

Glycerol on Lipid Enhancement and FAME Characterization in Algae for Raw Material of Biodiesel

Anwasha Khanra, Shrasti Vasistha, Monika Prakash Rai [‡]

Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Sector-125, Noida (Gautam Budh Nagar), U.P. (201313) India

(manali.anwasha@gmail.com, shrasti.vasistha@gmail.com, mprai@amity.edu)

[‡]Corresponding Author: mprai@amity.edu; monika1778@gmail.com

Telephone: +91-120-4392721, Mobile: +91-9313887818

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Abstract- To reduce the cost of media components and consecutively enhance the amount of lipid formation, the effect of crude glycerol on *Euglena gracilis* is reported for the first time. Presently, *E. gracilis* has been chosen because of excellent capability of its growth and lipid synthesis in mixotrophic culture condition on organic carbon sources (Glucose and Glycerol). Biochemical composition of the cell in presence of both organic carbon sources were compared with mixotrophic condition. Glycerol was selected as most suitable carbon source and the highest biomass concentration of 2.63 g L⁻¹ along with lipid accumulation of 27.64 % were observed. The effect of biodiesel derived crude glycerol on the growth of *E. gracilis* was found very positive. A significant increase in lipid accumulation (49.46%) was noticed in presence of crude glycerol by optimizing concentration and other culture parameters. Fatty acid methyl esters (FAMEs) produced from lipid biomolecules by following transesterification reaction, were analysed through GC-MS. FAMEs governed by 93.458% C16-C18 fatty acids with appropriate quantities of Saturated fatty acids (SFA) and Unsaturated fatty acids (UFA). Several requisite fuel properties were estimated and found to be in accordance with American and European biodiesel standards. Hence, this study focuses the improved lipid synthesis in *E. gracilis* utilizing crude glycerol for the preparation of raw biodiesel and it supports biorefinery approach.

Keywords Algae, Crude glycerol, *Euglena gracilis*, FAME, Mixotrophy.

1. Introduction

Since the last decades, alternative fuel sources have gained much attention due to the large uncertainty associated with future oil supplies [1, 2, 3]. Green biomass attracts huge interest among researchers as a possible feedstock for the production of eco-friendly biofuel, including biodiesel, bioethanol etc. [4, 5, 6]. Among photosynthetic microorganisms, algae have a great potential of capturing atmospheric CO₂ leading to carbon neutral biodiesel production [7]. Algae have a tendency to synthesize and store lipids mostly in the form of triacylglycerols (TAG) that can be extracted for the production of biodiesel [8, 9, 10]. Transesterification is the most commonly used method that involves the conversion of lipid into fatty acid methyl ester (FAME) using acid, base or enzyme as catalyst [11, 12]. In

the commercialization of algae based system, the accurate prediction of lipid productivity under various environmental conditions mainly mode of cultivation, operational light regime and fatty acid characteristics are crucial for the cost assessment and feasibility studies of full scale biodiesel production.

Most of the algal strains are photoautotrophic in nature where light energy is harvested and CO₂ is used as a carbon source [13]. However, photoautotrophic cultivation mode is quiet insufficient for high biomass and lipid production from algae as less light penetration and mutual shading of the algal cells cause light inadequacy and hence, growth rate decreases earlier [14]. In case of few algal species, like *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Scenedesmus sp.*, etc., mixotrophic culture condition exists where CO₂ and

organic carbon both are utilized simultaneously in the metabolic pathways of photosynthesis and respiration [15, 16, 17]. The cells are provided with organic carbon supplementation leading to enhance biomass production with improved lipid accumulation making them better source for biofuel application.

The cost of organic carbon sources like glucose, fructose etc. is very high for mixotrophic and heterotrophic mode of algae cultivation; to overcome the situation, cheap substrates like crude glycerol and sodium acetate can be used. Choi et al., (2015) reported that crude glycerol has been influenced for high biomass and lipid content in algae, because it is a byproduct obtained in biodiesel production; moreover it also supports the concept of biorefinery [18]. Biorefinery based integrated approach in algae not only leads the lipid accumulation for biodiesel production, but also produces various by-products promoting for pharmaceutical and nutraceutical applications [19].

In photoheterotrophic or mixotrophic cultivation condition, light is very important factor, because the ratio of carbon flux and the amount of light should be proportionate for high lipid yield. Few algal strains like *Scenedesmus sp.*, *Chlorella sp.*, *Nannochloropsis* have shown significant effect of light supply when grown under photoheterotrophic culture condition providing organic carbon sources in the media [17, 20].

In the process of biodiesel synthesis, the FAME profile and respective fuel properties are very essential parameters. Fuel properties like cetane number (CN), iodine value (IV), saponification value (SV), degree of unsaturation (DU), oxidative stability (OSI), long chain saturated factor (LCSF) and cold filter plugging point (CFPP) are requisite to check the quality of biodiesel production [21, 11]. These fuel properties are greatly dependent on FAME composition of algal lipid. Saturated fatty acids (SFAs) containing the chain length C12 to C18 possess some valuable features such as low viscosity, higher cetane number and less emission of pollutants (SO_x, NO_x etc.) that make them prominent for biodiesel production. Monounsaturated fatty acids (MUFA) like C16:1 and C18:1 are also very essential component for suitable biodiesel production, while the long chain polyunsaturated fatty acids (LC-PUFA) like arachidonic acid (ARA), docosahexanoic acid (DHA) etc. should be present in a limited quantity [22, 23].

In view of selecting algal strain as a promising and potential feedstock for biodiesel applications, the biomass, lipid content and FAME profile are to be needed for consideration. As best in our knowledge, various algal strains like *Chlorella sp.*, *B. braunii*, *Scenedesmus sp.* have been repeatedly reported for biofuel purpose [23], but the photosynthetic microbe *E. gracilis* has not been much explored, though this naturally occurring species generally possess high cellular lipid. *Euglena* species referred as a unicellular phytoflagellate protist, comes under eugleneaceae family that can easily grow under photoautotrophic, heterotrophic and mixotrophic culture condition in both aerobic and anaerobic mode [24]. Considering the physical property like pH, *E. gracilis* is

capable of growing at a wide range of pH (2-9) that shows its ability to easy scale up for various applications [25]. Furthermore, a strong acidification of the *Euglena* species assists less risk of contamination through fungal and bacterial strains except few acidophilic bacteria. Due to this strong physical make-up, *E. gracilis* is demonstrated as a favourable candidate for biorefinery aspect. In the work of Ramachandra et al. (2013), it has been reported that the cultivation of *E. gracilis* was performed successfully in wastewater resulting as cost efficient lipid production (24.6% w/w) along with biomass accumulation of 1.24g L⁻¹ dry weight by consuming organic carbon under photoheterotrophic mode [26]. Apart from lipid production, *E. gracilis* is also known for the enhanced synthesis of extracellular metabolites such as α -tocopherol, paramylon, tyrosine, wax ester [24].

In the present study, *E. gracilis* was chosen due to having intense ability of growth under mixotrophic condition as well as producing high lipid yield. In view of minimizing the cultivation cost, crude glycerol is used as an organic carbon source in mixotrophic mode for quality biodiesel production. Here, we report for the first time the effect of crude glycerol on the growth of *E. gracilis*, lipid production and biodiesel properties of the FAME produced.

2. Materials and Methods

2.1 Microbial strain and culture condition

Euglena gracilis NCIM 2710 obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India was cultured photoautotrophically in Hutner media containing 0.2g CaCl₂, 0.5g MgSO₄, 0.4g NH₄HCO₃, 0.4g KH₂PO₄; 2 ml of Stock A, composed of 2.2g ZnSO₄·7H₂O, 2g MnSO₄·4H₂O, 0.5g Na₂MoO₄·2H₂O, 0.04g CoCl₂·6H₂O dissolved with HCl and diluted to 50 mL with double distilled water; 1 mL of trace minerals stock B, composed of 0.078g CuSO₄·5H₂O, 0.057g H₃BO₃ dissolved in double distilled water and diluted to 100 mL. The Hunter medium was modified by removing the vitamins as no significant effect of media vitamins was found on cell growth (data not shown). Inoculation was set by using exponentially growing cells maintaining the initial optical density of 0.1 and culture was grown on atmospheric CO₂ in a temperature controlled orbital shaker at 28±1°C providing 16:8 light/dark illumination with light intensity of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and shaken intermittently.

2.2 Cultivation under mixotrophic mode

The alga was cultivated under mixotrophic condition providing organic carbon sources. In this study, two common organic carbon sources glucose and glycerol were chosen to estimate the biomass concentration, lipid accumulation and lipid productivity. The amount of carbon sources were calculated and added according to the number of carbon atoms present in each. Other media components and environmental conditions were kept constant as provided in photoautotrophic culture.

2.3 Growth analysis

Growth of *E. gracilis* was measured by dry cell weight method after every 24h for both photoautotrophic and mixotrophic cultures. The algal cells were harvested in the respective stationary phase by centrifugation (Eppendorf 5810R) at 894 x g for 15 min. The pellet was then washed twice to remove the debris and dried in hot air oven at 70°C to obtain the dry cell mass. Biomass concentration, productivity, specific growth rate (μ) and doubling time (D) were calculated by using following formulas [8, 27].

Biomass concentration (g L^{-1}) = mass of the culture/ volume

Biomass productivity ($\text{g L}^{-1} \text{d}^{-1}$) = mass of culture/volume x days

Specific growth rate (μ) = $\ln X_2 - \ln X_1 / T_2 - T_1$

where X_2 and X_1 show the final and initial biomass concentration respectively; T_2 and T_1 show the final and initial time respectively.

Doubling time (D) = $0.693 / \mu$

Where, μ is specific growth rate

2.4 Biochemical Composition

2.4.1 Lipid production

The total lipids present in cells were extracted by using organic solvents chloroform and methanol in the ratio of 2:1 as done in our previous study [27]. The dried microbial lipid content was calculated by using the following formulas [8].

Lipid production (g L^{-1}) = mass of lipid (g) / volume (L)

Lipid productivity ($\text{g L}^{-1} \text{d}^{-1}$) = mass of lipid (g) / volume (L) x cultivation time (d)

Lipid content (%) = mass of lipid (g) / mass of culture (g) x 100

2.4.2 Protein Estimation

The crude protein was determined by Lowry method [28]. The absorbance of the sample was checked and the concentration was determined using standard curve.

Total Protein Content = wt. of protein (from BSA curve) X 100/ dry cell mass (g)

2.4.3 Carbohydrate Measurement

The content of carbohydrate is estimated by the modified method of 3, 5-Dinitrosalicylic acid colourimetrically using 100 mg of dry algal powder [29]. The carbohydrate content was estimated using DNS reagent and optical density of the sample was determined against the blank at 540 nm in a UV-visible spectrophotometer.

Carbohydrate Content (%) = wt. of carbohydrate (from Glucose standard curve) X 100/ dry cell mass (g)

2.4.4 Estimation of Moisture and ash content

Moisture content present in algal cell was estimated gravimetrically after drying the samples at 105 °C for 1 h and desiccating for 30 min before weighing. On the other hand, the ash content was also determined after heating the dried biomass at 550 °C for 30 min and desiccating for 30 min [30].

2.5 Growth of *E. gracilis* on crude glycerol

In the first stage of experiment, among two organic carbon sources (glucose and glycerol), glycerol was found most suitable in view of biomass and lipid productivity. In the second stage of the experiment, technical glycerol was replaced by crude glycerol, obtained from acid catalysed transesterification of algal lipid. The effect of different concentration viz. 0, 1.5, 3, 4.5 and 5 % volume fraction of crude glycerol was recorded for biomass and lipid production.

2.6 Transesterification and FAME analysis

The total extracted lipids were transesterified using methanol and acid based catalyst to obtain FAME [27]. The FAME obtained after the completion of reaction, was washed properly by using double distilled water and recovered in organic phase. The FAME sample was subjected to dry at Rotary Evaporator (BUCHI R210) and measured gravimetrically. The composition of FAME was analysed by GC-MS and the components were identified by the comparison of retention time with those of the standard.

2.7 Evaluation of Biodiesel property

The FAME components produced from *E. gracilis* were evaluated for its biodiesel quality by determining the CN, SV, IV, DU, OSI, LCSF and CFPP using the following formulas [8].

$$SV = \sum (560 \times F) / MW \quad (1)$$

$$IV = \sum (254 \times F \times D) / MW \quad (2)$$

$$CN = (46.4 + 5458 / SV) - (0.225 \times IV) \quad (3)$$

$$DU = (\text{MUFA, wt \%}) + (2 \times \text{PUFA, wt \%}) \quad (4)$$

$$\text{LCSF} = 0.1 \times \text{C16} + 0.5 \times \text{C18} + 1 \times \text{C20} + 1.5 \times \text{C22} + 2 \times \text{C24} \quad (5)$$

$$\text{CFPP} = 3.1417 \times \text{LCFS} - 16.477 \quad (6)$$

$$\text{OSI} = Y = 117.9295 / x + 2.5905 \quad (7)$$

Where F = % of each fatty acid, MW = molecular weight of each fatty acid [31], D = no. of double bonds and x = weight % of oleate (C18:1) + weight % of linoleate (C18:2).

Statistical analysis: All the analyses were performed in triplicates and the mean values were plotted in the graphs.

3 Results and Discussion

3.1 Growth assessment of *E. gracilis* under different culture conditions

The present investigation revealed that *E. gracilis* has been grown in photoautotrophic and mixotrophic cultivation mode using Hutner media and the growth pattern is shown in Fig.1. In photoautotrophic mode, the biomass concentration of 0.096 g L⁻¹ has been obtained during 6th day of exponential phase and maximum biomass of 0.160 g L⁻¹ has been observed on 12th day of cultivation (Supplementary Fig 1). Comparatively a fast growth rate has been achieved in mixotrophic condition (providing glucose), where, the alga showed early stationary phase and highest cell density of 0.564 g L⁻¹ has been achieved on sixth day. Hence, the biomass yield in mixotrophic condition was found 3.5 folds higher in comparison to photoautotrophic culture on their maximum growth. According to Yu et al., 2011 *Anabaena sp* PCC7120 showed 1.6 fold increment in biomass under mixotrophic cultivation by utilizing exogenous glucose than in autotrophic mode [32]. Furthermore, two times higher growth of *C. sorokiniana* in mixotrophic growth condition was reported as compared to photoautotrophic and even heterotrophic mode [20]. It is reported that the algae can utilize light and CO₂ in mixotrophic condition by following photochemical pathway where the energy produced in the form of ATP, which escalate the glucose metabolism through TCA cycle in *E. gracilis* [25].

The algal growth was also scrutinised by measuring the specific growth rate (μ) which is defined as the increase in cell number per unit time, but the cell size remains constant. The specific growth rate and doubling time (D) on the 6th day of inoculation is represented in Table (1). The specific growth rate ' μ ' in mixotrophic culture condition has been found as 0.519 d⁻¹ which is 1.7 times higher in comparison to photoautotrophic mode (0.299 d⁻¹). Doubling time of *E. gracilis* in mixotrophic mode of cultivation has been recorded as 1.335 d that is significantly reduced than in photoautotrophic condition i.e. 2.318 d. As expressed in Table 1, the alga showed higher specific growth rate with shorter doubling time and higher biomass production under mixotrophic culture proves the suitability of it over photoautotrophic culture. All these properties contribute some important and desirable features like decreased chance of contamination and reduced production cost during mass cultivation [8].

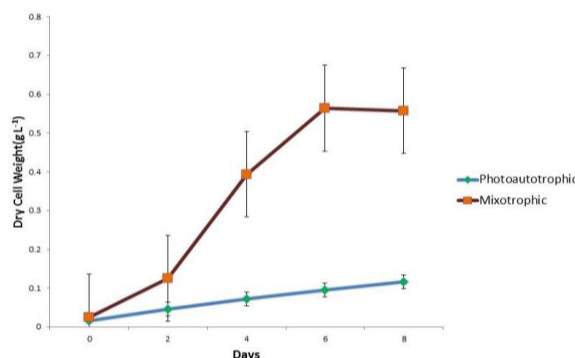


Fig 1 Growth analysis of *E. gracilis* in photoautotrophic and mixotrophic culture modes

Table 1. Comparison of Biomass concentration, specific growth rate and doubling time in photoautotrophic and mixotrophic mode

Cultivation	Biomass (g L ⁻¹)	Specific growth rate (d ⁻¹)	Doubling time (d)
Photoautotrophic	0.096 ± 0.002	0.299 ± 0.004	2.318 ± 0.002
Mixotrophic	0.564 ± 0.003	0.519 ± 0.003	1.335 ± 0.004

Data are expressed as mean ± SD, n=3

3.2 Selection of organic carbon source for biomass and lipid yield

To establish suitable organic carbon source for higher biomass density and lipid accumulation, *E. gracilis* was grown mixotrophically in Hutner media by applying technical glycerol as organic carbon source. The result shown in Table 2, depicts the maximum biomass production 2.68±0.005 g L⁻¹ and productivity 0.44±0.002 g L⁻¹ d⁻¹ in presence of glycerol whereas, glucose enriched medium has expressed low biomass productivity of 0.093 ± 0.002 g L⁻¹ d⁻¹. This is probably due to less permeability of glucose through *E. gracilis* cell membrane [33, 34].

Measurement of lipid biomolecules under various carbon supplementations is also described in Table 2. Highest amount of lipid production of 0.741 ± 0.006 g L⁻¹, lipid productivity 0.124 ± 0.004 g L⁻¹ d⁻¹ with maximum lipid content 27.64 % have been achieved in presence of glycerol containing medium. On the other hand, less lipid content has been observed with glucose (20.46%). Lipid accumulation in *E. gracilis* is found to decrease

significantly in absence of organic carbon and recorded nearly 11 %.

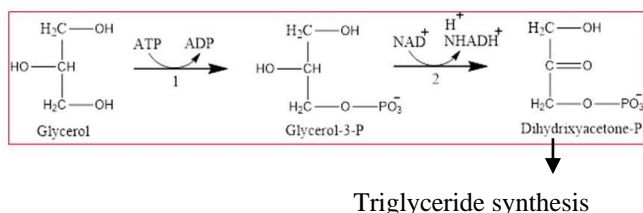
Table 2. Estimation of biomass concentration, biomass productivity, lipid production, lipid productivity and lipid content of *E. gracilis* utilizing carbon sources.

Carbon sources	Biomass concentration (g L ⁻¹)	Biomass productivity (g L ⁻¹ d ⁻¹)	Lipid production (g L ⁻¹)	Lipid productivity (g L ⁻¹ d ⁻¹)	Lipid content (%)
Without organic Carbon	0.089 ± 0.004	0.015 ± 0.001	0.009 ± 0.004	0.001 ± 0.004	10.95 ± 0.004
Glucose	0.561 ± 0.003	0.093 ± 0.002	0.120 ± 0.005	0.02 ± 0.003	21.39 ± 0.003
Glycerol	2.68 ± 0.005	0.44 ± 0.002	0.741 ± 0.006	0.124 ± 0.004	27.64 ± 0.002

Data are expressed as mean ± SD, n=3

As per the result obtained, it can be predicted that *E. gracilis* has the capability to assimilate varieties of carbon sources that were to be tested. Enhanced biomass and lipid content in glycerol containing medium is mainly due to the easy metabolism of glycerol to produce glycerol 3-phosphate (G3P) and dihydroxy acetone phosphate (DHAP) by two major enzymes glycerol kinase and glycerol phosphate dehydrogenase present in the microbe. DHAP is directly associated with triglyceride synthesis pathway as shown in following chemical reaction (A).

Reaction (A). Chemical reaction of DHAP production from glycerol towards triglyceride synthesis (1 and 2 represents glycerol kinase and glycerol phosphate dehydrogenase)



From the above chemical reaction, it is expected that glycerol plays an important role in lipid biosynthetic pathway. Glycerol is also utilized as one of the major organic carbon source to produce energy in the form of Adenosine di phosphate (ADP) that is assimilated by algal cell. Some previous reports also proved that glycerol is an excellent source for lipid production in algae like

Scenedesmus sp. CCNM1077, *Haematococcus sp.*, *Nanochloropsis sp.*, and *Chlorella sp.* [35].

3.3 Biochemical composition of *E. gracilis* in presence of different organic carbon

A comparative analysis of different macromolecules like lipid, carbohydrate and protein were described on the basis of organic carbon sources (glucose and glycerol), provided to the algal cell. Glycerol mediated culture condition shows maximum lipid and protein content as 27.25 and 28.32% respectively in compared with glucose rich culture mode. No significant difference has been shown on ash content of both glucose and glycerol rich medium.

Table 3: Biochemical Composition of cell in presence of glucose and glycerol in mixotrophic condition

Culture condition	Lipid (%)	Carbohydrate (%)	Protein (%)	Moisture Content (%)	Ash Content (%)
With Glycerol	27.25	33.40	28.32	9.3	2.0
With Glucose	19.18	45.28	22.54	11.4	2.2

3.4 Effect of crude glycerol concentration on biomass and lipid production

In this present study, influence of different concentration of crude glycerol has been evaluated in the medium to observe the biomass and lipid yield from *E. gracilis*. As shown in Fig 3, the highest biomass yield has been found as 3.25 g L⁻¹ utilizing 3% (v/v) crude glycerol in the medium; however the highest lipid production (1.573 g L⁻¹) and lipid content (49.46%) have been attained by providing 4.5 % (v/v) glycerol with slight effect on biomass yield (3.18 g L⁻¹). Least biomass and lipid production have been obtained in glycerol free medium and less production has also been recorded at highest glycerol concentration of 6 % (v/v). Our result shows that crude glycerol is a favourable organic carbon to support biomass and lipid yield in *E. gracilis*, but Triglyceride synthesis concentration of crude glycerol above 4.5 % (v/v) found to be inhibitory. In the study of Liang et al., (2009), the maximum growth of algae was obtained at 1 % glycerol while the productivity decreased by increasing the concentration of 2 % onwards, but the maximum lipid content was recorded also in 2 % and reduced afterwards [14]. Therefore, we can represent that after a certain

concentration of organic carbon sources, biomass of algal species is able to diminish due to substrate inhibition and this criteria is species dependent.

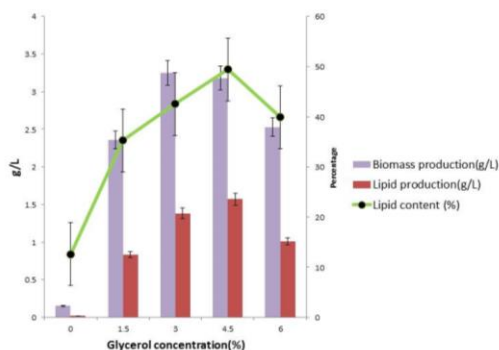


Fig. 2. Effect of different glycerol concentration on biomass concentration, lipid production and lipid content of *E. gracilis*

3.5 FAME Characterization and fuel properties of biodiesel from *E. gracilis*

The chemical composition of fatty acid methyl esters (FAME) obtained by GC-MS, shows the quality biodiesel properties as mentioned in Figure 3. According to the figure, it indicates the fatty acid profile of *E. gracilis* under mixotrophic cultivation mode using glycerol. Carbon chain length of C16 to C18 contributes 93.46 % of total fatty acid produced which are the most abundant fatty acids needed for biodiesel application. FAME profile depicts palmitic acid (C16:0) which is one of the major fatty acid with the existence of 24.17 % followed by Oleic acid (18:1) with 22.20 %. Apart from that, the other two essential fatty acids like linoleic (C18:2) and linolenic acid methyl ester (C18:3) are also present with the occurrence of 13.68 % and 11.61 % respectively. The sum of saturated and monounsaturated fatty acid components lying in the range of 70.88 % of the total FAME produced, shows quality fuel properties. According to European standard EN14214, the concentration of linolenic acid is necessary as less than 12% for biodiesel application (European standard 2004) and our current study displays its percentage within the limit (Table 4) .

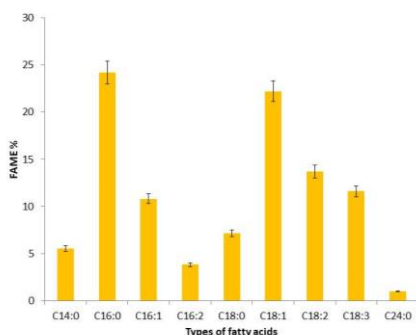


Fig. 3. Fatty acid (in %) composition *E. gracilis*

Table 4. Predicted biodiesel properties and comparison with other algal biodiesel feedstocks

Property(units)	<i>E. gracilis</i> (Present study)	<i>S. abundans</i> [22]	<i>Coelastrella sp. M-60</i> [7]	<i>Chlorella sp.V</i> [5]	European Standards (EN 14214)	US Standards (ASTM D6751)
CN	73.08	53.68	55.5	64.90	47(min)	51(min)
SV(mg KOH/g oil)	191.45	205.8	194.5	197.99	-	-
IV(g I ₂ /100g biodiesel)	9.680	85.07	84.9	39.85	120(max)	-
DU(wt%)	90.14	76.78	91.9	-	-	-
LCSF(°C)	8.012	16.12	19.3	6.43	*	-
CFPP(°C)	8.694	34.17	44.3	3.73	-	-
OSI(h)	3.065	5.99	-	-	8(min)	3(min)

*= Location and Season dependent

CN, an eminent fuel property of biodiesel, indicating the ignition quality is highly determined by FA profile. Enhanced CN shows better combustion, less nitrous oxide (NO_x) emission, well oxidative stability and easier start-up of engine [36]. The CN calculated for our present study is 73.08 which meet the minimum amount of 47 and 51 as for European and US standard.

IV is able to determine the unsaturation of biodiesel where more double bond in FA profile results higher IV for that oil content [36]. Increased IV represents polymerization of glycerides and deposition of lubricant in the engine, so the algal strains should have IV less than the set limit as 120 g I₂ / 100 g oil given by EN14214. Present work also shows the IV as 9.68, which ranges within the limit.

SV needs to saponify one gram of oil measuring as milligrams of KOH. Basically, SV is required to calculate the CN. According to European standard, SV is not included as a constrained property of biodiesel . In the present work, SV found to be 191.45 mg KOH g⁻¹ which is

nearby similar to *Coelastrella sp.* M60 that was recorded 194.5 mg KOH g⁻¹ [11].

DU is directly associated to OSI of the biodiesel, obtained from *E. gracilis*. Maximum amount of SFA and MUFA shows lower DU which significantly increases the OSI value. The DU (90.14%) in recent study is very similar with earlier study on *Coelastrella sp.* M60.

OSI is extremely affected by the presence of higher concentrations of saturated fatty acids. As can be seen in Table 4, the OSI value for *E. gracilis* has been calculated as 3.065 that reaches the maximum set limit created by the European standards.

LCSF and CFPP are another two most important parameters described as biodiesel property. Both of these properties are able to evaluate the high percentage of SFA along with flow properties depending on temperature. However, higher SFA content creates crystallization of fuel that may lead the fuel filters of engine under colder climatic situation. Therefore, the lowest suitable limit for CFPP is suggested at -5^o C to -13^o C [37]. Our work determines the CFPP as 8.694 due to the presence of a considerable content of PUFA which improve the flow properties. The fuel properties of *Euglena gracilis* is first time elaborated in the present study and the effect of crude glycerol found extremely beneficial with FAME profile and fuel characteristics for its application.

References

- [1] R. Arslan, Y. Ulusoi. "Utilization of waste cooking oil as an alternative fuel for Turkey", 5th International conference on Renewable Energy Research and Applications (ICRERA), Birmingham, UK, pp. 149-152, 20-23 Nov 2016. (conference paper)
- [2] A. El-Khair, B. El-Sayed, Manal, G. Mahamoud, Shima and R. Hamed "Complementary Production of Biofuels by the Green Alga *Chlorella vulgaris*" *IJREER*, vol.5, no.3, pp. 936-943, 2015.
- [3] A.E. Ozdemir, S.A. Oy, "Alternative renewable energy producing systems by utilizing piezoelectric transducers" 5th International conference on Renewable Energy Research and Applications (ICRERA), Birmingham, UK, pp. 59-62, 20-23 Nov 2016. (conference paper)
- [4] M. Aziz, T. Oda, T. Kashiwagi, Z. Hodai, "Novel power generation from microalgae : application of different gasification technologies" Processing residuals from microalga technology", 4th International conference on Renewable Energy Research and Applications (ICRERA), Palermo, Italy, pp. 745-749, 22-25 Nov 2015. (conference paper)
- [5] P. Yadav, A.Kumar Varma and P.Mondal "Production of Biodiesel from Algal Biomass Collected from Solani River using Ultrasonic Technique" *IJREER*, vol.4, no.3, pp. 714-724, 2014.
- [6] J. Bendfeld, S. Balluff, S. Krauter, "Green energy from the ocean: an overview of cost-effective and reliable measuring systems", 4th International conference on Renewable Energy Research and Applications (ICRERA), Palermo, Italy, pp 375-378, 22-25 Nov 2015. (conference paper)
- [7] B. Kiran, R. Kumar, D. Desmukh, "Perspectives of microalgal biofuels as a renewable source of energy", *Energ Converse Manage.*, vol. 88, pp. 1228-44, 2014.
- [8] S. Vidyashankar, V.S.K. Gopal, G.V. Swarnalatha, M.D. Kavitha, V.S. Chauhan, R. Ravi, A.K. Bansal, R. Singh, A. Pande, G.A. Ravishankar, R. Sarada, "Characterization of fatty acids and hydrocarbons of chlorophycean microalgae towards their use as biofuel source", *Biomass and Bioenerg.*, vol. 77, pp. 75-91, 2015.
- [9] B. Verma, C. Balomajumder, "Algal Biodiesel Production: Comparison, Characterization and Optimization of Various Extraction Processes" , *IJREER*, Vol.6, No.3, 2016, pp 1071-1075.

4 Conclusion

The present study concludes that the most positive method for cultivation of *E. gracilis* is mixotrophic culture condition using glycerol. 4.5 % volume fraction of crude glycerol was found most encouraging for high biomass and lipid production of 3.18 g L⁻¹ and 1.573 g L⁻¹ respectively. Cellular lipid content of 49.46% was observed under improved culture conditions providing crude glycerol. The FAME profile constitutes over 93% of total fatty acids ranging between 16-18 carbon chain lengths with suitable percentage of SFA, MUFA and PUFA. Appropriate fuel properties confirm the potential of *E. gracilis* as a resourceful feedstock for biodiesel production. This work proposes the utilization of crude glycerol, coming from biodiesel industry for large scale cultivation of *E. gracilis* economically.

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Supplementary material

Supplementary 1 is provided along with the manusc

- [10] E.K. Coban, C. Gencoglu, D. Kirman, O. Pinar, D. Kazan, "Assessment of the effects of medium composition on growth, lipid accumulation and lipid profile of *Chlorella vulgaris* as a biodiesel feedstock", 4th International conference on Renewable Energy Research and Applications (ICRERA), Palermo, Italy, pp. 793-796, 22-25 Nov 2015. (conference paper)
- [11] R. Karpagam, K.J. Raj, Ashok Kumar B, P. Varalakshmi, "Characterization and fatty acid profiling in two fresh water microalgae for biodiesel production: Lipid enhancement methods and media optimization using response surface methodology", *Bioresour Technol.*, vol. 188, pp. 177-184, 2015.
- [12] R. Valez-ojeda, M. Gonzalez- Munoz, R. Vs-Vazquez, J.N. Zapat, J.C. Chavarria- herandez, S.L. Adrian, F.B. Perez, T.T. Thompson, G.G. Solorzano, R.M.E.G. Medrano, "Characterization of five fresh water microalgae with potential for biodiesel production", *Algal Res*, vol. 7, pp. 33-44, 2015.
- [13] K. C. C. Morais, J. V. C. Vargas, A. B. Mariano, J. C. Ordonez, V. Kava, "Sustainable Energy via Biodiesel Production from Autotrophic and Mixotrophic Growth of the Microalga *Phaeodactylum tricornutum* in Compact Photobioreactors", IEEE Conference on Technologies for Sustainability (SusTech), Phoenix, AZ, USA DOI: 10.1109/SusTech.2016.7897177, 9-11 Oct. 2016. (conference paper)
- [14] Y. Liang, N. Sarkany, Y. Cui, "Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions", *Biotechnol Lett*, vol. 31, pp. 1043-1049, 2009.
- [15] M.P. Rai, S. Nigam, R. Sharma, "Response of growth and fatty acid compositions of *Chlorella pyrenoidosa* under mixotrophic cultivation with acetate and glycerol for bioenergy application", *Biomass and Bioenerg*, vol. 58, pp. 251-257, 2013.
- [16] S. Kim, J.E. Park, Y.B. Cho, S.J. Hwang, "Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions", *Bioresour Technol*, vol. 144 pp. 8-13, 2013.
- [17] I. Pancha, K. Chokshi, S. Mishra, "Enhanced biofuel production potential with nutritional stress amelioration through optimization of carbon source and light intensity in *Scenedesmus sp.* CCNM 1077", *Bioresour Technol.*, vol. 179, pp. 565-572, 2015.
- [18] H.J. Choi, S.W. Yu, "Influence of crude glycerol on the biomass and lipid content of microalgae", *Biotechnol Biotechnol Equip.*, vol. 29, no. 4, pp. 506-513, 2015.
- [19] J. Trivedi, M. Aila, D.P. Bangwal, S. Kaul, M.O. Garg, "Algae based biorefinery- How to make sense?" *Renew Sust Energ Rev.*, vol. 47, pp. 295-307, 2015.
- [20] B. Cheirsilp, S. Torpee, "Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation", *Bioresour Technol.*, vol. 110, pp. 510-516, 2012.
- [21] G. Knothe, "Improving biodiesel fuel properties by modifying fatty ester composition". *Ener Environ Sci*. vol. 2, pp. 759-66, 2009.
- [22] G. Knothe, "Production and properties of biodiesel from algal oils. In: Borowitzka MA, Moheimani NR (eds)," *Algae for biofuels and energy*. Springer 2013, pp. 207-221.
- [23] P. Neofotis, A. Huang, K. Sury, W. Chang, F. Joseph, A. Gabr, S. Twary, W. Qiu, O. Holguin, E.W.J. Polle, "Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation", *Algal Res*, vol. 15, pp.164-178, 2016.
- [24] T. Ogawa, M. Tamoi, A. Kimura, A. Mine, H. Sakuyama, E. Yoshida, T. Maruta, K. Suzuki, T. Ishikawa, S. Shigeoka, "Enhancement of photosynthetic capacity in *Euglena gracilis* by expression of cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase leads to increases in biomass and wax ester production", *Biotechnol Biofuels*, vol. 8, pp. 80-91, 2015.
- [25] Y. Yamane, T. Utsunomiya, M. Watanabe, K. Sasaki, "Biomass production in mixotrophic culture of *Euglena gracilis* under acidic condition and its growth energetics", *Biotechnol Lett.*, vol. 23, pp. 1223-1228, 2011.
- [26] V.T. Ramachandra, N.H. Chanakya, M.D. Mahapatra, "*Euglena sp.* as a suitable source of lipids for potential use as biofuel and sustainable wastewater treatment", *J Appl Phycol*, vol. 25, no. 3, pp. 855-865, 2013.
- [27] M.P. Rai, S. Gupta, "Effect of media composition and light supply on biomass, lipid content and FAME profile for quality biofuel production from *Scenedesmus abundans*", *Energy Converse Manage*, 2016, (online available May 2016).
- [28] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, "Protein measurement with the folin phenol reagent", *J Biol Chem*, vol. 193, pp. 265-275, 1951.
- [29] X. Miao, Q. Wu. "High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*," *J Biotechnol*. Vol.110, no. 1, pp. 85-93, 2004.
- [30] S. Leow, R.J. Witter, R.D. Vardon, K.B Sharma., S.J. Guest, J.T. Sarathmann, "Prediction of microalgae hydrothermal liquifaction products from feedstock biochemical composition", *Green Chemistry*, vol. 17, pp. 3584-3599, 2015.
- [31] Anders Moller, Danish Food information 2011, Sheppard A, *J Lipid Manual*, U.S. Food and Drug Administration 1992.
- [32] G. Yu, D. Shi, Z. Cai, W. Cong, F. Ouyang, "Growth and physiological features of Cyanobacterium *Anabaena sp.* strain PCC 7120 in a glucose-mixotrophic culture", *Chinese J Chem Eng.*, vol.19, pp. 108-115, 2011.
- [33] P. Nicolas, G. Freyssinet, V. Nigon, "Effect of light on glucose utilization by *Euglena gracilis*," *Plant Physiol*, vol. 65, pp. 631-634, 1980.
- [34] J.P. Schwarzshans, D. Cholewa, P. Grimm, U. Beshay, J.M. Risse, K. Friehs, E. Flaschel, "Dependency of the fatty acid composition of *Euglena gracilis* on growth

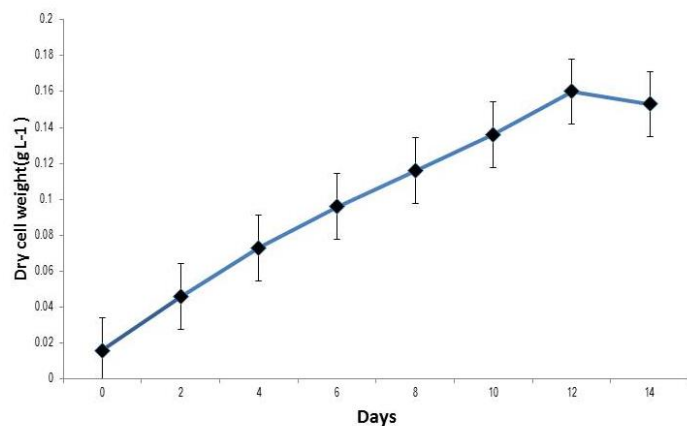
phase and culture conditions”, *J Appl Phycol*, vol. 27, no. 4, pp. 1389-1399, 2014.

[35] V. Andruleviciute, V. Makareviciene, V. Skorupskaite, M. Gumbyte, “Biomass and oil content of *Chlorella* sp., *Haematococcus* sp., *Nannochloris* sp. and *Scenedesmus* sp. under mixotrophic growth conditions in the presence of technical glycerol,” *J Appl Phycol.*, vol. 26, pp. 83-90, 2014.

[36] G. Knothe, “Fuel properties of highly polyunsaturated fatty acid methyl esters. Prediction of fuel properties

of algal biodiesel”, *Ener Fuel*, vol. 26, pp. 5265-5273, 2012.

[37] M.J. Ramos, C.M. Fernandez, A. Casas, L. Rodriguez, A. Perez, “Influence of fatty acid composition of raw materials on biodiesel properties”, *Bioresour Technol*, vol. 100, pp. 261-268, 2009.



Supplementary Fig. 1. Growth evaluation of *E. gracilis* in photoautotrophic mode of culture condition