strains under different cultivation conditions. There is a large number of reports on effects of micro and macro nutrients on growth and lipid productivity of microalgae (Chen et al., 2011). These macro and micro nutrients may have significance as growth supporting or lipid inducing at their different levels of concentrations (Karemore et al., 2013). Therefore, optimization of these media components turns necessary to enhance the biomass and lipid productivity. For instance, optimization of the carbon, nitrogen and phosphate sources for photoheterotrophic growth of *Ettlia texensis* resulted in highest biomass productivity of 0.97 g $L^{-1}$ $day^{-1}$ which was ~6.5-fold higher than the biomass productivity obtained from unoptimized media composition (Isleten-Hosoglu et al., 2012). An increase in lipid productivity up to 0.19 g $L^{-1}$ $day^{-1}$ was also observed when *Botryococcus braunii* was grown in the optimized lipid production media designed using response surface methodology (Tran et al., 2010). It is also important to note that microalgal growth and lipid production are two mutually exclusive phenomena and therefore, the optimized condition required for growth may not be suitable for oil production (Muthuraj et al., 2014). Two-stage cultivation strategies have been reported to achieve high cell density lipid rich algal biomass (Karemore et al., 2013). In the first stage of cultivation, cells were grown under optimal conditions for maximum biomass formation followed by their harvesting. The harvested cells were re-suspended in the nutrient starved medium for maximization of lipid accumulation during the second stage of the process. However, this two-stage process involves higher capital cost, additional harvesting and medium requirement (Xia et al., 2014). Therefore, a single-stage cultivation will be appropriate where biomass growth and lipid enrichment can take place in a single bioreactor.

In the present study, we report a novel indigenous microalgal isolate *Chlorella sorokiniana* FC6 IITG (hereafter mentioned as FC6) as a cell factory for biodiesel production. The strain was characterized for the effect of various physico-chemical parameters (temperature and pH) and nutritional conditions (carbon and nitrogen sources) on its growth and lipid productivity. These experiments were performed to identify the substrates critical for growth and/or lipid induction. Further, nutritional requirements of the strain were optimized in order to maximize the biomass titer. Effect of different cultivation conditions on biomass and lipid productivity of the strain were also studied by growing the cells under heterotrophic, mixotrophic and photoautotrophic mode in an automated bioreactor. Finally, a strategy has been demonstrated to achieve high cell density lipid-rich cultivation of the strain FC6 under single-stage fed-batch mixotrophic condition.

## 2. Methods

### 2.1. Isolation and identification of axenic microalgal strains

The fresh water samples were collected from the North East-India (90.93° E longitude and 26.71° N latitude) during the month of June 2010 and inoculated into BG11 medium. The BG11 media comprised of (g $L^{-1}$) $NaNO_3$ 1.5 as nitrogen source, $K_2HPO_4$ 0.04 as phosphate source, trace elements ($MgSO_4{\cdot}7H_2O$ 0.075, $CaCl_2{\cdot}2H_2O$ 0.036, $Na_2CO_3$ 0.02, citric acid 0.006, ferric ammonium citrate 0.006, and EDTA 0.001) and microelement 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). The cells were incubated in an orbital shaker maintained at 150 rpm, 28 °C under 30 μE $m^{-2}$ $s^{-1}$ light intensity with a light: dark cycle of 16:8 h. Individual algal cultures were isolated using conventional serial dilution and plating methods. The axenicity was confirmed through optical examination under light microscope (Eclipse E200, Nikon, Japan) and by spreading the culture on soya bean casein digest agar plates. The isolates were screened for neutral lipid accumulation using Nile-red based staining method and analyzed through confocal microscope (Detailed protocol in Supplementary material). The strains were identified based on the morphometric analysis using Field-Effect scanning electron microscope (FESEM, Carl Zeiss SIGMA VP, Germany) and molecular analysis by 18S rDNA sequencing (for details refer to Supplementary material).

### 2.2. Inoculum preparation

The seed culture was prepared by inoculating two loops full of slant culture into 250 mL Erlenmeyer flask containing 100 mL of BG11 medium and incubated photoautotrophically in a programmable incubator shaker (Multitron-Pro, Infors HT, Switzerland) at 150 rpm, 28 °C under 30 μE $m^{-2}$ $s^{-1}$ light intensity with a light: dark cycle of 16:8 h. 1% (v/v) of the grown culture with absorbance ($A_{690}$) of 1.0 was used as inoculum in all the experiments carried out in the present study.

### 2.3. Effect of pH, temperature, carbon and nitrogen sources on growth and lipid content of FC6

Effect of initial pH of the medium, temperature, carbon and nitrogen sources on growth and lipid content of FC6 was studied in order to identify the conditions that are growth supporting and/or lipid inducing. The strain was characterized at different initial medium pH (2, 4, 6, 8, 10 and 12) and at different temperatures (20, 28, 36 and 44 °C) under photoautotrophic condition. Screening of different nitrogen sources were carried out by replacing sodium nitrate in the original BG11 medium with equimolar concentration (0.018 M nitrogen) of potassium nitrate, sodium nitrite, ammonium chloride, urea, meat extract, glycine, yeast extract and peptone under photoautotrophic condition. Further, screening of carbon sources was carried out under both heterotrophic and mixotrophic conditions with six different carbon sources: glucose, fructose, sucrose, lactose, maltose and sodium acetate at an equimolar concentration of 6 M carbon. While light intensity of 30 μE $m^{-2}$ $s^{-1}$ with a light: dark regime of 16:8 h was provided under mixotrophic condition, heterotrophic cultivation was performed under complete dark condition. The biomass was monitored at regular intervals and the final lipid content was obtained by gas chromatography (GC) at the end of the each batch.