Impact of the nitrate concentration on the biomass growth and the fatty acid profiles of microalgae *Chlorella sorokiniana*

A Toumi and N A Politaeva
Laboratory of Industrial Ecology, Peter the Great St. Petersburg Polytechnic University, Saint Petersburg, Russia
E-mail: toumi.amira@hotmail.com

Abstract. *Chlorella sorokiniana* microalga is one of the species of interest that could be used for the production of valuable compounds such as lipids. Nitrogen stress is a common strategy used to enhance the lipid content in microalgal biomass. The present study compares the effects of several nitrate concentrations in the cultivation medium on the growth and fatty acid composition of the biomass of *Chlorella sorokiniana*. Results show that nitrogen starvation negatively impacts the growth of the biomass while nitrate repletion increased the biomass growth rates. The effect of initial concentrations of KNO$_3$ from 0.1 g/l to 0.3 g/l did not show significant differences on the biomass productivity. Higher concentrations of KNO$_3$ (0.4 g/l) are shown to decrease the yields of biomass. The highest yield of total lipids (25%) was obtained from the biomass grown under nitrogen deficiency, followed by the biomass grown in the medium containing 0.3 g/l of nitrates (23%). The study of the fatty acid profiles showed that nitrogen starvation decreased the accumulation of polyunsaturated fatty acids (PUFA), especially omega-3 (linolenic acid), and increased the concentration of trans and saturated fatty acids. This cultivation strategy could be more suitable for the production of biodiesel. For the food and feed industry, the presence of nitrogen in the cultivation medium could be preferable as; in this case, the biomass cumulates higher concentrations of linolenic acid.

1. Introduction

Our modern world is facing global challenges due to the rapid population growth and an increase in human activities. Climate change and the food crisis are undoubtedly among the issues that need to be tackled using new sustainable and holistic approaches.

Photosynthetic microalgae are efficient carbon dioxide assimilators and could be used to decrease the greenhouse effect [1]. Several microalgae species are promising sources of valuable compounds such as lipids, proteins, carbohydrates and antioxidants, and are the focus of several studies [2]. Microalgal lipids could be destined for the food industry (omega-3 fatty acids) as well as for the production of biofuels (fatty acids with 14–20 carbon chains) [3]. Microalga *Chlorella sorokiniana* is a species of interest that could be used for mass production of biofuels and other derived products. This photosynthetic organism has a fast division rate and contains high concentrations of protein, lipids and antioxidants [4, 5]. However, it is estimated that some microalgal products, especially biofuels are still too expensive to produce and thus, cost-ineffective. Currently, it is proposed to produce biomass-derived products applying the principles of circular economy; implementing cascade extractions and waste recycling [6, 7]. This would decrease the generated waste and increase the overall profits. Another strategy to decrease the production costs of valuable compounds is to use...
directed cultivation. This method deliberately focuses on using specific cultivation conditions for obtaining faster biomass growth rates and substantial accumulation of the desired compounds in the cells [8]. The difficulty lies on the variability of these outcomes among the various species and strains of microalgae. Also, several factors influence the metabolism of microalgae such as the quality of light and its intensity, temperature, the pH, the concentration of electrolytes and nutrients in the growth medium [9]. Nitrogen is regarded as one of the most limiting factor for the growth of microalgae and higher plants. It is an essential constituent of protein, nucleic acids and chlorophyll, making it crucial to all vital processes of the cells [10]. Kumar et al. 2017 studied the effect of a nitrogen source on the growth and lipid accumulation patterns of *Chlorella vulgaris*. It has been shown that nitrates (NO\textsubscript{3}\textsuperscript{-}) is a preferable source of nitrogen for this microalga. The biomass growth and accumulation of lipids decreased when using NO\textsubscript{2}, NH\textsubscript{4}, glycine and urea [11]. In the field of ecology, several microalgae (including strains of chlorella) are being studied for their possible applications for wastewater treatment, using their ability to effectively remove contaminants such as nitrates and nitrites from their growth media [12].

Nitrogen stress is a widely documented strategy for inducing lipid accumulation in microalgae [13,14]. However, this nutrient deficiency has been shown to decrease the growth of microalgae biomass as well as the chlorophyll and protein content which could make the biomass unlikely to be suitable for a cascade extraction of valuable compounds. In S. Nigam et al., 2011, it was shown that nitrogen starvation reduced the growth rate of the biomass of *Chlorella pyrenoidosa* while increasing the lipid content. The highest yield of lipids (26%) was reached using an initial nitrate concentration of 0.05 g/l of KNO\textsubscript{3} [15].

It is crucial to evaluate the relevance of using nitrogen starvation strategies in microalgae cultivation. Some studies have shown that nitrogen depletion increased the amount of saturated fatty acid and decreased the polyunsaturated fatty acids (PUFA) in green microalgae [16]. These results seem to be species specific and require a deep understanding of microalgae metabolism [17]. In the present study, it is proposed to compare the effect of the initial nitrate concentration on the growth and the fatty acid profile of *Chlorella sorokiniana* biomass.

2. Material and methods

Green microalga *Chlorella sorokiniana* (*C. sorokiniana*) strain 211-8k used in this study was obtained from the Goettingen University Culture Collection of Algae, Germany.

2.1. Cultivation conditions

For studying the effect of nitrates on the growth and lipid content of microalgae, the biomass of *C. sorokiniana* was cultivated under autotrophic conditions in controlled laboratory conditions. The cultivation process took place in closed photobioreactors (PBR). A growth medium deficient in nitrogen was used for studying the effect of nitrogen deficiency on microalgae (Table 1).

| Table 1. Composition of the growth medium. |
|------------------------------------------|
| Substance                  | Concentration, g/l |
| ZnSO\textsubscript{4}·7H\textsubscript{2}O | 10\textsuperscript{-4} |
| CuSO\textsubscript{4}·5H\textsubscript{2}O  | 10\textsuperscript{-5} |
| CoSO\textsubscript{4}·7H\textsubscript{2}O  | 10\textsuperscript{-4} |
| MnCl\textsubscript{2}·4H\textsubscript{2}O  | 5. 10\textsuperscript{-4} |
| H\textsubscript{3}BO\textsubscript{3}·WF      | 5. 10\textsuperscript{-5} |
| Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O | 10\textsuperscript{-4} |
| FeCl\textsubscript{3}·6H\textsubscript{2}O  | 4. 10\textsuperscript{-6} |
| Na\textsubscript{2}EDTA·2H\textsubscript{2}O | 6. 10\textsuperscript{-6} |
| KNO\textsubscript{3}                         | -                   |
| KH\textsubscript{2}PO\textsubscript{4}       | 0.1                 |
| MgSO\textsubscript{4}·7H\textsubscript{2}O  | 0.24                |
For studying the effect of nitrates on the growth of *C. sorokiniana*, nitrogen was introduced to the medium in the form of nitrates (KNO₃) at the first day of cultivation at four different concentrations (0 g/l (original medium), 0.1, 0.2 g/l, 0.3 g/l, 0.4 g/l). Before inoculation, the microalgae cells were washed twice with distilled water, ridding them from their previous medium. The inoculums were added to 2 liter capacity PBRs containing 1 liter of the nutrient medium each. Five concentrations of nitrates (from 0g/l to 0.4 g/l) were used, in duplicate. The initial biomass concentration in all the samples was about 4 million cells/ml (equivalent to an optical density at 750 nm of A₇₅₀= 0.200 ± 0.002). The biomass was cultivated for 9 days at constant temperature (23±1°C), under a 12-hour illumination (4.8 W/m, 5000 Lx, LED panel) and a constant aeration rate of 1.5 l/min. The cultures were shaken twice a day to avoid biofouling. The pH and temperature of the cultures were monitored daily using the I-160MI ionomer. All the experiments in the present work were done in triplicates.

2.2. Biomass growth estimation
The growth of the biomass of *C. sorokiniana* was assessed by measuring the optical density (OD) of the microalgae suspensions at 750 nm using a Shimadzu UV-1280 spectrophotometer. To avoid errors due to evaporation, the volume of the culture medium was leveled daily using distilled water. A calibration curve was constructed to determine the number of cells according to the absorbance at 750nm.

2.3. Biomass harvest and drying methods
At the end of cultivation, the biomass was harvested by vacuum filtration using membrane nylon filters Nylon 66 Membranes 0.45 × 47 mm (SUPELCO), with a pore diameter of 0.45 μm. The concentrated biomass was frozen without delay at -18°C and later freeze-dried using the AK-50N lyophilizer (Proflab).

2.4. Cell disruption conditions
In order to increase the yield of total lipids, samples of dry biomass were subjected to mechanical disintegration using a Silent Crusher M Homogenizer (Heidolph Instruments, Schwabach, Germany). For this purpose, 3 g of dry biomass samples was suspended into 10 ml of a hexane: ethanol solution at a 9:1 ratio then disintegrated for 10 minutes at a rotor speed of 20 000 rpm.

2.5. Lipid extraction and FAME analysis
Following the cell disruption, the extraction of lipids by Sohxlet was carried out using a Büchi E-812 SOX extraction unit. The disintegrated suspension was then placed in cellulose extraction thimble (25×100 mm). 90 ml of extraction solution (hexane: ethanol, 9:1) was necessary to conduct the extraction of lipids for 15 cycles. The yield of total lipids was then determined by gravimetric method. The analysis of 38 fatty acid methyl esters in the extracts was carried out by gas chromatography on a Kristal 5000 chromatograph according to the GOST 31663 2012, GOST 31665 2012 and RISO 5508-2010. (column dimensions: 105×0.25×0.25 μm, carrier gas: nitrogen, temperature of evaporator: 250°C, Column temperature: 140°C).

3. Results and discussion
The growth of microalgae biomass was monitored daily by measuring the optical density of the suspensions containing different nitrate concentrations (0 g/l, 0.1 g/l, 0.2g/l, 0.3g/l, 0.4 g/l). The results the triplicate experiments are presented in Figure 1. The mean and standard deviations were calculated using MS Excel.
Figure 1. Growth curves of *Chlorella sorokiniana* biomass obtained at different initial concentrations of nitrates (0–0.4 g/l).

Figure 1 shows a positive correlation between nitrogen concentration in the medium and the biomass growth. The growth profiles of *C. sorokiniana* obtained at the initial nitrate concentrations of 0.1 g/l; 0.2 g/l, 0.3 g/l, 0.4 g/l are very similar to each other and the biomass yields at the end of cultivation were practically the same (Figure 2). These media produced growth curves with very similar tendencies, i.e. a one day lag phase followed by a 7 days logarithmic phase. The samples containing 4 g/l of KNO₃ demonstrated a slightly shorter period of exponential growth (6 days) and lower biomass concentrations at the end of cultivation. The stationary phase was not clear in all the media due to the duration of the experiment.

In the absence of nitrates, the growth curve of the biomass showed a different progression. After a 3 day lag phase, a short logarithmic phase of only 2 days was followed by a 6 days stationary phase. The maximal biomass yields under nitrogen deprivation were significantly lower (by three times) than in the presence of a nitrogen source (Figure 2).

Figure 2. Maximal biomass production of *Chlorella sorokiniana* grown at different initial concentrations of KNO₃ (0-0.4 g/l).
It is also important to point out that the biomass turned yellowish and pale after a few days of cultivation in the media containing the lowest nitrate concentrations (0 g/l and 0.1 g/l). It is known that the biochemical composition of microalgae can be affected by physicochemical parameters of cultivation. Nitrogen is an important element for living organisms as it is a building block for amino acids, proteins and nucleic acids. Chlorophyll represents a nitrogen pool and was probably consumed by the cells to support growth after nitrogen exhaustion. Therefore, the chlorophyll content in microalgae cultures decreased at low concentrations of KNO₃. These results show that nitrogen is essential for the growth of microalgae biomass.

Nitrogen concentration in the nutrient medium is one of the most influencing factors on the accumulation of lipids in microalgae. Results of the yields of lipids after Soxhlet extraction are presented in Table 2.

Table 2. Yields of total lipids obtained from *Chlorella sorokiniana* biomass obtained at different initial concentrations of KNO₃ (0–0.4 g/l).

| Initial concentration of KNO₃, g/l | Yields of total lipids, % |
|-----------------------------------|--------------------------|
| 0                                 | 25.04 ± 0.50             |
| 0.1                               | 20.19 ± 0.40             |
| 0.2                               | 18.99 ± 0.37             |
| 0.3                               | 23.22 ± 0.46             |
| 0.4                               | 21.67 ± 0.43             |

According to Table 2, the highest yield of lipids was obtained from the biomass cultivated under nitrogen deficiency (25%). However, the growth rate of the biomass in this medium was the lowest. These results are in accordance to those expected from the data found in the bibliography.

The second highest yield of total lipids was obtained from the biomass grown at 0.3 g/l of nitrates (23%). At this KNO₃ concentration, the growth patterns of *C. sorokiniana* were adequate for a high biomass productivity.

Hereinafter, the lipid sample obtained from the biomass grown under nitrogen deficiency will be referred to as (-NO₃), and the sample obtained using 0.3 g/l as (+NO₃). In order to assess the effect of nitrates on the fatty acids accumulation patterns of *C. sorokiniana*, the fatty acid profile of the two samples was assessed. The FAME analysis has shown that *Chlorella sorokiniana* contains higher concentrations of long chains fatty acids (with a number of carbon atoms superior to 16), thus, the two samples have been compared on the basis of their content of higher fatty acids, as shown in Figure 3.
Figure 3. Fatty acid profiles of two lipids samples obtained with (+NO₃⁻) and without (-NO₃⁻) the addition of nitrates to the nutrient medium of *Chlorella sorokiniana* microalgae EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Results of the FAME analysis of both lipid samples show a predominance of C16-C18 fatty acids, namely C16:0, C18:1, C18:2, and C18:3. The most remarkable difference between the samples is the concentration of linolenic acid, an essential omega-3, which was the most abundant acyl moiety in the biomass cultivated with the addition of KNO₃ to the nutrient medium. Interestingly, this type of PUFA was almost 12 times lower in the lipid extract obtained from the biomass cultivated under nitrogen deficiency. The comparison of the fatty acids (FA) accumulation patterns is shown in Table 3.

Table 3. Types of fatty acids present in the biomass samples of *C. sorokiniana* cultivated with (+ NO₃-) and without (-KNO₃-) the addition of nitrates.

| Type of fatty acids (FA) | Concentration of FA, % (-NO₃⁻) | Concentration of FA, % (+NO₃⁻) |
|-------------------------|--------------------------------|--------------------------------|
| Saturated FA            | 26.576                         | 23.739                         |
| Unsaturated FA          | 73.824                         | 76.257                         |
| Unsaturated FAs (without trans fats) | 49.255                         | 65.154                         |
| Trans fats              | 24.569                         | 11.103                         |
| PUFA (cis)              | 37.651                         | 55.291                         |
| Omega 3 (cis)           | 32.520                         | 48.399                         |
| Omega 6 (cis)           | 6.130                          | 4.254                          |
| Omega 9 (cis)           | 11.049                         | 9.304                          |
| Eicosapentaenoic acid (EPA) | 1.151                          | 3.195                          |
| Docosahexaenoic acid (DHA) | 1.361                          | 10.607                         |
As shown in Table 3, microalgae *C. sorokiniana* has cumulated more saturated and trans fatty acids in the absence of nitrates in the medium. However, the PUFA content was lower in this biomass, which is consistent with the data found in literature. The variability of the trans fatty acids concentration in microalgae species has been rarely documented and is not broadly explain.

The degree of saturation of fatty acids depends on the activity of enzymes called desaturases. Long chain PUFA are produced from oleic acid (C18:1 cis-9) by series of desaturations and elongations. Studies show that the gene expression of several desaturases was affected differently by nitrogen stress. Assessing the effect of nutrients on the expression of genes should be one of the priorities of future researches for a better understanding of microalgae metabolism [17].

Long-chain PUFA (C20-C22) such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are recognized as highly valuable for their cardio-protective properties [18]. However, the two samples did not highly differ in their EPA and DHA content.

4. Conclusion

The present study shows that nitrogen could be used as a modulating factor for producing a biomass containing more or less of certain valuable compounds. The absence of nitrates negatively affected the growth of *C. sorokiniana* and decreased the chlorophyll content of the biomass. However, the addition of nitrates reaching 0.1g/l increased the biomass yields by three times. The comparison of the effect of different nitrates concentrations in the medium (0.1 g/l to 0.3g/l) did not show distinctive differences on the biomass growth patterns. An initial concentration of nitrates of 0.4 g/l seemed to give slightly lower biomass yields. This could indicate that this concentration is not adapted for the short term cultivation of *C. sorokiniana*.

The highest yield of total lipids (25%) was obtained from the biomass grown under nitrogen deficiency, followed by the biomass grown in the medium containing 0.3 g/l of nitrates (23%).

Nitrogen starvation importantly decreased the production of polyunsaturated fatty acids (PUFA), particularly omega-3 (linolenic acid), while increasing the concentration of trans and saturated fatty acids. For the biofuel production, high saturated/low unsaturated fatty acid content would be preferable for the transesterification process [16, 19]. Thus, nitrogen stress could be an interesting strategy for obtaining lipids destined for obtaining biodiesels.

The presence of nitrates in the nutrient medium seems preferable for producing a biomass destined for the food and feed industry. The presence of nitrogen has been shown in this study to enhance the concentration PUFA, more particularly, omega-3 fatty acids (linolenic acid). Also, this nutrient is crucial to microalgae cells as it is an important constituent of protein and enzymes. Increasing the amount of valuable compounds such proteins and lipids in microalgae biomass is one of the main focuses of researchers.

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