



## Evaluating the potential of green alga *Chlorella* sp. for high biomass and lipid production in biodiesel viewpoint

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### ABSTRACT

The present study assessed the potential of green alga *Chlorella* sp. for maximal biomass and lipid production in biodiesel aspect. Initially, growth of the tested strain was analyzed in terms of dry cell weight, optical density and then, lipid content, lipid yield and fatty acid composition were studied. The growth profile study was carried out from 0th day to 20th day of cultivation to find an optimal day for harvesting the biomass. The potential microalga *Chlorella* sp. was found to yield maximum biomass at about 1.1 g/L and cell density of 2.0 optical density on 16th day of growth. Further, in this study, bioprospecting of *Chlorella* sp. showed a lipid content of 11.5% on 16th day in standard medium without carbon dioxide purging and mechanical stirring. Adding to that, cells harvested from log and stationary growth showed same lipid content of 11.5%, whereas culture harvested from lag phase was found to reveal a marginally less lipid content of 10.5%. Further, the lipid yield was estimated from the test strain *Chlorella* sp. grown under log and stationary phases. Based on the data, it is observed that the total lipid of the strain grown under both the phases was found to yield similar lipid of 0.11 g/L, which substantiates the lipid content of the strain. Fatty acid profile analysis of the tested strain indicates the presence of major fatty acids such as palmitic, palmitoleic, oleic, and linolenic acid. With this in view, the *Chlorella* sp. was foreseen to be a potential feedstock for biodiesel production considering cell density, dry cell weight, lipid content, lipid yield and fatty acid composition at ambient condition.

### 1. Introduction

At present, energy used by human society is satisfied through fossil-based fuels, which is non-renewable in nature (Kwon et al., 2012; Mathimani et al., 2015). Due to their non-renewable feature, fossil fuels are depleting at alarming rate. Further, combustion of fossil fuels emit greenhouse gases, which are harmful to the environment (Mathimani et al., 2017b; Pugazhendhi et al., 2017). Adding to above, there are major issues currently with the use of fossil fuels (i) energy demand (ii) economic crisis (iii) increased fuel consumption (iv) hike in fuel price (v) emission of pollutant gases. These issues might create unfavorable changes (climatic change) in the environment like global warming (Mathimani et al., 2017b, 2017c; Shobana et al., 2017). To avert these problems, renewable energy sources should be explored or alternate fuel should be used (Anburajan et al., 2017; Mathimani et al., 2017a; Sivagurunathan et al., 2018). More specifically, unstable supply of oil

and the transition in petroleum based fuel prices would trigger an interest in finding new, sustainable and alternative fuel sources (Miriam et al., 2017), and these might rely upon environmental, geopolitical and economical aspects (Dellomonaco et al., 2010). In this scenario, biofuel – an alternative, renewable, ecofriendly fuel has been identified as a potential fuel alternate to diesel fuel (Koley et al., 2018).

Biofuel is of many types, bioethanol, biohydrogen, biodiesel, and biogas based on the end product of the reaction (Prabakar et al., 2018; Saravanan et al., 2018; Sivagurunathan et al., 2018). Various feedstocks have been used for biofuel production and based on feedstocks used, biofuel has been categorized into three generations. The first and second-generation feedstocks include edible and non-edible oils respectively. Though biofuel produced from different food crops is renewable, alternative and non-toxic, they pose numerous issues in utilizing them as a promising fuel source; those issues include food versus fuel controversy, surplus fertile land requirement for feedstock

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cultivation, and low lipid or oil production between the seasons (Elshaib et al., 2014; Mathimani and Mallick, 2018; Saravanan et al., 2018). Therefore, finding third generation feedstock has been started in which microalgae have been identified as promising third generation biofuel feedstock (Feng et al., 2011; Kumar et al., 2017) and particularly microalgae have been used for biodiesel production. Biodiesel is a long chain alkyl ester, non-toxic, carbon-neutral fuel that emits low concentration of CO, CO<sub>2</sub> (Mathimani et al., 2017b). The advantages of using microalgae for biofuel production include, high biomass and photosynthetic rate, low culturing land requirement etc., (Mathimani et al., 2015). In certain cases, marine strains are also used since they require seawater with minimal nutrients for large scale cultivation (Mathimani et al., 2018). However, choosing a pertinent strain with high biomass and high lipid content is still a challenge. Further, finding an optimal growth stage, which generates ample biomass and simultaneously accumulates lipid is a primary goal. Adding to the above, biomass productivity, lipid productivity and preferred fatty acid profile of the strain grown under different growth phases need to be studied prior to the selection of feedstock. Hence, this present work was taken to identify a promising microalgal strain with high biomass yield, maximum lipid production and preferable fatty acid composition towards economically viable biodiesel production.

## 2. Materials and methods

### 2.1. Culture purification and identification

Algal samples were initially observed under light microscope and subjected for purification procedures namely streak plate and serial dilution to obtain unialgal or pure cultures. During the purification, culture growth was monitored periodically and observed under microscope. Based on the morphology, algal isolate was identified as *Chlorella* sp. and it has been used for further studies.

### 2.2. Culture maintenance

The isolated microalgal strain *Chlorella* sp. was initially cultured in 50 mL conical flask containing bold basal medium and scaled up to 250 mL conical flask and incubated at 25 °C thermo-chamber with a light intensity of 2000 lx at 14/10 light/dark period.

### 2.3. Growth measurement

#### 2.3.1. Optical density

The growth of *Chlorella* sp. was assessed using dry cell weight and optical density of the culture suspension. The optical density (OD) of the culture suspension was read at 540 nm to record the biomass density at different days.

#### 2.3.2. Dry cell weight estimation

To estimate the dry cell weight of culture, a fixed volume of culture suspension was centrifuged at 5000 rpm for 15 min and the supernatant was discarded and biomass pellet was carefully collected (Converti et al., 2009). The obtained pellet was washed twice with distilled water to get rid of medium traces. Finally, the washed biomass pellet was dried until the constant weight was observed and kept for later analysis.

### 2.4. Lipid extraction

Total lipid from dried *Chlorella* cells was extracted as detailed in Mathimani et al. (2017c). For lipid extraction, 1 g dried biomass was taken and subjected for lipid extraction using binary solvent system. In this study, chloroform: methanol binary solvent system with 2:1 ratio was used for efficient lipid extraction. The total lipid extraction procedure was repeated until lipid was extracted from the cells completely. The crude lipid taken in separatory funnel was added with water, and

allowed to stand for phase separation. After the incubation period, lower chloroform layer containing total lipid was gently collected. The hydrophilic impurities dissolved in water were discarded carefully. The resulted chloroform-containing lipid was taken and solvent was evaporated to obtain purified lipid.

### 2.5. Lipid estimation

The collected lipid was calculated for percentage gravimetrically i.e., lipid was estimated in weighing balance until consistent weight was seen and the percentage of lipid in *Chlorella* sp. was calculated from the biomass taken for lipid extraction as given in the below equation (Mathimani et al., 2017c).

$$Y(\%) = \frac{W_L - W_P}{DCW} \times 100$$

Y – lipid content, W<sub>L</sub> – weight of lipid, W<sub>P</sub> – preweight of the vial, DCW – dry cell weight.

### 2.6. Transesterification and fatty acid profile analysis

Acid catalyzed transesterification method (Mathimani et al., 2015) was chosen to convert lipid into fatty acid methyl ester (FAME) and the reaction conditions are 2.5 h reaction time, 65 ± 1 °C reaction temperature, and 3.5% H<sub>2</sub>SO<sub>4</sub> concentration. At the end of the reaction, FAME layer was collected and purified and injected in a Gas Chromatography. The fatty acid profile of the strain was identified by using Supelco 37 FAME mix standard.

## 3. Results and discussion

Isolated strains were observed under light microscopy for their morphological characteristics, and subjected for conventional monoculture isolation techniques. In this present study, the collected sample was identified as unicellular green microalga *Chlorella* sp. based on its morphology, and it has been used for subsequent studies.

### 3.1. Optical density vs dry cell weight

The isolated monoculture *Chlorella* sp. was scaled up and grown in nutrient medium until high biomass was obtained to begin the experimentation. The fixed inoculum of *Chlorella* cells in conical flask was analyzed for time course growth kinetics from day 0 to 20. In this study, optical density at 540 nm, dry cell weight, biomass yield, were taken to measure the growth of alga during the course of its growth. Prior to estimate the growth of alga, a calibration curve between dry cell weight and optical density was plotted to ensure the linear correlation between the biomass weight and optical density of *Chlorella* cells. To substantiate, OD of cultures 0.5, 1, 1.5, 2 was taken and their respective dry cell weight was estimated from medium traces free biomass and then calibration plot was drawn with correlation coefficient (R<sup>2</sup>). Calibration plot presenting correlation between dry cell weight vs optical density was given Fig. 1. As evident from Fig. 1, the dry cell weight and optical density of algal suspension was linear and both are correlated well. Further, R<sup>2</sup> value denotes linear graph involving optical density vs dry cell weight. Particularly, R<sup>2</sup> of the graph is very close to 1, which implies that dry cell weight is linearly correlated with optical density of *Chlorella* culture suspension. Having plotted the calibration curve, growth pattern of *Chlorella* sp. was investigated to determine the optimal day for harvesting biomass for high dry cell weight and simultaneous lipid extraction.

### 3.2. Growth of *Chlorella* sp

Growth curve of *Chlorella* sp., from 0 to 20 days was displayed in Figs. 2 and 3. As seen from the figures, dry cell weight (g L<sup>-1</sup>) and

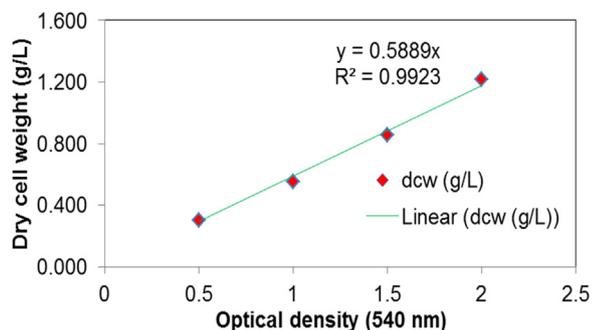


Fig. 1. Optical density vs. dry cell weight of *Chlorella* sp.  $R^2$  value is in preferable limit and the graph signified linear arrangement.

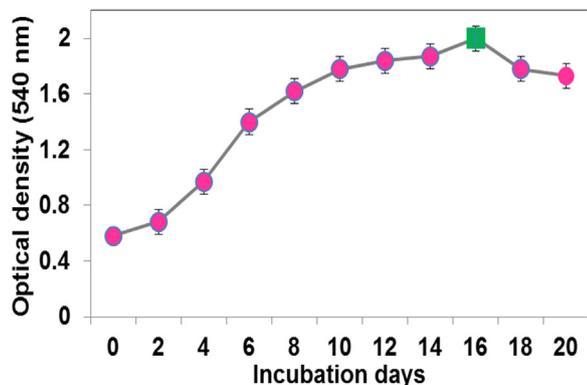


Fig. 2. Growth profile of *Chlorella* sp. measured in terms of optical density.

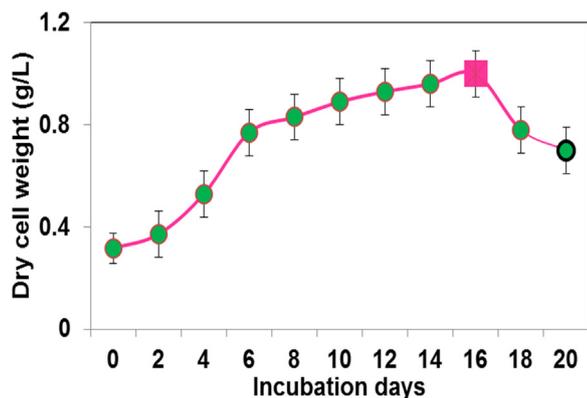


Fig. 3. Dry cell weight of tested microalga *Chlorella* sp.

$OD_{540\text{nm}}$  was estimated as growth metrics for the test alga using triplicate experiments. In concern with optical density, the initial OD of the culture was 0.51 on day 0 and the OD got increased during the growth. The high density culture (maximal OD) was seen on cultivation day 16 which is 2.0 OD, and thereafter, the OD got decreased as the culture growth declines. From the growth curve of *Chlorella* sp. plotted using OD it is understood that maximal growth of alga was noticed on 16th day of experimentation.

### 3.3. Dry cell weight of *Chlorella* sp. grown at ambient condition

In addition to cell density estimation through optical density, dry cell weight of the culture was also done to monitor the growth of alga from 0 day to 20th day of cultivation. Microalgal biomass is thought to be the next generation biofuel feedstock but commercial scale cultivation of potent microalgae for fuel production is not successful economically (Markou and Nerantzis, 2013). Further, dried microalgal

biomass possess 49.2% carbon, which is mostly present in carbohydrates and lipids from the photosynthetic fixation of carbon dioxide (Mirón et al., 2003). Hence, the total biomass content of *Chlorella* sp. was estimated in terms of dry cell weight basis. The dry cell concentration of *Chlorella* sp. from lag phase to decline phase was displayed in Fig. 3. Dry weight is an important parameter to estimate the biomass content of the alga, which could be extended for biodiesel application. As presented in Fig. 3, the dry weight of the culture was 0.31 g/L on day 0, and it has been increased to 0.51 g/L on day 5 of cultivation. The biomass content was linearly increasing with incubation days and the maximal dry cell weight of 1.1 g/L was obtained on 16th day and then it started decreasing upto 20th day. The biomass obtained in this study was higher since the culture was grown in ambient condition without  $CO_2$  purging and no stirring was carried out either mechanically or manually.

High growth was recorded on day 16 in terms of cell density and dry weight. for *Chlorella* sp. and meanwhile, the unicellular green alga *Chlorella* sp. was found to yield very low biomass after 16th day and prior to 4th day of cultivation. For all the estimation involving test strain, initial lag phase was noticed for about 2 days. From this growth profile analysis of the strain, it is deduced that *Chlorella* sp. was found to unveil high dry weight with rapid doubling rate on day 16. Therefore, it is imperative to study the biomass yield, lipid content and lipid yield of the strain to evaluate its feasibility for biodiesel.

### 3.4. Biomass yield of *Chlorella* sp. under log and stationary phases

Using growth curves plotted with dry cell weight, biomass yield of *Chlorella* sp. was estimated from the culture harvested in log and stationary phases. The purpose of choosing only log and stationary phases is that the biomass density is higher in those two phases of growth as seen in optical density. The results obtained in dry cell weight was reflected in biomass yield as well. The biomass yield of *Chlorella* sp. cultivated at ambient condition (log and stationary phase) was given in Fig. 4. The biomass concentration or biomass yield was represented in g/L as already explained in dry cell weight of the test alga. Harvesting the strain grown under log phase found to yield less biomass of 0.98 g/L and then it got increased with the progression of cultivation. Under stationary phase, the highest biomass yield of 1.1 g/L was obtained, which substantiates the dry cell weight and cell density data obtained in Figs. 2 and 3. The growth metrics of *Chlorella* sp. disclosed that higher biomass can be produced on 16th of cultivation and however, no information on lipid content has been retrieved, which is considered to be an important parameter for biodiesel production. Evaluating the suitability of the selected strain for biodiesel, lipid was extracted, estimated and analyzed.

### 3.5. Examining *Chlorella* sp. for higher lipid content

Lipid content of *Chlorella* sp. was studied to check its suitability as a

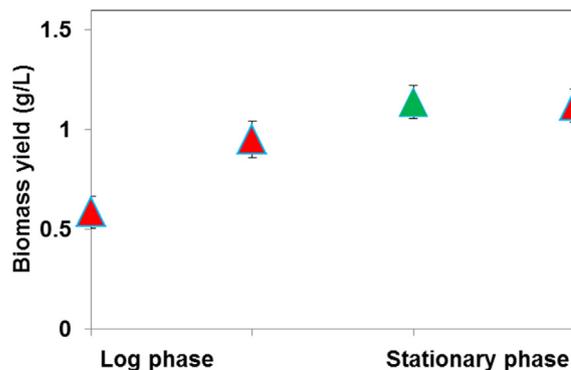


Fig. 4. Biomass yield of *Chlorella* sp. harvested from log and stationary phases.

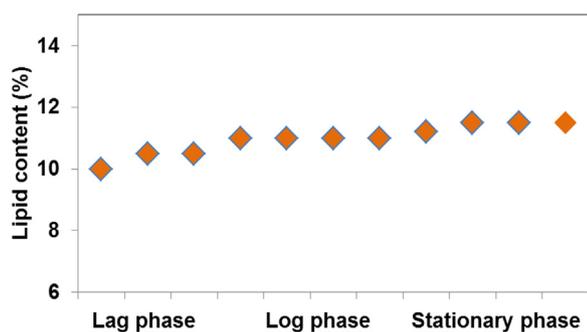


Fig. 5. Lipid content of *Chlorella* sp. harvested from lag, log and stationary phases of its growth.

promising feedstock for alternative fuel production. Microalgae with high lipid content are desirable for high quality biodiesel production (Chisti, 2007; Mathimani and Nair, 2016). However, lipid content is highly variable between the organisms/species. In general, microalgae isolated from different sources contain lipid content, ranging from 5% to 77% (Chisti, 2007). To achieve the sustainable microalgal biodiesel production, bioprospecting algal strains for high lipid content is essential (Mathimani et al., 2018). With in view, the *Chlorella* sp. was explored for their total lipid content. The lipid content of *Chlorella* sp. was studied under three different phases of growth namely lag, log and stationary phases to evaluate the effect of growth phases on lipid content.

Total lipid content of *Chlorella* biomass harvested from lag, log and stationary phases of growth was presented in Fig. 5. The lipid content of *Chlorella* sp. was marginally low as 10% in lag phase harvested cells whereas in log phase cultures, the lipid content of the strain was high as 11.5%. Eventually, *Chlorella* cells harvested from stationary phase was found to show higher lipid content of 11.6% compared to lag phase cultures. In this part of the study, it is concluded that both log and stationary phase growth cultures showed similar and higher lipid content about 11.5%. This is in consistent with the report that *Chlorella* cells showed lipid content in the range between 5–22% due to different habitats (Mathimani et al., 2018). In this study, particularly green alga only taken for investigation since cyanobacteria lack oil bodies or oil droplets for triglycerides storage and they encompass only building block lipids (Wahlen et al., 2011). It is to be noted that the lipid content reported here in this study is from the alga grown under ambient CO<sub>2</sub> without any nitrogen and phosphate stress. This nutrient stress study will be carried out in future to increase the lipid content since nutrient stress is reported to increase the lipid accumulation in algal strains and deprivation or limitation of nitrogen or phosphate or certain metal components in media was found to enhance the microalgal lipid yield (Mujtaba et al., 2012; Sharma et al., 2012). Further, considering the lipid composition, the fast growing microalgae with short life cycle synthesize triglycerides in late exponential phase, which occupies 20–30% per dry cell weight at standard culture conditions (Chisti, 2007; Yang et al., 2013).

### 3.6. Lipid yield of the test strain *Chlorella* sp

Another parameter that has to be considered to check the suitability of *Chlorella* sp. as a better biodiesel feedstock is lipid yield (g/L). For that, culture grown under log phase and stationary phase was harvested and subjected for total lipid extraction and estimation. Using lipid content of the strain, the lipid yield was calculated from triplicate estimations. Here in this study, culture from log and stationary phases only taken as in the previous lipid content study log and stationary phases only has produced high lipid content of 11.5% compared lag phase. As shown in Fig. 6. The lipid yield of *Chlorella* sp. was estimated to be 0.11 g/L in log phase and similarly in stationary phase, it was

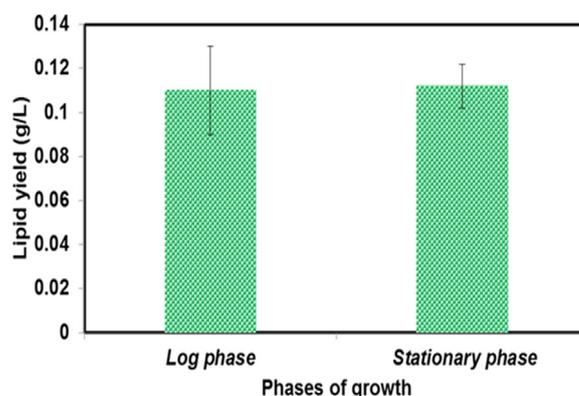


Fig. 6. Lipid yield of the tested strain *Chlorella* sp. harvested from log and stationary phases.

0.12 g/L. Culture grown under log and stationary phases showed same lipid yield. In general, lipid in microalgae is synthesized through various metabolic machinery and the key organelles for lipid synthesis is chloroplast (plastid) and endoplasmic reticulum (Bellou et al., 2014). The fatty acid acyl-CoA synthesized in chloroplast was translocated to endoplasmic reticulum in which it passes through various enzymatic biochemical reactions in “kennedy pathway” to produce triacylglycerol (Bellou et al., 2014). From the data presented in this study, it is concluded that the tested green alga *Chlorella* sp. can be cultured in mass scale to produce biodiesel as the strain has generated high biomass and lipid at ambient condition.

### 3.7. Fatty acid composition

The fatty acid composition of the tested strain *Chlorella* sp. was given in Table 1. The fatty acid profile analysis of the tested alga *Chlorella* sp. showed the presence of 11 identified fatty acids such as capric acid, lauric acid, palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, cis-11,14-eicosadienoic acid, cis-11,14,17-eicosotrienoic acid, tricosononic acid, cis-13,16-docosadienoic acid. Among the identified fatty acids, the saturated fatty acid palmitic acid (C16:0) was found to be present in higher concentration about 20.2%. Further, other major fatty acids are oleic acid (17.2%), palmitoleic acid (12.04%), and linolenic acid (11.12%). In concern with the fuel properties of the FAME, Fatty acid profile of the strain decides the quality of biodiesel. Hence, the strain with desired fatty acid composition needs to be chosen to produce high-quality biodiesel in commercial scale. Considering these criteria, the fatty acid composition of the tested strain has been assessed in biodiesel viewpoint to use as a fuel in engine. In general, palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), and linolenic acid (C18:3) are the key fatty acids, which improves the fuel properties of biodiesel (Mathimani et al. 2018). With

Table 1  
Fatty acid composition of the tested strain *Chlorella* sp.

S. no.	Fatty acid	Composition (%)
1.	Capric acid (C10:0)	0.88
2.	Lauric acid (C12:0)	0.97
3.	Palmitic acid (C16:0)	20.2
4.	Palmitoleic acid (C16:1)	12.04
5.	Oleic acid (C18:1)	17.21
6.	Linoleic acid (C18:2c)	8.98
7.	Linolenic acid (C18:3)	11.12
8.	Cis-11,14-eicosadienoic (C20:2)	1.47
9.	Cis-11,14,17-eicosotrienoic (C20:3)	2.04
10.	Tricosononic (C23:0)	0.68
11.	Cis-13,16-docosadienoic (C22:2)	1.05
12.	Unidentified	23.35

reference to the properties, oxidative stability and cetane number of FAME depends on the saturated fatty acids. As shown in Table 1, the *Chlorella* sp. contains high levels of saturated fatty acid i.e., palmitic acid, which indicates improved oxidative stability. Further, the linolenic acid concentration is in desirable limit i.e., (11.12%). On the other hand, polyunsaturated fatty acids present in low concentration compared to saturated and mono unsaturated fatty acids.

#### 4. Conclusion

Microalgae are potential and superior candidate for biofuel production specifically biodiesel. The microalga *Chlorella* is widely used and commercialized in variety of applications like food, fuel and products. The higher biomass and lipid producing capability of *Chlorella* makes it foremost candidate by researcher for biodiesel application. In this connection, the present work attempted to provide a clear view or opinion on utilizing *Chlorella* sp. for maximal biomass and lipid yield towards successful biodiesel production. From the result, high biomass yield of 1.1 g/L and lipid yield of 0.11 g/L was observed on 16th day of growth at ambient conditions. In addition, fatty acid composition of the tested strain includes certain anticipated fatty acids namely palmitic, palmitoleic, and oleic, which signifies favorable fuel properties of the fatty acid methyl ester. Though the tested strain *Chlorella* sp. was able to yield higher biomass and lipid, the produced biodiesel has not been tested for fuel properties, which will be carried out in our future studies.

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