Microbiol. Biotechnol. Lett. (2019), 47(1), 11–19
http://dx.doi.org/10.4014/mbl.1801.01004
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Characterization of the Growth, Total Lipid and Fatty Acid Profiles in Microalga, *Nannochloropsis oceanica* under Different Nitrogen Sources

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Received: January 10, 2018 / Revised: March 7, 2018 / Accepted: May 31, 2018

The properties of microalgae as bioresources for biodiesel production can be improved by adding nitrogen sources into the culture medium. Thus, *Nannochloropsis oceanica* CCAP 849/10 was cultured in f/2 media supplemented with five different forms of nitrogen at 0.88 mmol-N l^{-1} each: ammonium bicarbonate (NH_4HCO_3), ammonium sulfate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3), and urea. The cell density, lipid content, and fatty acid profile of the microalga were determined after 15 days of cultivation. The growth of *N. oceanica* based on cell number was lowest in the medium with NH_4NO_3, and increased significantly in the medium with NH_4HCO_3. Cells treated with $(NH_4)_2SO_4$, and NH_4NO_3 produced the highest total lipid contents (i.e., 65% and 62% by dry weight, respectively). The fatty acid profiles of the microalga were significantly different in the various nitrogen sources. The major fatty acids detected in cultures supplemented with NH_4HCO_3, $(NH_4)_2SO_4$, NH_4NO_3, or urea were C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:5, and C22:6. However, the C16:1 content in the $NaNO_3$-supplemented culture was very low. This study highlights that the nitrogen source can strongly influence lipid production in *N. oceanica* and its fatty acid composition.

Keywords: **Biodiesel, Chlorophyll a, nutrient, microalga, urea**

Introduction

It seems that continual trust on energy of fossil fuel resources is labile, due to both reduction of world reserves and the emission of greenhouse gases connected with their application. Hence, there are strong studies aimed at replacing a renewable resource, containing potential biofuels, as energy sources. Recently, biodiesel fuel has received significant concern, because biodiesel is a biodegradable, reproducible and also nontoxic fuel. This fuel emits neither net carbon dioxide nor sulfur to the atmosphere and exhale less gaseous impurity than common diesel [1]. Microalgae are single-celled photosynthetic aquatic organisms. They use sunlight as energy and make various components with carbon dioxide and other elements. These organisms have a higher photosynthetic efficiency than plants for the production of biomass [2]. Various studies showed that biofuels specifically derived from microalgae are considered to be a technically viable alternative resource of energy [3–7]. Microalgae with high lipid contents are new reproducible resources for biofuel production, and pilot production of biofuel from microalgae has been documented [8].

However, the commercial production of biofuel from these organisms has not begun due to its high cost. The main factors contributing to the lipid production of microalgae are: the growth rate, cell density and lipid content. Currently, the selection of microalgae with

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higher lipid contents and improvements to the quantity and quality of the lipids produced by microalgae are considered for the technology of biodiesel production from microalgae. Previous studies [9–11] have revealed that it is possible to manipulate the lipid yield and lipid properties of algal cells by the optimizing microalgae culture conditions (e.g. temperature and also light intensity) or features of nutrient medium (nitrogen, phosphates as well as iron concentrations). Microalgae biomass and biofuels production are changed by various physicochemical factors such as nutrients, light intensity, temperature, pH and salinity [12, 13]. Especially between diverse nutritional agents, nitrogen is one of the most critical nutrients for algae growth, because this nutrient is a precipitant in all structural and also functional proteins such as enzymes, peptides, chlorophylls, energy transfer molecules, and genetic materials in algal cells [14, 15]. Wang *et al.* [16] reported that the nitrogen concentration in culture medium strongly affects both cell growth rate and cellular biochemical compositions in microalgae. In addition, numerous investigations have demonstrated that when the nitrogen is restricted in culture medium, microalgae decrease cell growth rate and raise their lipid or carbohydrate content, reducing protein synthesis [17].

Many microalgae species prefer ammonium, science less energy is needed for its assimilating into amino acids. In contrary, some microalgae such as *Botryococcus braunii* and *Dunaliella tertiolecta* prefer nitrate over ammonium for growth [18, 19]. Recent studies confirmed that some species of *Chlorella* also prefer nitrate rather than ammonium for growth, and these species also effectively use a variety of organic nitrogen sources such as urea, glycine, yeast extract (YE) and peptone [20, 21]. The results of Norici *et al.* [22] investigations proved that relevancy on the nitrogen source, biochemical composition can also be changed. For instance, protein content of *Dunaliella salina* was 2-times higher with ammonium than nitrate supplementation. In contrary, the lipid amount of *Chlorella sorokiniana* was over 2-folds more with ammonium than urea or nitrate supplementation [23]. Since the desirable source of nitrogen for growth varies from species to species, and the biochemical composition also can be differed by the supplemented nitrogen sources, it is essential to measure different nitrogen sources and take the most suitable source for each taxon in order to increase the efficiency of the goal product, like lipid and carbohydrate for biodiesel and bioethanol, respectively. Therefore, comprehension of the nitrogen sources effect on growth and also lipid value will ameliorate lipid yield and help large-scale commercial producers in selecting an appropriate fertilizer [24].

In this study, a oily microalga, *Nannocholoropsis oceanica*, was selected for lipid production. The effects of various nitrogen forms such as nitrate, ammonium, and organic nitrogen (urea) on the cell growth, and the biochemical composition of *N. oceanica* were analyzed. Also, the microalgal lipid was converted to fatty acid methyl esters (FAME), and the fatty acid composition was assessed for measuring the effect on the resulted biodiesel properties.

Materials and Methods

Organism and culture treatments

Nannochloropsis oceanic CCAP 849/10 was purchased from the Culture Collection of Algae and Protozoa at Scotland. The microalga was grown in 500 ml flasks with f/2 medium [25] and continues aeration with air. *N. oceanic* was grown at 23 ± 1℃ and 100 μmol photons $m^{-2}s^{-1}$ light intensity (L/D = 14:10). In order to study the effect of different nitrogen forms on the cell growth and biochemical composition of *N. oceanica*, sodium nitrate ($NaNO_3$, 0.88 mmol N L^{-1}) in F/2 medium was replaced by different nitrogen sources, including ammonium nitrate (NH_4NO_3), ammonium bicarbonate (NH_4HCO_3), ammonium sulfate ($(NH_4)_2SO_4$) and urea ($CO(NH_2)_2$). Initial nitrogen concentration was the same, at 0.88 mmol N L^{-1} and pH was adjusted to 8 after addition of each nitrogen source.

The cultures were grown for 15 days. The biomass, based on cell number and lipid quantity and quality of microalga were then assessed. All of the experiments were carried out in triplicate.

Cell density

The cell densities of the cultures were measured by hemacytometer cell counter.

The chlorophyll a content

The chlorophyll a (Chla) was determined spectropho-

http://dx.doi.org/10.4014/mbl.1801.01004

tometry according to Mackinney [26]. Briefly, a volume of 2 ml culture sample was withdrawn. Cells were centrifuged at 3000 rpm for 10 min. The supernatant was removed and cells were then resuspended in 2 ml of distilled water to remove any salts and centrifuged. This washing process was repeated twice. Then, cells were resuspended in 2 ml of methanol (99.8%) with strong vortex mixing for 15 s. After 20 min, the cells were harvested via centrifugation at 4000 rpm for 5 min and the supernatant absorbance was read at the wavelength of 665 nm.

Fluorescence microscopy

Nile Red (NR) staining is a rapid diagnostic method to measure the amount of biodiesel-convertible lipid that the cells accumulate. Its fluorescence features made NR a natural candidate for lipid staining and quantification. For lipids fluorescence microscopy analysis, the cells were stained with 0.5 mg/ml NR (Sigma, USA) stock solution after fixing cells with 5% paraformaldehyde. Next the stained cells viewed under a fluorescent microscope (Olympus, IX70) with 100 × objective lens was used to visualize the fluorescent yellow-gold lipid in microalgal cells. Images were taken with a cooled CCD camera at the same exposure time. NR emission was observed with 460 ± 10 nm excitation and 560–640 nm band pass emission filters.

Lipid content

For determining lipid content, the algal cells were collected after 15 days by centrifugation at 4,000 rpm for 15 min, and pellets freeze-dried at -46℃. The modified method of Bligh and Dyer [27] was used for lipid extraction. In the method, chloroform-methanol (2:1, v/v) solution was applied for extracting lipid of the freeze-dried microalga cell. For this, 0.2 g of freeze-dried microalgae was added in 50 ml of chloroform-methanol solution over 24 h, furthermore at this time, sonicated (70-Hz) twice per each 30 min. The obtained suspension was filtered and washed twice with a KCl solution. Then, the lower phase was transferred into a pre-weighed glass vial. The chloroform-methanol solution was vaporized to dryness at 40℃ under vacuum. The content of lipid was determined gravimetrically and estimated by the follow equation:

$$Y(\%) = W_L/W_{DA}$$

Where, W_L is the weight of the extracted lipid and W_{DA} is the dry algae biomass.

Transesterification and FAME analysis

The extracted total lipid was used for testing its fatty acid profile. Fatty acid methyl esters (FAMEs) were trans-sterificated with 0.4 M KOH-methanol. FAMEs were analytically verified by gas chromatography analyses. Fatty acid methyl esters were declared using flame ionization detection after injecting the sample into an Agilent 6890N gas chromatograph equipped with a column of Omega wax 320, 30 m × 0.32 mm I.D., 0.25 μm. Both temperatures of the injector and detector were 260℃. The temperature of the column was gained from its initial value of 60 to 170℃ at a rate of 50℃ min^{-1}, pursued by an increment of 180℃ at 2℃ min^{-1}. The temperature retained stable for 2 min, then, it was increased to 230℃ at 2℃ min^{-1} and preserved fixed for 1 min; eventually, the temperature was augmented to 240℃ at 1℃ min^{-1}, wherein the temperature was stable until all FAMEs had been washed. The washing gas was helium, and the flow speed was 30 ml min^{-1}. The obtained Peaks were characterized by comparing retention times with known standards (Sigma Chemical Co., USA). The percent of fatty acids was defined using the normalization approach.

Data analysis

Two-way analysis of variance analyses (2-way ANOVA) were employed to assess the significance of lipid content variation between groups. When ANOVA confirmed significant variation, manifold comparisons among means value were done with Duncan's test. SPSS v16 was used for statistical analyses.

Results and Discussion

Cell density in various nitrogen sources

Cell density with ammonium bicarbonate reached the highest value of 25.5×10^6 after 15 days. Sodium nitrate showed the second highest cell concentration of 23.3×10^6, followed by ammonium sulfate (16.7×10^6), urea (14.3×10^6), and ammonium nitrate (8×10^6). How-

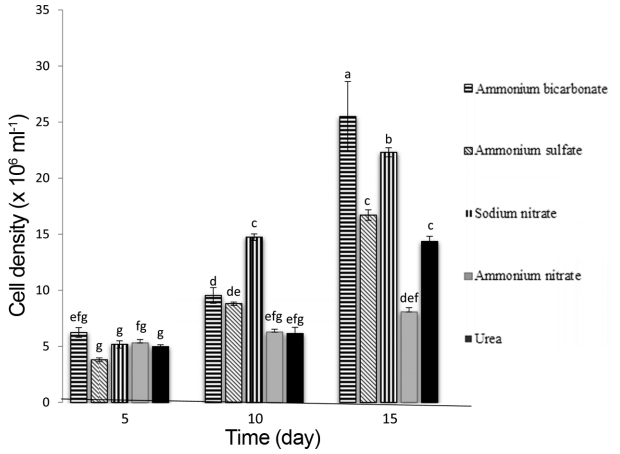

Fig. 1. Effects of different forms nitrogen supplementation of the culture medium on the cell density of *Nannochloropsis oceanica*. Data represent the mean values of triplicates ± standard deviation (SD). Values with the same lower case letter are not significantly different at $p = 0.05$ significance level based on Duncan's multiple range test.

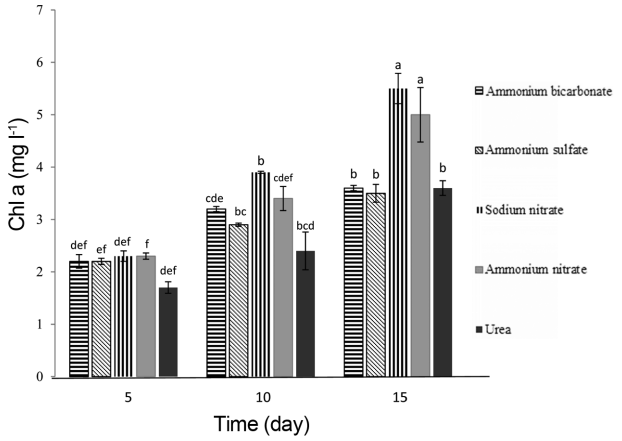

Fig. 2. Effects of different forms nitrogen supplementation of the culture medium on the chlorophyll a contents *Nannochloropsis oceanica*. Data represent the mean values of triplicates ± standard deviation (SD). Values with the same lower case letter are not significantly different at $p = 0.05$ significance level based on Duncan's multiple range test.

ever, in culture supplemented with ammonium nitrate, cell growth was slowed down, resulting in a lower cell density (Fig. 1).

Chlorophyll a is often used as an estimate of algal biomass. However, Chla content was lower in media supplemented with ammonium bicarbonate (3.5 ± 0.30 mg l^{-1}) than sodium nitrate (5.53 ± 0.32 mg l^{-1}) (Fig. 2). It seems algal biomass estimated by Chla content was not matched with cell density in microalga. Many species of microalgae are able to use different forms of nitrogen, containing nitrate, nitrite, ammonium and other organic nitrogen sources like urea [28]. Each nitrogen source is primary reduced to the ammonium form, then assimilated into amino acids via a diversity of pathways.

Although ammonium bicarbonate was the fastest consumed nitrogen source by *Nannocholoropsis* cells, it seems that the resulted highest cell density is not merely due to the nitrogen, as the ammonium bicarbonate also contains bicarbonate as carbon source. *Nannocholoropsis* seemingly utilizes both nitrogen and bicarbonate compounds, and as a result, the cell growth was more triggered. While the initial nitrogen concentration was similar in all cultures, the initial concentration of total carbon in the medium with ammonium bicarbonate was higher than that in medium with other N sources.

Urea, as organic nitrogen source, is relatively energetically cost effective than other nitrogen sources and also be easily utilized after being converted to ammonium and bicarbonate by urease in most microalgae species [29]. *Nannocholoropsis oceanica* grew fine in culture supplemented with urea, and the final cell concentration as well as biomass productivity was higher than observed with ammonium nitrate supplementation. While, Campos *et al.* [30] stated that *Nannochloropsis salina* grows better in the presence of urea rather than nitrate or ammonium. *Isochrysis galbana* was also found to achieve to the highest cell concentration in urea rather than nitrate or nitrite [31]. These results indicate that the preference for nitrogen source and the ability of nitrogen utilization were changed from species to species.

Experiments confirmed that many microalgae usually prefer ammonium rather than other sources of nitrogen such as nitrate or nitrite. Because, ammonium is the reduced form of nitrogen and can be directly assimilated into amino acids, while other forms (e.g. nitrate or nitrite) must first be reduced to ammonium within the cells before its consumption [32]. In the present study, it was found that ammonium bicarbonate but not ammonium nitrate is more favorable for the growth of *Nannocholoropsis* cells (Figs. 1 and 2). But, many species of microalgae, for example, *D. tertiolecta*, *I. galbana*, *Neochloris oleoabundans*, *C. sorokiniana*, and *Botryococcus braunii*, prefer nitrate over ammonium for their growth

and development [18, 20, 28, 33, 34].

The growth of *Nannocholoropsis* cells was slowed by ammonium nitrate supplementation, thus it seem had a toxic effect on the cell growth, at the certain concentration of 0.88 mM. Ramanna *et al*. [35] suggested that the negative effect on cell growth is due to the fact that the excessive transport of ammonium to the cells can prevent the ATP formation in the chloroplast, leading to photosynthesis inhibition. According to Norici *et al*. [22] findings, the transport of nitrate is more regulated in algal cells, while the influx of ammonium is not easily controlled, especially when the extracellular ammonium concentration is high. Some investigators [33, 36] reported that ammonium oversaturation in the medium can strongly decreased the pH by releasing H^+ ions, resulting in preventing growth of the cell and even causing cell lysis.

Changes in lipid content

The lipid content of *Nannocholoropsis oceanica* obtained after 15 days of incubation with different nitrogen forms is shown in Fig. 3. The level of increase in lipid content was very different, depending on the supplied N sources. In the cultures supplemented with ammonium sulfate and ammonium nitrate, the cells mostly produced higher lipid contents than those with other nitrogen sources (The highest total lipid contents were 65 and 62 %, respectively).

In cultures supplemented with organic-N (Urea) and nitrate, the lipid content moderately increased up to 51% and 42% respectively on the 15th day, while the supplementation with ammonium bicarbonate, resulting in the lowest lipid content (26%) on the final day (Fig. 3). As there is a reverse relation between microalgal growth and cellular lipid content, thus in culture supplemented with ammonium bicarbonate, the lowest lipid content was resulted. Although more lipid content was obtained from the cultures with ammonium sulfate, the lipid productivity was lower than that of nitrate- or organic-N supplementation due to a lower biomass production, resulting from the cell growth inhibition. Simi-

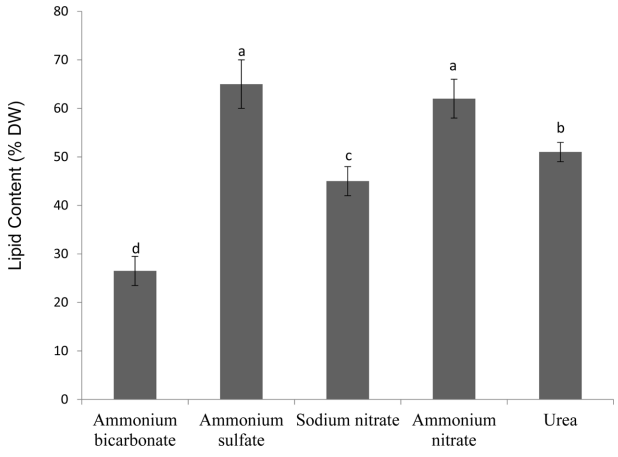

Fig. 3. Effects of different nitrogen source supplementation of the culture medium on the total lipid contents of *Nannochloropsis oceanica*. Data represent the mean values of triplicates ± standard deviation (SD). Values with the same lower case letter are not significantly different at $p = 0.05$ significance level based on Duncan's multiple range test.

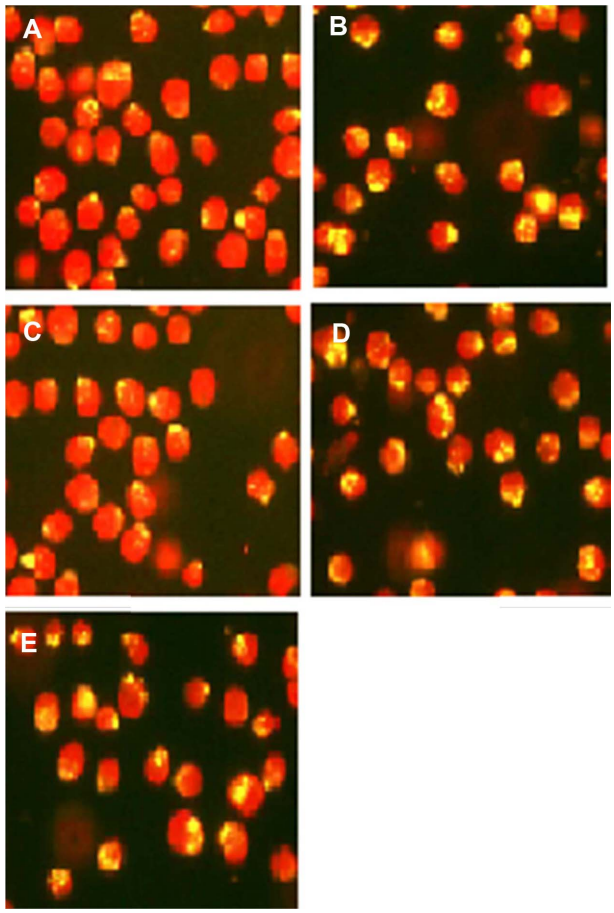

Fig. 4. Fluorescence micrographs of *Nannochloropsis oceanica* stained with Nile Red fluorescence dye and screened under 1000X magnification with different N sources: (A) Ammonium bicarbonate, (B) Ammonium sulfate, (C) Sodium Nitrate (D) Ammonium nitrate and (E) Urea. The lipid droplets appear as yellow color.

larly, some algae species such as *I. galbana* and *C. sorokiniana* accumulate higher lipids when supplemented with ammonium as a nitrogen source rather than nitrate or urea, however, it is important to know that the final biomass concentrations were very low, because ammonium inhibits the cell growth [23, 34].

Fluorescence microscopic analysis of Nile Red-stained cells

To visually observe the effect of nitrogen sources on lipid accumulation in cells, we collected and stained cells with Nile Red and observed by fluorescence microscopy. Microscopy results were in strong agreement with our previous results, as lipid accumulation (increased size and number of cytoplasmic lipid droplets) in ammonium sulfate and ammonium nitrate was higher than the control (sodium nitrate) and ammonium bicarbonate (Fig. 4A–E).

Fatty acid profiles

The fatty acid profiles of *Nannochloropsis oceanica* grown under different nitrogen sources are shown in Table 1. In cultures supplemented with ammonium bicarbonate, ammonium sulfate, ammonium nitrate and urea, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:5 and C22:6 were detected. However, in culture supplemented with sodium nitrate, C16:1 had very small peak. Other small peaks were detected using all nitrogen sources. From the area of the main peak, the fatty acid component ratio was calculated (Table 1). The relative

Table 1. Fatty acid methyl ester (FAME) profile of *Nannochloropsis oceanica* cells cultivated under different nitrogen sources. Values are presented as mean ± SD (n = 3).

FAME (%)	Nitrogen Sources				
	Ammonium bicarbonate	Ammonium sulfate	Sodium nitrate	Ammonium nitrate	Urea
C12	0.2 ± 0.0	n.d.	n.d.	0.5 ± 0.1	n.d.
C14	5 ± 0.8	5.6 ± 1	3.8 ± 0.7	4.4 ± 0.75	5.1 ± 1.2
C14:1T	n.d.	n.d.	n.d.	3.6 ± 0.9	n.d.
C14:1C	0.9 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	n.d.	0.6 ± 0.0
C16	38.2 ± 2.8	26 ± 3.1	33.8 ± 3.5	34.8 ± 2.9	32.2 ± 2.9
C16:1C	32 ± 2.3	29.7 ± 4.3	0.7 ± 0.0	29.2 ± 4.3	30.7 ± 3.7
C17	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	n.d.
C17:1	0.3 ± 0.0	1 ± 0.0	n.d.	0.3 ± 0.0	0.5 ± 0.0
C18	1.7 ± 0.3	0.2 ± 0.0	2.3 ± 0.6	2.2 ± 0.4	1.6 ± 0.3
C18:1C	9.6 ± 1.6	6.1 ± 1.1	9.6 ± 1.9	6.8 ± 1.1	6.9 ± 1.5
C18:2C	1.3 ± 0.0	2.6 ± 1.0	2.1 ± 0.0	2 ± 0.0	2.1 ± 0.0
(γ)C18:3	0.2 ± 0.0	0.2 ± 0.0	n.d.	0.2 ± 0.0	n.d.
(α)C18:3	n.d.	n.d.	0.2 ± 0.0	n.d.	n.d.
C20	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.d.	n.d.
C20:1	n.d.	n.d.	0.2 ± 0.0	n.d.	n.d.
C20:2	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.d.	n.d.
C20:3	n.d.	n.d.	0.2 ± 0.0	0.2 ± 0.1	n.d.
C20:4	n.d.	n.d.	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
C20:5	2.3 ± 0.9	4.1 ± 0.5	3.6 ± 0.3	3.3 ± 0.9	4.4 ± 1.0
C22	n.d.	0.2 ± 0.0	n.d.	n.d.	n.d.
C22:1	0.1	n.d.	n.d.	n.d.	n.d.
C22:6	7.7 ± 1.2	21.2 ± 4.5	12.6 ± 2.3	11.6 ± 3.0	14.6 ± 2.9
C24:1	n.d.	0.4 ± 0.0	0.1 ± 0.0	n.d.	n.d.
Others	0.1 ± 0.0	0.8 ± 0.1	29.1 ± 4.5	0.5 ± 0.0	1 ± 0.0

n.d.= not detected

http://dx.doi.org/10.4014/mbl.1801.01004

abundance of main fatty acid products is different between microalga grown under different nitrogen sources. The main fatty acid composition of nitrate-grown alga comprised 3.8%, 33.8%, 2.3%, 9.6%, 2.1%, 3.6% and 12.6% of C14:0, C16:0, C18:0, C18:1, C18:2, C20:5 and C22:6. In contrast, main fatty acid composition of urea-grown alga comprised 5.1%, 32.2%, 30.7%, 1.6%, 6.9%, 2.1%, 4.4% and 14.9% of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:5 and C22:6. Also, the main fatty composition of ammonium-grown algae consisted C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:5 and C22:6. In addition, a clear difference can be seen in C16:1 and C22:6 for cells grown in different nitrogen sources. Thus, it is obvious that different nitrogen sources can affect the composition of fatty acid in microalgae. Campos *et al*. [30] reported the same FAME fraction of lipid in *N. salina* irrespective of nitrogen source. Furthermore, the main fatty acids of *N. oceanica* IMET1 when grew in the modified f/2 medium were C16:0 and C16:1 [37].

One of the most prominent factors that affects the properties of biodiesel is profile of fatty acid, because the molecular features of FAMEs, including length of carbon chain and the double bond number, directly influence some characteristics of biodiesel such as: the viscosity, ignition quality, oxidative constancy, and property of cold flow [38, 39].

Different factors, such as various nutritional conditions, physicochemical conditions as well as growth phases can change the composition of fatty acid [40, 41]. Serrano *et al*. [42] found that oxidation stability and cold flow performance have reverse relationships to variations in composition of fatty acids.

For example, the raise in unsaturated fatty acids (UFAs) would improve the cold flow performance, while decreasing the oxidative stability. Inversely, the increase of saturated fatty acids (SFAs) could result in better oxidative stability but poor cold flow property.

Lapuerta *et al*. [43] stated that the great fraction attendance of unsaturated fatty acids outcome in a small cetane number of biodiesel, fathering a poor ignition state. Because the UFA fraction is lower in all forms of nitrogen, quality of ignition would be better. However, higher fraction of SFA can result in an inferior cold-flow property; it is possible to achieve the fuel quality by using some additives [38].

In this research, the effects of different nitrogen sources were examined on the different physiological parameters such as the growth, lipid production and composition of *N. oceanica* CCAP 849/10. The obtained data confirmed that *N. oceanica* produces a much higher lipid content when cultivated with ammonium sulfate than with other nitrogen sources. In the presence of ammonium sulfate, higher amount of C22:6 fatty acid yield in comparison with other nitrogen sources. Ammonium bicarbonate-grown cells have higher cell number than other nitrogen source-grown cells. Therefore, we concluded that replacement of nitrate in f/2 medium with ammonium bicarbonate will have a negative effect on lipid content but boost cell growth. Thus we suggest that ammonium sulfate is a better nitrogen source with respect to lipid productivity as biodiesel in *Nannochloropsis oceanica*.

Acknowledgments

This research was financially supported (No. 94/11138) by Research and Technology office of Arak University and authors thank Arak University.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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