Evaluation of Mixotrophic Cultivation of *Euglena gracilis* for Lipid Synthesis and FAME Characterization towards Biodiesel Application

A Khanra and M P Rai*

Amity Institute of Biotechnology, Amity University, Noida, Sec-125 Uttar Pradesh-201313, India

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The lipid enhancement of *Euglena gracilis* has been investigated in mixotrophic condition by altering the concentration of technical glycerol. The lipid yield (45.15%) was found more than three times higher in contrast to photoautotrophic cultivation (11.28%). The role of essential abiotic factors, light intensity (30, 60.5, 100, 150 μmol m⁻² s⁻¹) and photoperiod (24:0, 16:8, 8:16, 0:24 Light: dark) were also investigated. Fatty acid methyl esters (FAMEs) were synthesized and analysed through GC-MS. It possesses appropriate quantities of saturated fatty acids (SFA) and unsaturated fatty acids (UFA), in accordance with biodiesel standards. Henceforth, our present study focuses the improved lipid synthesis from *E. gracilis* for biodiesel application.

**Keywords:** *Euglena gracilis*, Fatty Acid Methyl Esters, Glycerol, Mixotrophy

**Introduction**

In the commercialization of algae based system, the lipid productivity and fatty acid compositions are crucial factors for the cost assessment and feasibility studies of full scale biodiesel production. Mixotrophic growth of algae, where light, CO₂ and organic carbon are simultaneously utilized, found more promising over photoautotrophic growth. In the present study, photosynthetic microbe *E. gracilis*, was utilized, having high cellular lipid, but has not been much explored in the past for FAME production. *Euglena* species referred as a unicellular phytoflagellate protist comes under class Euglenoidea and family Euglenaceae that can easily grow under photoautotrophic and heterotrophic culture condition in both aerobic and anaerobic mode. Due to this strong physical make-up, *E. gracilis* is demonstrated as a favourable candidate for various applications. Apart from lipid production, *E. gracilis* is also known for the enhanced synthesis of extracellular metabolites such as α- tocopherol, paramylon, wax ester. In view of minimizing the cultivation cost, glycerol has been used as an organic carbon source in mixotrophic mode for quality biodiesel production. The effect of light supply on mixotrophic growth of the alga was also observed for better performance. Here, we report for the first time the effect of technical glycerol on the growth, lipid production and FAME characteristics of *E. gracilis*.

**Materials and methods**

**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. 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 μmol m⁻² s⁻¹.

**Cultivation under mixotrophic mode**

The alga was cultivated under mixotrophic condition providing different organic carbon sources. In this study, four varieties of carbon sources like glucose, glycerol, sodium acetate and sucrose were used 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.

**Estimation of biomass and lipid**

Growth of *E. gracilis* was measured by dry cell weight method after every 24h for both photoautotrophic and mixotrophic cultures. The total lipids present in cells were extracted by using organic
solvents chloroform and methanol in the ratio of 2:1 and the biomass productivity, lipid productivity and lipid content were calculated.

**Growth of E. gracilis on glycerol**

In the first stage of experiment, among different organic carbon sources (glucose, glycerol, sodium acetate and sucrose), glycerol was found most suitable in view of biomass and lipid productivity. In the second stage of the experiment, the effect of different concentration viz. 0, 0.5, 1, 1.5, 2, 2.5, 3 % volume fraction of glycerol was recorded for biomass and lipid production.

**Optimization of light intensity and photoperiod**

Light supply is an important parameter in the photoheterotrophic growth of algae; hence it was optimized by changing the light intensity range from 30, 60.5, 100 and 150 μmol m-2 s-1. The experiment was set by providing optimized concentration of glycerol, and the variation in light intensity was achieved by controlling the distance between flasks and fluorescent lamp (40W). The light intensity was measured by using light meter (MEXTEC, LX 1010B) and the other growth parameters remained constant. Four different levels of light regime were utilized by altering illumination time (h) of cell cultivation in a regular manner like 24:0, 16:8, 8:16, 0:24 (light / dark). The microbial cells were harvested during stationary phase and the production of biomass and lipid biomolecules were calculated.

**Transesterification and FAME analysis**

The total extracted lipids were transesterified using methanol and acid based catalyst to obtain FAME. 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. Statistical analysis: All the analyses were performed in triplicates and the mean values were plotted in the graphs.

### Results and Discussion

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

Table 1 depicts the comparison between growth of alga in photoautotrophic condition and mixotrophic culture providing various organic carbon sources. Relatively very fast growth rate has been achieved in mixotrophic condition compared to photoautotrophic mode, where, the alga showed early stationary phase with higher cell density. The maximum biomass yield of 2.58 g/L was found in presence of glycerol i.e. 28 times higher in comparison to photoautotrophic culture (0.092 g/L). 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 carbon metabolism through TCA cycle. Table 2 also represents the result obtained utilizing other organic carbon sources sodium acetate, sucrose and glucose. A very similar biomass productivity of 0.237 ± 0.001 g L⁻¹ d⁻¹ and 0.253 ± 0.002 g/L/d have been observed in presence of sodium acetate and sucrose respectively. The glucose enriched medium has expressed the lowest biomass productivity of 0.115 ± 0.005 g L⁻¹ d⁻¹. This is probably due to less permeability of glucose through E. gracilis cell membrane. Measurement of lipid biomolecules under various carbon supplantations is also described in Table 1. Highest amount of lipid production of 0.662 ± 0.006 g L⁻¹, lipid productivity 0.132 ± 0.004 g L⁻¹ d⁻¹ with maximum lipid content 25.66 % have been achieved in presence of glycerol containing medium. On the other hand, less lipid content has been observed with rest of the three tested carbon sources viz. glucose, sodium acetate and sucrose showing 20.46, 18.69 and 21.12 % respectively. Lipid accumulation in E. gracilis is found to decrease significantly in absence of organic carbon and recorded nearly 12 %. The lipid

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>Biomass concentration (g L⁻¹)</th>
<th>Biomass productivity (g L⁻¹ d⁻¹)</th>
<th>Lipid production (g L⁻¹)</th>
<th>Lipid productivity (g L⁻¹ d⁻¹)</th>
<th>Lipid content (%)</th>
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<td>Without organic Carbon</td>
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<td>Glycerol</td>
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<td>Sodium acetate</td>
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Data are expressed as mean ± SD, n=3

Table 1 — Estimation of biomass concentration, biomass productivity, lipid production, lipid productivity and lipid content of E. gracilis utilizing four different carbon sources

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production in presence of glycerol is observed remarkably high, where more than hundred times productivity has been observed compared to photoautotrophic cultivation. 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, where DHAP is directly associated with triglyceride synthesis 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.\textsuperscript{2,12}.

**Effect of glycerol concentration on biomass and lipid production**

In this present study, influence of different concentration of glycerol has been evaluated in the medium to observe the biomass and lipid yield from *E. gracilis*. As shown in Figure 1, the highest biomass yield has been found as 2.53 g L\textsuperscript{-1} utilizing 1% (v/v) glycerol in the medium; however the highest lipid production (0.895 g L\textsuperscript{-1}) and lipid content (39.77 %) have been attained by providing 1.5 % (v/v) glycerol with slight effect on biomass yield (2.25 g L\textsuperscript{-1}). Least biomass and lipid production have been obtained in glycerol free medium and very less production has also been recorded at highest glycerol concentration of 3 % (v/v). Our result shows that glycerol is a favourable organic carbon to support biomass and lipid yield in *E. gracilis*, but concentration of glycerol above 1.5 % (v/v) found to be inhibitory. In our previous study, the maximum growth of *C. pyrenoidosa* was obtained at 0.5 % glycerol while the productivity decreased by increasing the concentration of glycerol 1 % onwards, but the maximum lipid content was recorded also in moderate concentration (0.5%) and reduced afterwards\textsuperscript{13}. 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 criterion is species dependent.

**Response of light intensity and photoperiod on biomass and lipid production**

Light is an essential environmental factor for governing the cell growth to enhance its biomass and
lipid production. In the present study, the effect of light intensity on biomass is not much significant while effect on lipid accumulation was found very promising. There is minor change in biomass yield was observed with increasing light intensity from 30-150 µmol m$^{-2}$s$^{-1}$ as shown in Figure 2. The highest biomass concentration measured at 100 µmol m$^{-2}$s$^{-1}$ was 2.35 g/L, which found very similar to the biomass (2.18 g/L) obtained at lowest light intensity of 30 µmol m$^{-2}$s$^{-1}$. Light intensity beyond 100 µmol m$^{-2}$s$^{-1}$ growth is slightly decreases. The findings of lipid production at 100 µ mol m$^{-2}$s$^{-1}$ was found in parity with the biomass productivity of the cell, where with high biomass, lipid production is also found maximum. The highest lipid production of 0.974 g L$^{-1}$ with lipid content of 41.45 % has been observed at 100 µ mol m$^{-2}$s$^{-1}$ of light intensity. The lipid content has been decreased considerably beyond the light intensity of 100 µ mol m$^{-2}$s$^{-1}$. Most of the previous studies revealed that the high light intensity found unfavourable for biomass production, stimulates the lipid accumulation in the cell, while our result showed contrast with these findings. In the present experiment, growth and lipid production from *E. gracilis* was found maximum under moderate light intensity of 100 µ mol m$^{-2}$s$^{-1}$ shows a favourable condition for particular species. In the study of *Nannochloropsis*, similar kind of result were obtained at various light intensities, where 100 µ mol m$^{-2}$s$^{-1}$ was found optimum for maximum growth and lipid content in comparison to lower (50 µ mol m$^{-2}$s$^{-1}$) and higher (150 µ mol m$^{-2}$s$^{-1}$) light intensities. The findings of George *et al.* (2014) also supports that the lower light intensity (60 µ mol m$^{-2}$s$^{-1}$) was found more suitable for high lipid accumulation than higher light irradiance (150 µ mol m$^{-2}$s$^{-1}$) in case of *A. falcatus*. Decrease in lipid synthesis under high light intensity may be because of oxidative damage, caused by reactive oxygen species. Hence, the optimum light intensity is required for favourable growth and lipid productivity in algae and the range may vary from species to species. The response of photoperiod optimization on biomass production and biomass productivity is depicted in Figure 3. In our present work, the maximum cell density of 2.27g L$^{-1}$ and biomass productivity of 0.378 g L$^{-1}$d$^{-1}$ have been achieved by providing 24 h continuous light at intensity of 100 µmol m$^{-2}$s$^{-1}$. The lowest biomass production 0.86 g L$^{-1}$ and biomass productivity 0.143 g L$^{-1}$d$^{-1}$ have been recorded at 24 h dark period.

![Fig. 2 — Response of different light intensities on Biomass concentration, lipid production and lipid content in *E. gracilis*](image)

![Fig. 3 — Biomass concentration and biomass productivity of *E. gracilis* under various light regime](image)
The cell growth has been reduced gradually by increasing the dark phase in this experiment. Goncalves et al. (2014) reported that *Chlorella vulgaris*, grown under continuous light illumination (24h) achieved maximum biomass productivity. This result proves that higher light exposure leads to enhance the biomass production and productivities; on the other hand, by increasing the dark phase, the cell growth reduces because the cell multiplication is inhibited in the absence of light. The light regime also promotes the lipid production of *E. gracilis* as shown in Figure 4. 16 h light and 8h dark period have been observed the most desirable for maximum lipid content of 45.15 %, lipid production of 0.998 g L⁻¹ with lipid productivity of 0.166 g L⁻¹ d⁻¹. In contrast, the lipid productivity is slightly decreased providing continuous illumination. Periodic dark phase is equally important because few enzymes which are essential for CO₂ fixation remained inactive during light phase. It might be the reason why light/dark regime is more preferable over continuous illumination and also the lower lipid storage in continuous dark phase occurs due to poor cell density. Most of the algae showed lipid production in the range of 16 h to 12 h light period and the selection of light/dark cycle for suitable lipid accumulation vary from species to species.

**FAME characterization**

The chemical composition of fatty acid methyl esters (FAME) obtained by GC-MS, shows the quality biodiesel properties as mentioned in Table 2. 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. These 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 persists less than 12% for biodiesel application and our current study displays its percentage within the limit. In the previous study, *Euglena* sp. demonstrated the PUFA content 46% including very high amount of C18:3 (24.43%) that led to less oxidative stability to the fuel. The current study of glycerol mediated *E. gracilis* culture supports the production of SFA, MUFA and PUFA in a requisite amount for quality biodiesel production.

**Conclusion**

The oleaginous alga *E. gracilis* can be cultured using technical glycerol as organic carbon source in mixotrophic culture condition. 1.5 % volume fraction of glycerol with the light intensity of 100 μmol m⁻² s⁻¹ and light regime of 16:8 light/dark was found most encouraging for high biomass and lipid production of 2.25 g L⁻¹ and 0.998 g L⁻¹ respectively. Cellular lipid content of 45.15 % was obtained under improved culture conditions. 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 FAME composition confirms the potential of *E. gracilis* as a resourceful feedstock for biodiesel production. This work proposes the low cost cultivation of *E. gracilis* with glycerol, which is a byproduct of biodiesel industry.

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