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Lipid enhancement through nutrient starvation in *Chlorella* sp. and its fatty acid profiling for appropriate bioenergy feedstock

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ABSTRACT

Selection of suitable microalgal candidate for biodiesel relies upon its biomass, lipid productivity and fatty acid profile. In this perspective, the key objective of the present research is to assess the biomass and lipid productivities of the *Chlorella* sp. under ambient condition and further, culture the strain under nitrate and phosphate deprivation conditions to induce the lipid accumulation. Under the standard BG-11 medium, biomass and lipid productivity of the tested strain was estimated to be 46 mg L^{-1} d^{-1} and 5.1 mg L^{-1} day^{-1} respectively. With this in view, nutrient starvation was carried out to escalate the lipid content of *Chlorella* sp. Under nitrate deprivation, the lipid content was 21.8 %, which was comparatively higher than standard BG -11 medium grown cells (control) i.e., 11.5 %. Similarly, under phosphate starvation, the lipid content was 13.9%. In a view to formulate cost-effective medium, sodium nitrate and dipotassium hydrogen phosphate in the medium was replaced by urea and superphosphate and lipid content, fatty acid profile of urea and superphosphate starved cells were studied. The lipid content under urea and superphosphate starvation was found to be 21.1% and 11% respectively. Further, the fatty acid profile of the *Chlorella* sp. grown under sodium nitrate and urea deprivation showed the predominant fatty acids like C16:0, C16:1, C18:1, C18:2, and C18:3, and notably, they were increased under nutrient starved conditions. In addition to that, fuel properties of fatty acid profile of *Chlorella* sp. complies EN 14214 enabling its suitability as a fuel.

1. Introduction

Energy is obligatory and its need is limitless for many sectors, which includes transportation, industries, residence and commercial, driven by both non-renewable (fossil fuels) and renewable (geothermal, wind, solar) sources (Owusu and Asumadu-sarkodie, 2016). Despite the known fact that the fossil fuels are under depletion, population increase in the recent times craves for more renewable energy sources to combat the demands established by modernization, urbanization, and industrialization (Chandra and Singh, 2017). On the other hand, Greenhouse gas (GHG) emissions are proportionally increasing by the increase of global energy consumption annually. At present, CO_2 reached a historic high of 33.1 Gt which is 1.7% highest growth rate since 2013 (IEA report, 2018). Moreover, over-exploitation of non-renewable fossil fuel resources for energy consumption in various sectors by humans led to i) undeniable climate change such as environmental pollution, global warming, etc. and ii) depletion of existing fossil fuel sources (Tang, 2013). Hence, subsequent introduction of various renewable feedstock classified as first (edible crops), second (non-edible crops, forest residues) and third generation (microalgae) feedstock for meeting up energy demand has been prioritized in the recent times (Chandra and Singh, 2017). As far as energy demand is concerned, plausible and alternative energy sources are required for sustainable outcome in the energy sector (Kumar et al., 2019; Rawat et al., 2013).

Among the various energy feedstock available, algae are more compatible, which can mitigate CO_2 by biological fixation through photosynthesis with the efficiency of 10–50 times greater than land plants (Mondal et al., 2017). In addition, algae seem to be the prominent feedstock for biofuel production owing to their intrinsic properties (high lipid content and high photosynthetic rate) and massive production rate than crop plants (Brennan and Owende, 2010; Mathimani and Pugazhendhi, 2018). In fact, the microalgal biomass productivity is around 4.5–7.5 ton $ha^{-1}y^{-1}$ which is comparatively higher than the oil bearing land crop Jatropha (4.1 ton $ha^{-1}y^{-1}$) (Raheem et al., 2018). By exploring the inherent capability of microalgae, biofuels such as biodiesel, bioethanol, biohydrogen can be produced efficiently from algae

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(Tiwari and Kiran, 2018). Among these biofuels, biodiesel is considered as non-toxic, biodegradable and environmentally sustainable fuel to substitute diesel fuel (Dickinson et al., 2017; Faried et al., 2017; Mofijur et al., 2019). Besides, biodiesel from algal source is considered as quasi-carbon neutral, which are much ahead of conventional diesel in terms of lower levels of hydrocarbons, carbon monoxide, SO_X emissions (Singh et al., 2017). Biodiesel comprises of alkyl esters of fatty acids obtained from transesterification of triglyceride and alcohol in the presence of catalyst and notably, triglyceride content required for biodiesel production is significantly higher in microalgae than oilseeds (Wahlen et al., 2013; Yang et al., 2017). Furthermore, in the recent times, enrichment of TAG accumulation in microalgae by manipulation at the molecular level through genetic transformation, gene silencing and genome editing using ZFNs, TALENs, CRISPR/Cas9 are being undertaken (Jagadevan et al., 2018; Min et al., 2018). Though there are various microalgal species, choosing a strain that is i) easily cultivable and acclimatize to surrounding environment ii) having high biomass and lipid productivity iii) having high proportions of characteristic fatty acids suitable for biodiesel production is of much importance from a commercial point of view. Hence, in this study *Chlorella* sp. was used to evaluate its potential as third generation feedstock for cost-effective biodiesel production by evaluating its biomass, lipid productivity and fatty acid composition under ambient and nutrient stress conditions.

2. Materials and methods

2.1. Culture maintenance

The isolated microalgal strain *Chlorella* sp. was cultivated in BG-11 media with specified culture conditions at 25 ± 2 °C, 14h light: 10h dark photoperiod, with a light intensity of 2000 lux. The mother culture was maintained without any contamination under these specified culture conditions.

2.2. Biomass productivity

The biomass productivity of *Chlorella* sp. was determined through gravimetric analysis of dry cell weight at stipulated time period using the formula as mentioned (Mathimani et al., 2017a). briefly, the microalgal cells were centrifuged at 6000 rpm for 5 min and the pellet was dried in hot air oven at 60 °C and was measured gravimetrically. It is expressed as mg L^{-1} d^{-1}.

$$Biomass\ productivity\ (mg\ L^{-1} day^{-1}) = \frac{X_e - X_i}{t_e - t_i} \tag{1}$$

Where X_e is the concentration of biomass (mg L^{-1}) at the end of the run (t_e) and X_i and t_i are the concentration of biomass (mg L^{-1}) and time at the beginning of the run respectively.

2.3. Lipid content and lipid productivity

The lipid extraction for the dried microalgal biomass was performed using Bligh and Dyer method (Bligh and Dyer, 1959). The dried microalgal powder was ground using mortar and pestle with the addition of chloroform: methanol (2:1) binary solvent system followed by phase separation with water. The lower hydrophobic chloroform layer containing the lipids were collected and measured gravimetrically after evoporating the solvent. The total lipid content and lipid productivity were determined by the following formula (Mathimani et al., 2017a; Thuy et al., 2019).

$$Y\,(\%) = \frac{W_L - W_P}{DCW} \times 100 \tag{2}$$

Where Y is lipid content, W_L is weight of lipid, W_P is pre weight of the vial and DCW is dry cell weight

$$Lipid\ productivity\ (mg\ L^{-1}\ day^{-1}) = \frac{WL_e - WL_i}{t_e - t_i} \tag{3}$$

Where WL_e and t_e are concentration of lipid (mg L^{-1}) and time at the end of the run respectively and WL_i and t_i are the concentration of lipid (mg L^{-1}) and time at the beginning of the run respectively.

2.4. Lipid enhancement under inorganic and fertilizer grade nutrients deprivation condition

The tested microalga *Chlorella* sp. was subjected to nutrient deprivation condition to increase the lipid accumulation. For this, Nitrate ($NaNO_3$) deprived BG-11 media and Phosphate (K_2HPO_4) deprived BG-11 media was prepared keeping BG-11 complete nutrient media as control. Another study was performed with commercial fertilizing nutrients by replacing the nitrate ($NaNO_3$) source by urea and phosphate (K_2HPO_4) source by superphosphate in BG-11 media (named as modified BG-11 media) to compare the lipid enhancement through inexpensive commercial analogue of inorganic nitrate and phosphate sources. For this, urea deprived modified BG-11 media and superphosphate deprived BG-11 media were prepared by keeping modified BG-11 media as control. An equal amount of initial inoculum of *Chlorella* sp. was added to all the test conditions. All the experiments were performed in triplicates.

2.5. Transesterification and GC analysis

Homogeneous acid catalysed transesterification (TE) was performed for the conversion of extracted lipids into Fatty acid methyl ester (FAME). The reaction temperature of 65 ± 1 °C for the time period of 2.5 h with the addition of 3.5% acid catalyst (H_2SO_4) was adopted for performing efficient transesterification (Mathimani et al., 2017a). After TE, the purified FAME was given for gas chromatography (PerkinElmer Clarus 500) analysis for fatty acid profiling. The optimum operating conditions kept for GC analysis are 260 °C at injection port, carrier gas (nitrogen) flow rate of 1 mL min^{-1}, oven temperature of 140–240 °C, detector temperature at 260 °C and 45 mins of total run time. Using the fatty acid profile, the fuel quality parameters such as Degree of Unsaturation (DU), Long Chain Saturation Factor (LCSF), Cold Filter Plugging Point (CFPP) were calculated as described by Mathimani et al. (2018).

3. Results and discussion

3.1. Evaluation of Chlorella sp. for biomass and lipid productivity

As the biomass and lipid productivity determines the prospective feedstock for efficient biodiesel production, the chosen green microalgal strain was evaluated for its biomass productivity (mg L^{-1} day^{-1}) and lipid productivity (mg L^{-1} day^{-1}) under nutrient repletion condition. Under complete nutrient medium, the biomass productivity of *Chlorella* sp. was 46 mg L^{-1} day^{-1} (Fig. 1). This was in accordance with the results of Hamedi et al. (2016) where the biomass productivity of *Chlorella vulgaris* was found to be 40.9 mg L^{-1} day^{-1} in SH4 medium. The lipid productivity of isolate, *Chlorella* sp. in BG-11 media (under complete nutrient medium) was found to be 5.1 mg L^{-1} day^{-1}. On a similar note, *Chlorella vulgaris* cultivated in Basal media under photoautotrophic condition showed lipid productivity of 2.7 ± 0.5 mg L^{-1} day^{-1} whereas in modified Bristols media under photoautotrophic condition the same organism showed lipid productivity of 3.7 ± 0.4 mg L^{-1} day^{-1} (Yeh and Chang, 2012).

3.2. Lipid content under inorganic nitrate and phosphate starvation

In general, microalgae tend to accumulate lipid under varied stress conditions such as light intensity, temperature, nutrient starvation,

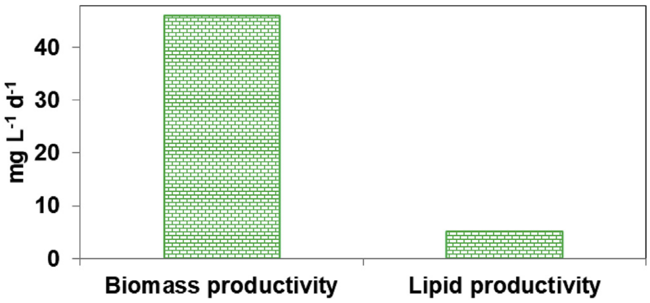

Fig. 1. Biomass and lipid productivity of the tested microalgal strain *Chlorella* sp.

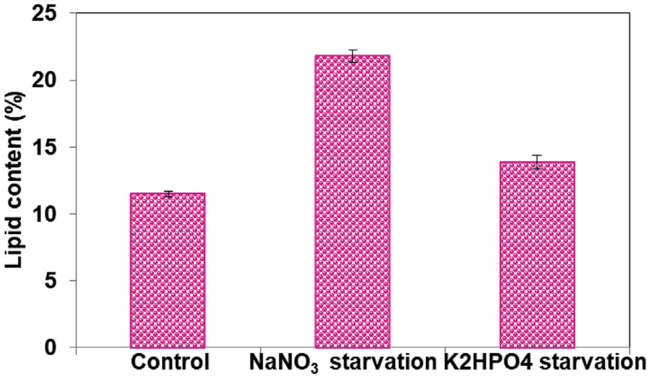

Fig. 2. Total lipid content of the tested microalgal strain *Chlorella* sp. cultivated under Control (standard BG-11 medium), $NaNO_3$ starvation, and K_2HPO_4 starvation.

salinity and metal stress (Zhu et al., 2016). In such a way, the nitrate and phosphate deprivation in BG-11 media showed a considerable increase in the lipid content of *Chlorella* sp. Under nitrate deprivation, the lipid content was 21.8 %, which was comparatively higher than standard BG-11 (control) i.e., 11.5 % lipid content. However, in a study conducted by Mujtaba et al. (2012), the final lipid content of *C. vulgaris* in BG-11 media under nitrogen starvation was found to be 24.6%. However, as shown in Fig. 2, under phosphate starvation, the lipid content was 13.9%, which is comparatively higher than the lipid content under control conditions (11.5%). This is in agreement with most of the reported studies where the phosphorus limitation in the media resulted in high cellular lipid content (Liang et al., 2013; Sharma et al., 2012; Yang et al., 2018). Briefly, the increased lipid content is due to the alteration in the lipid biosynthesis pathway where the membrane polar lipids are converted to storage form of neutral lipids under stress conditions (Fakhry and El Maghraby, 2015). Besides, the molecular elucidation of neutral lipid accumulation under N stress in the metabolic pathway brings out many possibilities such as i) Inhibition of gluconeogenesis and redirecting carbon towards triacylglycerol (TAG) accumulation ii) Up-regulation of glycolytic pathway where conversion of pyruvate to acetyl-CoA is activated after which the acetyl-CoA enters the TCA cycle and build up precursors to ACCase for biosynthesis of fatty acids iii) Up-regulation of Kennedy pathway where the final committed step in TAG biosynthesis occurs by the conversion of diacylglycerol to TAG by diacylglycerol acyltransferase (Mathimani et al., 2018; Yang et al., 2013). On the other hand, phosphorus is a vital compound present in various biomolecules such as DNA, proteins, RNA, phospholipids etc. However under phosphate limitation, cell division ceases and carbon gets accumulated in the form of TAGs which was substantiated at the molecular level by the down-regulation of genes involved in purine and pyrimidine synthesis, DNA replication and formation of ribosomal proteins (Mühlroth et al., 2017). From this study, it was observed that the lipid content of *Chlorella* sp. had a noticeable increase under nitrate and phosphate starvation conditions.

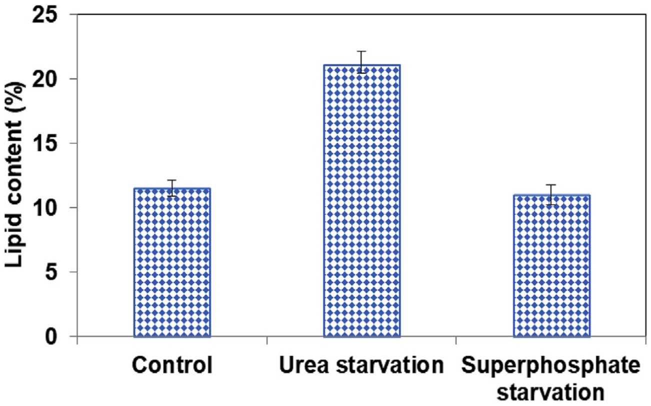

Fig. 3. Total lipid content of the tested microalgal strain *Chlorella* sp. cultivated under Control (modified BG-11 medium), Urea starvation, and Superphosphate starvation.

3.3. *Lipid content under urea and superphosphate starvation*

As a commercial point of view, any subsequent reduction of cost in the process leads to economically viable biodiesel (Sing et al., 2013). In this study, commercially available nutrients that are cheaper than inorganic nutrients such as urea and superphosphate were used by replacing sodium nitrate ($NaNO_3$) and di-potassium hydrogen phosphate (K_2HPO_4) in the standard BG-11 media (named as modified BG-11). The lipid content under urea starvation and superphosphate starvation were found to be 21.1% and 11% whereas in modified BG-11 media, it was found to be 11.5% lipid content in *Chlorella* sp. (Fig. 3). In another study, higher lipid content of 34.13 ± 0.15 % was observed in *Chlorella* sp. isolated from Huang gang pond, China in urea starved BG-11 media (Zhou et al., 2013). This lipid content was higher than the result obtained in this study. However, on comparing both the nutrient starvation condition in this study, both nitrate and urea starved *Chlorella* sp. showed higher lipid content than the phosphate starved *Chlorella* sp. Hence considering the high lipid content, the nitrogen starved *Chlorella* sp. (both $NaNO_3$ and urea starved cells) was further analysed for fatty acid profiling.

3.4. *Fatty acid profiling*

The influence of nitrogen starvation on *Chlorella* sp. for fatty acid profiles were elucidated through gas chromatography. The fatty acid composition of *Chlorella* sp. under control, nitrate starved, and urea starved conditions were represented as pie chart (Fig. 4). The fatty acid profile of *Chlorella* sp. showed varied concentrations of saturated fatty acids and unsaturated fatty acids. C16:0, a saturated fatty acid and C18:1, a monounsaturated fatty acid was predominantly detected in FAME profile of *Chlorella* sp. under three different conditions but with slight variation in concentrations as seen in abundant proportion in most of the microalgal fatty acid composition (Chen et al., 2018; Sinha et al., 2016). Majority of the fatty acids were unsaturated (MUFA and PUFA) but the composition of C16:0 was high in the total FAME content under all the three conditions. The presence of major fatty acids like C16:0 (Hexadecanoic acid), C16:1 (9-cis-hexadecenoic acid), C18:1 (Octadecanoic acid), C18:2 (octadecadienoic acid), C18:3 (Octadecatrienoic acid) and C20:3 (eicosatrienoic acid) were noticed from the FAME profiling through GC analysis. In specific, under sodium nitrate and urea starved conditions, 23.8% and 23.67% of C16:0 were observed respectively which were higher than the control (20.2%) and also predominant over other fatty acids. It is also noteworthy that Cetane number increases with high proportions of saturated fatty acids leading to good quality biodiesel (Nabi and Heimann, 2013). Similarly, C18:1

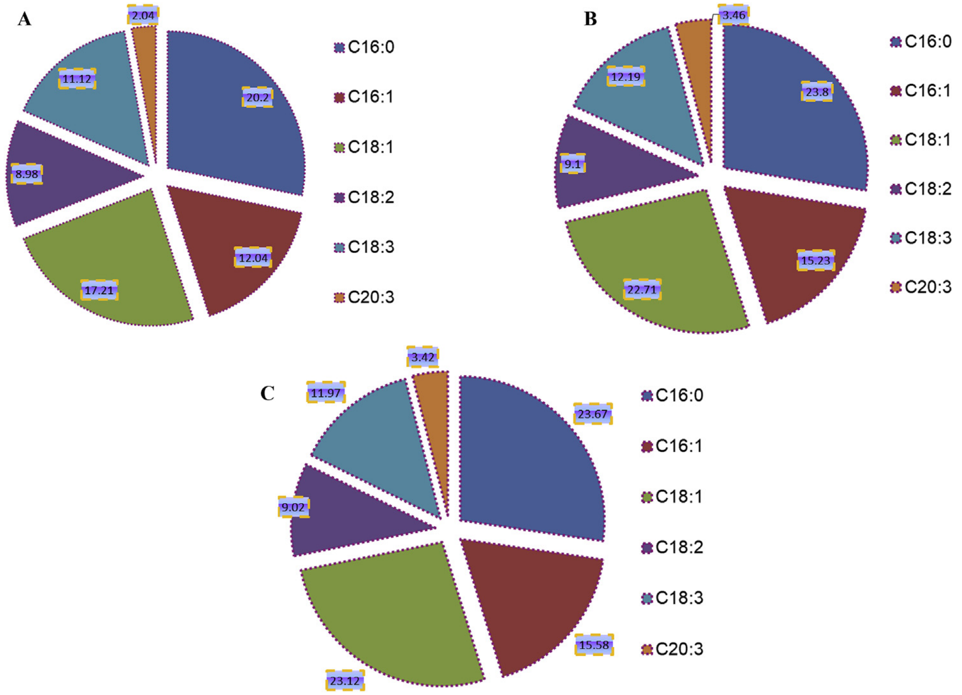

Fig. 4. Major fatty acids identified in the *Chlorella* sp. A. standard medium B. sodium nitrate starvation, C. urea starvation.

Table 1
Biodiesel fuel properties of *Chlorella* sp. grown under i) standard BG-11 media ii) Sodium nitrate starvation iii) Urea starvation.

Parameters	Standard BG-11 media	Sodium nitrate starvation	Urea starvation
DU	69.45	80.52	80.68
LCSF	2.002	2.38	2.367
CFPP	−10.187	−9.001	−9.042

was observed to be 22.71% and 23.12% under sodium nitrate and urea starved conditions respectively which were even higher than control (17.21%). The other major fatty acids like C18:2, C18:3 and C20:3 were spotted to be at lower concentrations than C16:0 and C18:1. In fact, for high quality biodiesel production there should be optimal balance between saturated and unsaturated fatty acids (Sinha et al., 2016) and less concentration of C18:3 ($< 12\%$) in the total FAME content is desirable as *bis-allylic* carbon in C18:3 is more prone to oxidation and thereby reducing the stability of biodiesel (Mathimani et al., 2017b). In this study, C18:3 in the total FAME content was found to be at low concentration i.e., 11.12%, 12.19%, and 11.97% under control, nitrate starved, urea starved conditions. From these results, it is obvious that nitrate and urea starved cells are capable of deriving fatty acids that are suitable for biodiesel production.

3.5. *Assessment of biodiesel quality*

The biodiesel quality is greatly influenced by the presence of saturated and unsaturated fatty acids of algae. Fuel quality parameters DU, LCSF, CFPP were determined in this study and are presented in Table 1.

DU is the total amount of MUFAs and PUFAs indicating the number of double bonds present in the fatty acid chain which when present in higher concentration leads to decrease in the oxidative stability (Benjumea and Agudelo, 2011). In this study, DU under standard, sodium nitrate starvation, and urea starvation (69.45, 80.52, and 80.68 respectively) were in accordance with the standard fuel quality parameters. According to European standard EN 14214, the LCSF should not be > 5 and the CFPP should be $< 0\,^{\circ}\mathrm{C}$ (Mathimani et al., 2018). In this study, the LCSF values under standard, sodium nitrate starvation, and urea starvation (2.002, 2.38, and 2.367 respectively) were in congruent with EN 14214. Similarly, the CFPP values under standard, sodium nitrate starvation, and urea starvation (−10.187, −9.001, and −9.042 respectively) comply with European standard. Hence, the biodiesel obtained from *Chlorella* sp. have met the specifications laid by EN 14214, substantiating its use as a fuel in diesel engines.

4. Conclusion

Microalgae with high biomass and lipid productivity are suitable for biodiesel application. Based on the results of this study, our isolate *Chlorella* sp. have shown higher biomass productivity of $46\,\mathrm{mg\,L^{-1}\,day^{-1}}$ and higher lipid content of 21.8% and 21.1% under sodium nitrate and urea starvation condition than control. To aid a significant path in process economization, this study imparts the use of affordable farm fertilizers such as urea and superphosphate. The fatty acid composition analysis of the produced FAME showed the presence of medium chain fatty acids containing high proportions of C16:0 and C18:1 suitable for high quality biodiesel. Moreover, the fuel quality parameters such as DU, LCSF, and CFPP obtained from the fatty acid profile of *Chlorella* sp. were in accordance with the European standards EN 14214. Hence, this study suggests the use of *Chlorella* sp. as a potential third generation feedstock for biodiesel production.

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