Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production

Attilio Converti*, Alessandro A. Casazza, Erika Y. Ortiz, Patrizia Perego, Marco Del Borghi

Department of Chemical and Process Engineering “G.B. Bonino”, University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy

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**A B S T R A C T**

A possible source of biological material for the production of biodiesel is represented by microalgae, in particular by their lipid content. The aim of the present work was to study the effects of temperature and nitrogen concentration on the lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* in view of their possible utilization as novel raw materials for biodiesel production. In addition, various lipid extraction methods were investigated. The extracted lipids were quantitatively and qualitatively analyzed by gravimetric and gas chromatographic methods, respectively, in order to check their suitability according to the European standards for biodiesel. The lipid content of microalgae strongly influenced by the variation of tested parameters; indeed, an increase in temperature from 20 to 25 °C practically doubled the lipid content of *N. oculata* (from 7.90 to 14.92%), while an increase from 25 to 30 °C brought about a decrease of the lipid content of *C. vulgaris* from 14.71 to 5.80%. On the other hand, a 75% decrease of the nitrogen concentration in the medium, with respect to the optimal values for growth, increased the lipid fractions of *N. oculata* from 7.90 to 15.31% and of *C. vulgaris* from 5.90 to 16.41%, respectively.

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1. Introduction

The microalgae are unicellular photosynthetic organisms that use light energy and carbon dioxide, with higher photosynthetic efficiency than plants for the production of biomass [1,2]. They may be destined to different applications, such as biofuel production, purification of wastewater under either autotrophic or mixotrophic conditions [3,4], extractions of high added value foods and pharmaceutical products, or as food for aquaculture [5].

The lipids from microalgae could be used in different processes for energy exploitation, including the simple combustion in boiler or in a diesel engine. However, the best possible use of this oil is certainly its transformation to a biofuel, especially biodiesel [6].

Moreover, high added value compounds can be extracted from microalgae, such as fatty acids (γ-linolenic, arachidonic, eicosa-pentaenic, docosahexaenoic acids, etc.) [7,8], pigments (carotenoids and ficoliproteins), biochemically stable isotopes [6,9–11] and vitamins such as biotin [12], vitamins C [13] and E [14,15]; also some metabolites appear to have some pharmacological activities, among others the anticholesterolic, antitumoral, immunomodulatory, antibacterial and antymycotic ones.

Finally in aquaculture, the microalgae are used as main food of rotifers, and as very important live food for larvae of marine fish, filter-feeding invertebrates, etc. In 1999, the production of microalgae for aquaculture was 1000 tons: 62% for mollusks, 21% for shrimps and 16% for fish [16].

Microalgae biomass contains approximately 50% of carbon on a dry weight basis [17], and all the carbon present in cell is usually from carbon dioxide. With the production of 100 tons of microalgae biomass, around 180 tons of CO₂ can be disposed using natural or artificial light.

The microalgae for biodiesel production can potentially use part of carbon dioxide from industrial plants [18,19]; from this point of view the microalgae can also be seen as simple CO₂ sequestrants to use in plants for greenhouse gas emissions control (ET—Kyoto Protocol).

Given the high productivity, justified by the shorter generation time and higher oil content compared to crops (up to 80% on dry weight) [20], it is clear the potential contribution that microalgae can give on large scale fuel oils production. A further benefit is that microalgae may also be grown on arid lands unsuitable for conventional agriculture, such as desert areas, or in large reservoirs of saline water.

Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions (temperature and light intensity) or nutrient media characteristics (concentration of nitrogen, phosphates, and iron) [21,22].
This work is a first attempt to increase the lipid content in microalgae as a result of variations in temperature and concentration of nitrogen in two different species of microalgae: *Nannochloropsis oculata* H. (from aquaculture of Rosignano’s Society of Marine fish, Italy) and *Chlorella vulgaris*. The lipids extracted from the two microalgae grown in Erlenmeyer flasks under conditions favoring lipid content in the cell have been analyzed quantitatively and qualitatively, by gravimetric and gas chromatographic methods, and different extraction methods have been investigated to maximize the yield in biodiesel.

2. Materials and methods

2.1. Microalgae

Two microalgal species were used in this study, specifically *N. oculata* H. (from aquaculture of Rosignano’s Society of Marine fish, Italy) and *C. vulgaris* CCAP 211 (Culture Collection of Algae and Protozoa, Argyll, UK). Both microalgae are eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure [23]. Because of their small size ranging from 2 to 4 μm, they are considered to be part of phytoplankton. *C. vulgaris* was grown in the Bold’s Basal Medium and *N. oculata* on the Guillard f2 one [24,25] using the CO2 contained in air (about 300 ppm) and NaNO3 as the sole sources of carbon and nitrogen [26], respectively.

2.2. Culture system

Growth experiments were done at different temperatures and concentrations of nitrogen in 2.0 L-Erlenmeyer flasks. The medium and flasks were sterilized in an autoclave for 20 min at 121 °C in order to prevent any contamination during the early stages of growth.

The growth was done using a refrigerated incubator series 6000 (Termaks, Milan, Italy) equipped with artificial lighting, and the air, and consequently the CO2 contained in it, was injected continuously through pumps M2K3 (Schego, Offenbach, Germany) after passing through a cotton trap.

Each autotrophic batch cultivation was carried out in duplicate for 14 days at a continuous photon flux density of 70.0 μE m−2 s−1, which was measured by a Radiometer HD9021 (Delta OHM, Padua, Italy). The duration of cultivations and light intensity were established on the basis of literature [25,27,28].

Temperature and nitrogen concentration in the medium were selected as the independent variables. The central values of temperature, 20 °C for *N. oculata* and 30 °C for *C. vulgaris*, were chosen on the basis of data reported in the literature [28,29]. Then, additional experiments were done either increasing or reducing the growth temperature by 5 °C intervals with respect to the above optima. Moreover, *C. vulgaris* was also grown at 38 °C to evaluate the effect of high temperature on the lipid content of the microalgae. Based on these considerations, the temperature values tested for *N. oculata* were 15, 20 and 25 °C and those for *C. vulgaris* 25, 30, 35 and 38 °C.

Because the literature suggested that nitrogen limitation could exalt the lipid content of many microalgae [23], the central concentrations of nitrogen in medium (0.300 g L−1 for *N. oculata* and 1.50 g L−1 for *C. vulgaris*) were selected according to Guillard [25], and additional cultivations were run at 0.150 and 0.075 g L−1 with *N. oculata* and at 0.750 and 0.375 g L−1 with *C. vulgaris*, respectively.

2.3. Microalgal biomass concentration

The microalgae concentration was determined daily by absorbance measurements at 625 nm by a UV–vis spectrophotometer, model Lambda 25 (PerkinElmer, Milan, Italy). All measures were carried out in triplicate, and biomass concentration was related to optical density by the equation $y = 2.416 \times (R^2 = 0.994)$ for *N. oculata* and $y = 4.203 \times (R^2 = 0.990)$ for *C. vulgaris*, respectively.

After growth, biomass was separated from the medium by centrifugation at 5000 rpm for 15 min, using a centrifuge model 42426 (ALC, Milan, Italy). Biomass was dried at 105 °C for 48 h, pulverized in a mortar and stored at −20 °C for later analysis.

2.4. Lipids extraction

The lipid fraction was extracted by

- Classic extraction, using petroleum ether (EtP) as a solvent and an extraction time of 4 h;
- Soxhlet extraction [30] under the same conditions;
- Folch method [27] that makes use of a mixture of chloroform and methanol (2:1, v/v) for 1.5 h;
- The methodology described by Krienitz and Wirth [31] that consists in the Folch method combined to the use of ultrasounds (mod. UP100H, Hielscher, Teltow, Germany) and increasing the extraction time from 1.5 h to 6.0 h;
- Ultrasonic extraction using petroleum ether as a solvent [32] and the same instrumentation as in the preceding method.

All extractions were performed on dry biomass. A further test was also carried out on wet biomass by the Folch method combined to ultrasounds to assess the possible influence of water on the action of ultrasounds.

2.5. Kinetic and yield parameters

- The specific growth rate was calculated by the equation:
  \[ \mu = \frac{1}{t} \ln \left( \frac{X_t}{X_0} \right) \]  
  (1)
  where $X_m$ and $X_0$ are the concentrations of biomass at the end and at the beginning of a batch run, respectively, and $t$ is the duration of the run.
- The lipid productivity was calculated by the equation:
  \[ v = \frac{C_l}{t} \]  
  (2)
  where $C_l$ is the concentration of lipids at the end of the batch run and $t$ is the duration of the run.
- The yield of the microalgae lipids was calculated by the equation:
  \[ Y (\%) = \frac{W_L}{W_{DA}} \]  
  (3)
  where $W_L$ and $W_{DA}$ are the weights of the extracted lipids and of the dry algae biomass, respectively.

2.6. Lipids analysis

To remove residual microalgae, the extraction products were filtered on membranes Type FH (Millipore, Bedford, MA) with 0.50 μm mean pore diameter. After washing twice with the extraction solvent and its complete evaporation, gravimetric analysis was done. Part of the lipid fraction was transesterified through the method described by Zunin et al. [33] and qualitatively characterized by a gas chromatograph, model Ultra Trace (Thermo Finnigan, Milan, Italy) equipped with ZB Vax column and FID detector (ThermoScientific, Milan, Italy) using appropriate reference standards (Sigma–Aldrich, Milan, Italy).
3. Results and discussion

3.1. Selection of lipid extraction methodology

The first part of this study dealt with the extraction of lipids from the cell, in order to determine the percentage of this component in the microalgae dry mass. Different methods of extraction were compared so as to select the one able to extract the highest oily fraction and to increase the reliability of results on lipid content comparisons due to changes in growth conditions. For those purposes, *N. oculata* was initially grown under the conditions suggested by the literature (20 °C, 70 μE m−2 s−1 and 0.3 g L−1 NaNO3) to get a total amount of biomass (about 20 g) sufficient to perform all the extraction tests.

Fig. 1 shows that the most effective extraction method, among those taken into consideration, was the combination of ultrasound with the Folch method, which led to a yield (>20%) that by far exceeded those obtained by the traditional Soxhlet (7%) and Folch (8%) methods. However, such a methodology did not show significant differences when applied to dried and wet biomass, the maximum lipid extraction yields being 24.3 and 22.8%, respectively.

![Fig. 1. Lipid yield (g lipids/100 g mass) obtained by different techniques of extraction from *N. oculata* grown at 20 °C, 70 μE m−2 s−1 and 0.3 g L−1 NaNO3. (■) Wet biomass; (▲) dry biomass. S (classic extraction), SL (Soxhlet), UAE (Ultrasound-assisted extraction), and F (Folch method).](image)

In the second part of this work, we studied the effects of temperature and nitrate concentration on batch growth of the two selected microalgae.

*C. vulgaris* growth appeared to be affected at temperatures above 30 °C (Table 1). At 35 °C, this microalga did in fact exhibit a 17% decrease in its growth rate (μ) when compared to 30 °C. Further increase in temperature (38 °C) led to an abrupt interruption of microalgal growth, and later the cells dead. This result was easily visible because of color change of the cells from green to brown, and consequently the microalgal growth rate was even negative.

As far as *N. oculata* is concerned, changes from the optimal conditions of growth (20 °C) also resulted in significant changes in the microalgae growth rate (Table 2). For temperatures below this threshold, growth rate more than halved, falling from 0.13 to

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>μa (days−1)</th>
<th>Yb (g lipids/100 g dry algae)−1</th>
<th>ʋc (mg lipids L−1 days−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.14 ± 0.00</td>
<td>14.71 ± 0.30</td>
<td>20.22 ± 0.60</td>
</tr>
<tr>
<td>30</td>
<td>0.14 ± 0.00</td>
<td>5.90 ± 0.42</td>
<td>8.16 ± 0.65</td>
</tr>
<tr>
<td>35</td>
<td>0.12 ± 0.01</td>
<td>6.60 ± 0.59</td>
<td>8.21 ± 0.17</td>
</tr>
<tr>
<td>38</td>
<td>−0.01 ± 0.01</td>
<td>11.32 ± 0.20</td>
<td>−2.72 ± 1.62</td>
</tr>
</tbody>
</table>

* Specific growth rate.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>μa (days−1)</th>
<th>Yb (g lipids/100 g dry algae)−1</th>
<th>ʋc (mg lipids L−1 days−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.06 ± 0.00</td>
<td>14.92 ± 0.82</td>
<td>9.11 ± 0.30</td>
</tr>
<tr>
<td>20</td>
<td>0.13 ± 0.00</td>
<td>7.90 ± 0.21</td>
<td>10.01 ± 0.22</td>
</tr>
<tr>
<td>25</td>
<td>0.07 ± 0.01</td>
<td>13.89 ± 0.61</td>
<td>10.10 ± 2.05</td>
</tr>
</tbody>
</table>

* Specific growth rate.

Table 1

Main parameters of growth and lipid production of *C. vulgaris* at different temperatures.

Table 2

Main parameters of growth and lipid production of *N. oculata* at different temperatures.
0.06 day⁻¹. A sharp drop in microalgae growth rate was also noticed at higher temperature (25 °C), as reported in the literature [28].

Using the same microalga, an additional investigation was carried out on the effect resulting from a reduction of the nitrogen concentration in the medium. Nitrogen limiting conditions were in fact reported to significantly increase the lipid fraction of many microalgae [21]. For this purpose, the concentration of nitrate in both media for *N. oculata* and *C. vulgaris* batch growth was reduced to half and quarter of the standard media described in Section 2 while the light intensity and the air flux were kept the same throughout the experiments.

The effect of a reduction of NaNO₃ concentration on *C. vulgaris* growth is summarized in Table 3. Whereas its specific growth rate was not significantly affected, a threefold increase in lipid content took place.

In contrast, *N. oculata* showed a gradual decrease in the growth rate accompanied by almost a duplication of the lipid content (Table 4).

These results as a whole suggest that, to perform an effective biodiesel production from microalgalae, the optimum compromise between a slowdown in growth and the increase in the lipid fraction should be achieved.

### Table 3

Main parameters of growth and lipid production of *C. vulgaris* at different NaNO₃ concentration in the growth medium.

<table>
<thead>
<tr>
<th>NaNO₃ (g L⁻¹)</th>
<th>μ (days⁻¹)</th>
<th>Y (g lipids (100 g dry algae)⁻¹)</th>
<th>υ (mg lipids L⁻¹ days⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.500</td>
<td>0.14 ± 0.00</td>
<td>5.90 ± 0.42</td>
<td>8.16 ± 0.65</td>
</tr>
<tr>
<td>0.750</td>
<td>0.14 ± 0.01</td>
<td>14.37 ± 0.64</td>
<td>20.44 ± 0.75</td>
</tr>
<tr>
<td>0.375</td>
<td>0.13 ± 0.00</td>
<td>15.31 ± 0.51</td>
<td>20.30 ± 0.40</td>
</tr>
</tbody>
</table>

* Specific growth rate.
* Lipid yield using Folch method and ultrasound.
* Lipid productivity.

### Table 4

Main parameters of growth and lipid production of *N. oculata* at different NaNO₃ concentration in the growth medium.

<table>
<thead>
<tr>
<th>NaNO₃ (g L⁻¹)</th>
<th>μ (days⁻¹)</th>
<th>Y (g lipids (100 g dry algae)⁻¹)</th>
<th>υ (mg lipids L⁻¹ days⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.300</td>
<td>0.13 ± 0.00</td>
<td>7.88 ± 0.21</td>
<td>10.01 ± 0.16</td>
</tr>
<tr>
<td>0.150</td>
<td>0.10 ± 0.00</td>
<td>13.01 ± 0.39</td>
<td>13.61 ± 1.10</td>
</tr>
<tr>
<td>0.075</td>
<td>0.10 ± 0.00</td>
<td>15.86 ± 0.59</td>
<td>16.41 ± 0.11</td>
</tr>
</tbody>
</table>

* Specific growth rate.
* Lipid yield using Folch method and ultrasound.
* Lipid productivity.

### 3.3. Effect of temperature and concentration of nitrogen source on the quantity and composition of the lipid fraction

Variations of temperature and decrease in the concentration of nitrate in the medium resulted in a significant change in cell composition, favoring the accumulation of lipid components in both microalgae during the batch growths (Tables 1–4).

A decrease in the growth temperature from 30 to 25 °C led to an increase in the lipid content of *C. vulgaris* from 5.9 to 14.7%, while the rate of growth remained unchanged and, as a result, lipid productivity (υ) increased from 8 to 20 mg L⁻¹ day⁻¹ (Table 1).

Even in *N. oculata*, there was an increase in the lipid component passing from 25 °C (13.9%) to suboptimal temperature conditions.
(7.9% at 20 °C and 14.9% at 15 °C). However, also the growth rate was significantly affected by changes in temperature, thus leading a lipid productivity which was approximately the same for all three growth runs (Table 2).

The reduction of the concentration of nitrate in the growth medium increased the lipid fraction in both species, despite of an almost constant growth rate, thereby doubling the productivity of the oil (Tables 3 and 4). The lack of NaNO3 limited the protein biosynthesis [25] thus increasing the lipid/protein ratio.

The final phase of the research was the qualitative analysis of the lipids contained in the oily fraction by gas chromatography, in order to assess their quality for the production of biodiesel according to the European standards (EN 14214 and EN 14213) [34].

Analysis of fatty acid methyl esters (FAME) produced by the reaction between fatty acids and methanol (transesterification), yielded very high values of palmitic acid (16:0), which constituted about 60% (mol/mol) of the overall lipid fraction in C. vulgaris (Fig. 3) and N. oculata (Fig. 4).

In accordance to literature [35] no significant change in the amount of palmitic acid was observed varying the concentration of NaNO3 (Figs. 3 and 4). However, the concentration of linolenic acid (18:3) in C. vulgaris makes the biodiesel derived from this microalga able to meet the level of European legislation for transportation use (12%, mol/mol) [34]. On the other hand, that coming from N. oculata requires additional treatment of catalytic hydrogenation or the use in mixture with a biodiesel richer in saturated fatty acids.

Finally, it is noteworthy the fact that increasing the temperature over the optimum value lead to increased content of oleic acid, which was particularly high for C. vulgaris (up to 34% at 38 °C).

4. Conclusions

The method combining chloroform/methanol and ultrasounds allowed the complete extraction of the microalgae fatty component. The variation of parameters tested (temperature and concentration of nitrogen) strongly influenced the lipid content of microalgae. Almost all the stress conditions investigated led not only to the accumulation of lipids, but also to a reduction in microalgae growth, thereby affecting the lipids productivity. In particular, whereas the growth of C. vulgaris was not significantly influenced by temperature, a decrease from 30 to 25 °C brought about lipid content 2.5 times higher. For N. oculata the lipid productivity seems not to depend on temperature as a result of increased lipid content and reduced growth.

Of particular interest was the influence of the concentration of NaNO3. A doubling of lipid content was in fact observed in both microalgae when nitrate concentration in the medium was reduced by 75%, although the growth practically kept unchanged.

The qualitative analysis of fatty acids showed very high values of palmitic acid (around 60%, mol/mol of the overall lipid fraction) in both microalgae. The concentration of linolenic acid in C. vulgaris met the requirement of European legislation for biodiesel, whereas the higher content of this acid in N. oculata requires an additional treatment to this purpose.

As far as the possible developments of this research are concerned, the next effort will deal with the growth of both microalgae in appropriate and efficient photobioreactors under the stress conditions identified in this study, and varying the light intensity.

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