Effect of sodium nitrate concentration on the lipid content of *Chlorella vulgaris*

Efeito da concentração de nitrato de sódio no conteúdo lipídico de *Chlorella vulgaris*

DOI:10.34117/bjdv5n12-392

Recebimento dos originais: 30/11/2019
Aceitação para publicação: 27/12/2019

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ABSTRACT

Microalgae can be used in the large-scale production of biofuels because of their ease of cultivation, high growth speed and high lipid content. Alterations in cultivation conditions change their cell composition and may affect the production of biomass, lipids, proteins, carbohydrates and pigments. This study evaluated the biomass and lipid production of *Chlorella vulgaris* cultivated with different concentrations of sodium nitrate in Guillard f/2 medium. Five treatments were performed in triplicate, with 15, 30, 45, 60 and 75 mg L\(^{-1}\) of sodium nitrate, keeping constant the concentrations of the other nutrients in Guillard f/2 medium. The stationary cultivation was performed in 12 L containers and was monitored daily by cell density with the determination of the nitrate concentration performed at the beginning, middle and end of the cultivation. The separation of the cells from the culture medium was performed by chemical flocculation (2N NaOH). The biomass was dried in a drying oven and quantified. Both 15 and 30 mg L\(^{-1}\) of sodium nitrate treatments with a biomass production of 4.35 ± 0.46 and 3.84 ± 0.36 g (\(p<0.05\)), respectively, and total lipids of 1.26 ± 0.22 and 1.41 ± 0.24 g (\(p<0.05\)), respectively, were significantly higher than the other treatments. The reduction of sodium nitrate in the culture medium resulted in a better recovery of biomass and production of lipids in *C. vulgaris*.

**Keywords:** Biomass; Microalgae; Cell; Lipid.

RESUMO

As microalgas podem ser utilizadas na produção de biocombustíveis em larga escala, em decorrência da facilidade de cultivo, acentuada velocidade de crescimento e alto teor lipídico. Alterações nas condições de cultivos mudam sua composição celular, podendo alterar a produção de biomassa,
lipídeos, proteínas, carboidratos e pigmentos. O trabalho avaliou a produção de biomassa e lipídeos de *Chlorella vulgaris* cultivada com diferentes doses de nitrato de sódio no meio Guillard f/2. Foram realizados 5 tratamentos, em triplicatas, com 15, 30, 45, 60 e 75mg L\(^{-1}\) de nitrato de sódio, mantendo constante as concentrações dos outros nutrientes do meio Guillard f/2. O cultivo estacionário foi realizado em recipientes de 12 L e foi acompanhado diariamente por densidade celular, sendo a determinação da concentração de nitrato realizada no início, meio e fim dos cultivos. A separação das células do meio de cultivo foi realizada por flocação química (NaOH 2N). A biomassa foi seca em estufa e quantificada. Os tratamentos com 15 e 30 mg L\(^{-1}\) de nitrato de sódio com uma produção de biomassa, com peso médio de 4,35 ± 0,46 e 3,84 ± 0,36 g *(p<0,05)*, respectivamente, e de lipídeos totais com 1,26 ± 0,22 e 1,41 ± 0,24 g *(p<0,05)*, respectivamente, foram significativamente maiores que os demais tratamentos. A redução de nitrato de sódio no meio de cultivo resultou em uma maior recuperação da biomassa e produção de lipídeos em *C. vulgaris*.

**Palavras-chave:** Biomassa; Microalga; Célula; Lipídeo.

### 1 INTRODUCTION

Currently, the focus for biodiesel production is moving towards the use of inedible food, such as the reuse of cooking oil, the use of low-quality animal fat and the cultivation of microalgae (DEVAPPA *et al*., 2010). Some species of microalgae are promising sources for large-scale biodiesel production, with higher productivity than some oilseeds, being an economically and environmentally viable solution as an excellent alternative to fossil fuels (GAO *et al*., 2010; SYDNEY *et al*., 2011, ZENG *et al*., 2011). Moreover, its production does not compete with food growing, since microalgae can be grown in non-agricultural lands (SUBHADRA; EDWARDS, 2010).

Large scale production of microalgae presents some challenges such as the use of inorganic nutrients, the need for a large volume of water and the difficulty of separating the smaller cells (0.5-30 μm) from the liquid culture medium (MARKOU; GEORGAKAKIS, 2011). A possible solution to overcome the high cost of microalgae cultivation is the replacement of potable water by sea water or by rural, urban and industrial wastewater, which is rich in nutrients (CHO *et al*., 2011), as well as using combustion gases from industries and thermo power plants as a source of CO\(_2\) to accelerate photosynthesis (MORAIS; COSTA, 2007).

The formulation of the culture medium is important in the cultivation of microalgae to obtain a high final cellular concentration. In addition, the components of the medium must meet the basic requirements for production and accumulation of cell metabolites, providing an adequate energy supply for cell biosynthesis and maintenance (AZMA *et al*., 2011).

Changes in the conditions of microalgae cultivation such as temperature (MEINERZ *et al*., 2009), light intensity (GUEDES *et al*., 2010) and nutrient composition of the medium (YANG *et al*., 2011) may promote biomass growth and influence the production of lipids, proteins, carbohydrates, pigments and other constituents (RADMANN; COSTA, 2008). There are several culture media, some...
of which are specific to certain microalgal groups and species (VOLKMANN et al., 2008). Thus, the preparation of the culture medium will depend on the target compound to be obtained, which will also define the use of a specific extraction method, either for proteins, lipids, carbohydrates or pigments (MULBRY et al., 2009).

Cell density in a microalgal culture tends to increase since its inoculation in the culture medium due to nutrient assimilation and appropriate physical and chemical conditions (CHENG et al., 2006). On the other hand, the concentrations of nutrients (N, P, Si, Fe, Co, etc.) dissolved in the culture medium are reduced with cell increment, decreasing considerably at the end of the cultivation (JIANG et al., 2011).

Different nitrogen concentrations may change the composition and total lipid content of microalgae (MATA et al., 2010). According to Scott et al. (2010), higher lipid content and high growth in Chlorella vulgaris can be achieved with the natural reduction of nitrogen in the medium, which does not occur when the microalgae is inoculated into a totally nitrogen-free medium. Dragone et al. (2011) found higher starch accumulation in C. vulgaris and Costa et al. (2006) also found higher lipid yield in C. vulgaris and C. minutissima when using the lower amount of nitrate in the media. However, the reduction of nitrogen in the medium may reduce the final cell density of the culture.

The microalga C. vulgaris Beyerinck (1890) belongs to the class Trebouxiophyceae, order Chlorellales, family Chlorellaceae (GORS et al., 2010). It is a unicellular, cosmopolitan, spherical species, without movements and with diameters ranging from 2.0 to 10.0 μm (PHUKAN et al., 2011). This species is widely used in the food industry, in human food and aquatic organisms feed, as well as in the pharmaceutical industry (WANG et al., 2010). It is also intensively used in effluent treatment and biofuel production (LIM et al., 2010; WIDJA et al., 2009). The potential uses of C. vulgaris are due to its high nutritional value, rapid growth, resistance to contamination in larger cultivation, as well as a constant biomass production with excellent biochemical composition (JANCZYK et al., 2007). The present study aimed to evaluate biomass recovery and lipid production of the microalgae C. vulgaris cultivated at different levels of sodium nitrate.

2 MATERIALS AND METHODS

The microalga Chlorella vulgaris was obtained from the Laboratory of Planctology of the Department of Fisheries Engineering of the Federal University of Ceará (Fortaleza, Ceará, Brazil), where it is kept in Guillard f/2 medium (GUILLARD, 1975). The microalgae were grown in a stationary way in triplicate, using 12 L containers, and the material used and the culture medium were sterilized in an autoclave for 15 minutes at 120 °C to avoid any kind of contamination. The culture conditions remained constant, with a temperature of 28 ± 1 °C, light intensity around 60 μE cm⁻² s⁻¹
having as a source two 40 W fluorescent lamps, and aeration provided by a compressor with a flow of 3.0 ± 1.0 L air min⁻¹.

Five treatments were performed varying the amount of sodium nitrate of the Guillard f/2 medium in 15, 30, 45, 60 and 75 mg L⁻¹, keeping the concentrations of other nutrients constant. Initially, the inoculum was acclimated to the different levels of sodium nitrate used in the experiment in 9 L containers under the same conditions of temperature, luminosity and aeration for three days. In each treatment the microalgae were inoculated at a density of 4.10 ± 0.02 x 10⁶ cell mL⁻¹ and, daily, the cell density was determined to monitor the development of the cultures, using a Neubauer chamber.

The concentration of nitrate-n in the culture medium was determined at the beginning, middle and end of the experiment by spectrophotometry at 500 nm. For this, samples of 100 mL were centrifuged at 3,000 X g for 5 min for each repetition. Then, the NitraVer 5 Nitrate reagent was added in 25 mL of each sample and, after 5 minutes, the samples were measured in the spectrophotometer and the nitrate concentration was expressed in mg L⁻¹.

With the cell density (CD) data, the growth rate was calculated in divisions per day (K) on the day of highest yield of the cultures (OHSE et al., 2008). This parameter was obtained according to equation 1, described by Lourenço (2006):

\[
K = \log_2 \left( \frac{N_f}{N_0} \right) / D_t
\]  

(1)

Where: \( K \) = doubling growth rate per day (divisions day⁻¹); \( N_0 \) and \( N_f \) = cell density at the beginning and on the day when the cultivation obtained the maximum cell concentration, respectively; \( D_t \) = time of cultivation in days.

The separation of the biomass of \( C. vulgaris \) from the culture medium was performed in the senescence phase of the cultures to better evaluate the maximum removal of nitrate during cultivation (SEBASTIEN; GRANJA, 2006). For this separation, the cultures were subjected to chemical flocculation induced by the pH increase of the medium, using a 2 N sodium hydroxide solution (NaOH) (CHEN et al., 2011a).

After complete flocculation, the supernatant was discarded, and the flakes were washed with distilled water to remove the flocculant agent and traces of the culture medium. Subsequently, the biomass recovered from the microalgae was dried in a drying oven with air renewal at 60 °C for 24 hours and weighed in an analytical balance.
The Bligh and Dyer method was used for lipid extraction (BLIGH; DYER, 1959). Five grams of dry microalgae biomass were added in triplicate in a 250 mL Erlenmeyer, 25 mL methanol, 12.5 mL chloroform and 5 mL water. The Erlenmeyer flask was closed and taken to sonication in an ultrasonic bath with 40 kHz frequency and 80 W power for 10 minutes. Then another 12.5 mL of chloroform and 5 mL of water were added, and another sonication was performed for 5 minutes. The solid part was vacuum filtered and then dried in a drying oven for 24 hours at 105 °C and weighed on an analytical balance to determine the lipid yield.

The lipids productivity and yield were determined according to equations 2 and 3, respectively, described by Converti (2009):

\[ v = \frac{C_L}{t} \]  
(2)

Where: \( v \) = productivity in g L\(^{-1}\) day\(^{-1}\); \( C_L \) = lipid concentration at the end of the experiment in g L\(^{-1}\); \( t \) = duration of the culture in days.

\[ Y = \left( \frac{W_L}{W_{DB}} \right) \times 100 \]  
(3)

Where: \( Y \) = yield in g 100 g of dried algae\(^{-1}\); \( W_L \) = weight of extracted lipid in g; \( W_{DB} \) = weight of dried biomass at the end of the cultivation.

The data obtained in this study were submitted to analysis of variance (ANOVA) and, in the case of significant differences, submitted to the t-test for means at the level of 5% using the BioEstat 5.0 software.

3 RESULTS

The growth curves and daily growth rates of Chlorella vulgaris cultivated with different concentrations of sodium nitrate (15, 30, 45, 60 and 75 mg L\(^{-1}\)) are shown in Figures 1 and 2, respectively. In the present study, with the increase in the algae population, the depletion of sodium nitrate became evident, regardless of the amount of this nutrient in the culture medium (Figure 3). When the cultures reached the senile phase, the average consumption of sodium nitrate was approximately 91%, showing the removal of this nutrient from the culture medium by the microalgae. The kinetic and lipid yield parameters of C. vulgaris cultured with different amounts of sodium nitrate showed that the reduction of this nutrient in the culture medium resulted in increased dried algae production, daily lipid productivity and lipid yield (Table 1 and Figure 4).
4 DISCUSSION

*Chlorella vulgaris* cultures with 45, 60 and 75 mg L\(^{-1}\) presented the five distinct growth phases (Figure 1). However, the induction phase was only one day for the culture performed with the highest amount of sodium nitrate, moving to 2 and 3 days in cultivation performed with 45 and 60 mg L\(^{-1}\) of sodium nitrate, respectively. According to Hernadez *et al.* (2009), after inoculation of microalgae in a nutrient-enriched culture medium, population growth over time presents a curve with five distinct phases: induction, exponential, deceleration, stationary and death. The end of the induction phase of these cultures is characterized by a tendency to increase their respective growth rates, which then decrease with their development (Figure 2). On the other hand, in cultures with 15 and 30 mg L\(^{-1}\) it was no longer possible to evidence the exponential growth phase (Figure 1). Moreover, these cultures had the lowest growth rates, which were similar, and in the case of the latter, this rate was practically constant from the moment of inoculation of the microalgae until the final phase (Figure 2). The cultivation with 60 and 75 mg L\(^{-1}\) of sodium nitrate reached the highest cell densities more quickly, with duration of 4 and 5 days, respectively. Although the culture conducted with 45 mg L\(^{-1}\) also occurred faster, the maximum cell density was significantly lower \((p<0.05)\) than those observed in the cultivation with higher sodium nitrate doses, being similar to those observed in the cultivation performed with 15 and 30 mg L\(^{-1}\). These cultures presented a longer duration, showing a long induction phase due to the low amount of sodium nitrate in the culture medium, which is insufficient to trigger an exponential growth of the microalgae population (Figure 1). The culture with 60 mg L\(^{-1}\) presented a growth curve with the five well-defined phases, demonstrating that this is the ideal dosage of sodium nitrate in the culture medium for the development of the microalgae (Figure 1). Studies such as Li *et al.* (2011) and Kong *et al.* (2010) report a deceleration of daily growth rate in *Chlorella* sp. cultivation as the consumption of nutrients occurs, mainly nitrates and phosphates, which are essential for algae growth.

Nutrient consumption in microalgae cultivation can be monitored by determining nitrate concentration in the growing medium (FIERRO *et al.*, 2008). Pereira and Branco (2007), cultivating the microalgae *Schizomeris leibleinie*, found that the algae density was lower when the nitrate dosage in the medium was reduced by 10 times, from 33.2 ± 2.1 µg cl a mL\(^{-1}\) in the highest amount of nitrate to 5.9 ± 0.4 µg cl a mL\(^{-1}\). Chen *et al.* (2011b) evaluated the effects of nutrient concentration variation on the growth of the *Dunaliella tertiolecta* microalgae and found an increase in algae density when they increased the amount of sodium nitrate in the culture medium by 10 times. Jiang-Ming *et al.* (2010) cultivated *C. vulgaris* in various concentrations of potassium nitrate (5.0, 3.0, 1.0 and 0.2 mM) in the culture medium and, noting that since the increase in potassium nitrate concentration from 0.2 to 5.0 mM, biomass was raised from 0.4 to 1.2 g L\(^{-1}\).
With the rupture of the cell membrane, all the internal content of the microalgae became available for extraction and, subsequently, for determination of biomass recovery and lipid production. The sonication method has high efficiency and low energy requirement, making it viable. This technique has been widely used to extract several low molecular weight substances and for bioactive vegetable compounds (MACÍAS-SÁNCHEZ et al., 2009).

The cultivation with 60 and 75 mg L\(^{-1}\) of sodium nitrate in the medium showed higher cell density (CD) (Figure 3) and lower algae biomass (Table 1), since when the amounts of sodium nitrate were reduced to 15 and 30 mg L\(^{-1}\) the opposite occurred, there was a decrease in CD and an increase in algae biomass due to lipid accumulation in the cells caused by stress, resulting in an increase in individual cellular mass.

The culture with the lowest amount of sodium nitrate in the medium (15 mg L\(^{-1}\)) presented the best result, since it can significantly increase \((p<0.05)\) the content of lipids in the dry biomass of the microalgae (Figure 4), but the time required to reach maximum cell productivity was longer, reaching 7 days. Thus, the amount of sodium nitrate most indicated for a possible larger scale cultivation is 45 mg L\(^{-1}\), because it presented a lipid production similar \((p>0.05)\) to the treatment with 15 mg L\(^{-1}\), but reached the maximum cell productivity in only 3 days, decreasing the possibility of contamination of the cultivation by other organisms, as well as the costs with nutrients. The cultures performed with these sodium nitrate dosages in the medium showed higher productivity than other oilseeds used as source for biodiesel production (Table 2).

Colla et al. (2007) observed that the sodium nitrate concentration in Zarrouk’s medium could be reduced by up to 4 times without loss of productivity in cultivation, decreasing the production costs of *Spirulina platensis* on a large scale. The reduction in the amount of nitrate in the culture medium can be considered an excellent strategy for the lipid increase of *C. vulgaris*, since the microalgae under stress possibly accumulated lipids as an energetic source for its survival (Figure 4). Stress caused by nutrient depletion allows an accumulation of energy in the form of lipids by microalgae (CHEN et al., 2011b).

Feng et al. (2011a) studied the stress effects on the microalgae *Isochrysis zhangjiangensis* with sodium nitrate depletion in Guillard f/2 medium, providing the nutrient at 24, 48 and 72 h intervals for nine days. The highest lipid yield was obtained in the 72 h interval with 27%, while the lowest lipid content was obtained in the 24 h interval with a yield of 20%. Thus, when the microalgae were submitted to a lower amount of sodium nitrate it suffered a nutritional stress and possibly accumulated lipid as an energy source.

Costa et al. (2006) obtained higher lipid content (8.0%) in the microalgae *C. minutissima* when they reduced the amount of sodium nitrate in the culture medium by 50%, while Converti et al. (2009)
evaluated the effect of nitrogen concentration on the lipid content of *Nannochloropsis oculata* and *C. vulgaris* microalgae for biodiesel production. When the nitrogen concentration in the culture medium was reduced by 75%, a significant increase in the lipid fractions of the microalgae was observed, increasing from 7.9 to 15.3% in *N. oculata* and from 5.9 to 16.4% in *C. vulgaris*.

Rodolfi *et al.* (2009) used 110 L photobioreactors for the cultivation of the microalgae *Nannochloropsis* sp. with nitrogen depletion and under direct solar radiation for 8 days. According to the authors, from day 3 until the end of the experiment, under normal conditions of nitrogen concentration (10%) in the cultures, the lipid content was 32% on average. However, when the reduction in the amount of this nutrient in the cultures was 2.5 and 1.2%, the oil yield reached more than 60% of biomass on average for both nitrogen concentrations.

Yeesang and Cheirsilp (2011) evaluated the lipid content of 4 green microalgae identified as *Botryococcus* spp., which were cultivated in a nitrogen-rich environment, achieving as the highest result a lipid yield of 25.8%, while in the cultivation with deficiency of the same nutrient, the highest yield rose to 35.9%. Feng *et al.* (2011b) obtained a lipid content of 42% when cultivating *C. vulgaris*, using an artificial medium with residuary waters. According to the authors, these microalgae proved to be very promising as a raw material to produce biodiesel.

Matos *et al.* (2009) tested several methods of lipid extraction from the dry biomass of *C. vulgaris* cultivated in Guillard f/2 medium, being the Bligh and Dyer method the most efficient for the extraction of these compounds, which reached a content of 52.5%. Li *et al.* (2011) found a content of fatty acid methyl ester of 11.0% in the dry biomass of *Chlorella* sp., providing a yield of 0.12 g of biodiesel per liter of culture.

Doan *et al.* (2011) conducted a study to evaluate the potential of some marine microalgae as raw material for biodiesel production. The results revealed that the microalgae *Nannochloropsis* sp. had both the highest concentration of dry biomass (0.4 ± 0.003 g L-1) and the highest lipid yield (44.9%), making it a very advantageous source for biodiesel production.

5 CONCLUSIONS

With the accomplishment of the present study it became evident the existence of a direct relation between the amount of sodium nitrate in the culture medium and the cellular density. On the other hand, an inverse relation between the amount of sodium nitrate and the lipid yield was observed, indicating that the reduction of nutrients for the culture of *C. vulgaris* is an excellent way to increase its biomass recovery and the production of lipids. This strategy can be used in a large-scale production, which makes the species a very promising candidate to produce biodiesel.
Figure 1 Growth curves of *C. vulgaris* cultivated with different amounts of sodium nitrate (15, 30, 45, 60 and 75 mg L$^{-1}$). Different letters show significant differences between maximum cell densities (CD).

Figure 2 Daily growth rates of *C. vulgaris* cultivated with different amounts of sodium nitrate (15, 30, 45, 60 and 75 mg L$^{-1}$).
Figure 3 Growth and nitrate depletion curves in *C. vulgaris* cultivation with different amounts of sodium nitrate (15, 30, 45, 60 and 75 mg L\(^{-1}\)). Different letters show significant differences in sodium nitrate depletion during cultivation development.

Table 1 Mean data and standard deviation of kinetic and lipid yield parameters of *C. vulgaris* cultivation in different amounts of sodium nitrate (15, 30, 45, 60 and 75 mg L\(^{-1}\)) in the culture medium.

| NaNO\(_3\) (mg L\(^{-1}\)) | Time (days) | Algae Biomass (g) | Lipid production (g) | Algae productivity (g L\(^{-1}\) day\(^{-1}\)) | Lipid productivity (g L\(^{-1}\) day\(^{-1}\)) |
|----------------------------|-------------|------------------|----------------------|-----------------------------------------------|---------------------------------------------|
| 15                         | 8           | 3.84 ± 0.36\(^a\) | 1.41 ± 0.24\(^a\)   | 0.041 ± 0.004\(^a\)                          | 0.015 ± 0.003\(^a\)                          |
| 30                         | 8           | 4.35 ± 0.46\(^a\) | 1.26 ± 0.22\(^a\)   | 0.046 ± 0.005\(^a\)                          | 0.013 ± 0.002\(^ab\)                          |
| 45                         | 5           | 2.77 ± 0.13\(^b\) | 0.95 ± 0.13\(^b\)   | 0.047 ± 0.003\(^b\)                          | 0.016 ± 0.002\(^a\)                          |
| 60                         | 4           | 2.29 ± 0.20\(^b\) | 0.64 ± 0.05\(^bc\)  | 0.049 ± 0.004\(^b\)                          | 0.013 ± 0.001\(^ab\)                          |
| 75                         | 5           | 2.55 ± 0.23\(^b\) | 0.58 ± 0.02\(^c\)   | 0.043 ± 0.002\(^b\)                          | 0.010 ± 0.001\(^b\)                          |

Different letters show significant differences between treatments.
Table 2 Comparison of some sources of biodiesel*.

| Source          | Oil yield (L ha) |
|-----------------|------------------|
| Corn            | 172              |
| Soy             | 446              |
| Canola          | 1.190            |
| Physic nut      | 1.892            |
| Coconut         | 2.689            |
| Palm oil        | 5.950            |
| Microalgae\(^a\) | 58.700           |
| Microalgae\(^b\) | 136.900          |
| C. vulgaris 15 mg L\(^{-1}\) | 50.000 |
| C. vulgaris 45 mg L\(^{-1}\) | 53.333 |

\(^a\)30% of oil of total dry weight.  
\(^b\)70% of oil of total dry weight.  
*Modified from Chisti (2007).

Figure 4 Lipid yield (LY) expressed in grams (g) for each 100 g of dried algae (DA) grown with different amounts of sodium nitrate (15; 30; 45; 60 and 75 mg L\(^{-1}\)). Different letters show significant differences of DA and LY between treatments.

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