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Cultivation of marine microalga *Nannochloropsis gaditana* under various temperatures and nitrogen treatments: effect on growth, lipid and pigment content

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Abstract

Microalgae based biofuels are getting attention due to energy crisis and environmental protection. There is potential to increase yields by manipulating environmental factors, which cause stress for microalgae. Sources of stress include manipulating environmental conditions such as salinity, pH, temperature, and nutrients. In the present study, we observe how various nitrogen treatments and temperatures can impact the growth, lipid and pigments accumulation on *Nannochloropsis gaditana*. We used five different nitrogen treatments; ammonium chloride, ammonium hydroxide, sodium nitrate, urea, a mixture of all these sources and three different temperatures (20°C, 25°C, 30°C). The highest biomass growth was found (0.278$d^{-1)}$ in ammonium chloride treatment and 25°C (0.224 d^{-1}). The lipid content was examined using a modified method of Zhu *et al.* (2002) and found better in CH_4N_2O nitrogen source (36.63%). Among temperature, the maximum lipid content (28 %) was found in case of 25°C. The pigments of microalgae biomass was maximum in 25°C (3.64 ± 0.11µg ml^{-1} of chlorophyll a and 0.232 ± 0.03 µg ml-1 of carotenoid) and NH_4Cl (5.57 ± 1.39 µg ml^{-1} of chlorophyll a and 0.337 ± 0.16 µg ml^{-1} of carotenoid). Our results suggest that tradeoffs between growth, pigments and lipid yields as well as culture success can ultimately decide what nitrogen sources and temperature to use.

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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 (Miao and Wu 2006). High added value compounds can be extracted from microalgae, such as fatty acid (Cardozo *et al*., 2007), pigments (carotenoids and ficobiliproteins), biochemically stable isotopes (Chisti, 2007) and vitamins (Bremus *et al*. 2006); also some metabolites appear to have some pharmacological activities, among others the anticholesterolemic, antitumoral, immunomodulatory, antibacterial and antimycotic ones. Some microalgal species, including some *Chlorella* sp, *Nannochloropsiss* sp, *Dunaliella* sp (Babu and Binnal, 2015) and *Scenedesmus* sp (Dittamart *et al*., 2014) have been reported to accumulate large quantities of lipids up to 50% by weight of dry biomass. These lipids can be converted into biodiesel by a chemical process called transesterification. However, the growth characteristics and lipid accumulation of microalgae are known to significantly depend on the cultivation conditions. (Grobbelaar, 2007). According to previous studies the environmental parameters, temperature, CO_2 aeration fixation, salinity, light intensity and nutrient supply were found to be vital factors for growth improvement and induction of lipid and pigment in microalgae (Harun *et al*., 2014).

Importantly, compared to the other microalgal species, relatively high biomass productivity and lipid content of *Nannochloropsis*. *gaditana* have suggest edits potential for biodiesel production. *N gaditana* is a marine microalga belonging to the class of Eustigmatophyceae. The genus is widely used in aquaculture due to its relatively high growth rate, resistance to mixing and contamination together with high nutritional values and high lipid content (Olofsson *et al*., 2012). Thus, various *Nannochloropsis* species have become popular model systems. However, we still have limited knowledge and experience on the possible applications of *N. gaditana* as a biofuel organism. In this study, we aimed to obtain the important experimental data for future application of *N. gaditana* to biofueltechnology and High added value compounds (pigments).

To do so, we test the effect of different nitrogen sources (ammonium chloride (NH_4Cl), ammonium hydroxide (NH_4OH), sodium nitrate ($NaNO_3$), urea (CH_4N_2O), and a mixture of all these sources) and three differents temperatures (20°C, 25°C, 30°C) on growth, lipids and pigments accumulation of *Nannochloropsis gaditana*.

Materials and methods

Source of the microorganisms

A culture of *Nannochloropsis gaditana obtained* from the company: Partisano biotech Algeria in Oran was used for the purpose of this study.

Microalgae cultures and medium

The strains selected were studied in axenic batch cultures, under non-nutrient limited conditions, the cultures were grown in 1 L erlenmeyer flasks with 500 mL f/2 medium (Guillard, 1975) using the atmospheric CO_2 as carbon source and kept under controlled environmental conditions of approximately light intensity (100 µmol m^{-2} s^{-1}) and cantinuous illumination at (23 ± 1°C). Each treatment consisted of triplicate flasks and continuous aeration was provided.

Effect of temperature on growth, lipid and pigments accumulation

To study the impact of temperature on the growth rate, experiments were performed at three temperatures, the temperature values tested were from 20 to 30°C with five degree intervals. All experiments were performed at the same incident light intensity of 100 µmol m^{-2} s^{-1}, pH 8 for 12 days

Effect of various nitrogen treatments on the growth, lipids and pigments accumulation

Growth experiments were done at f/2 medium, with an alternative source of nitrogen, we had a total of five nitrogen treatments (NH_4Cl, NH_4OH, $NaNO_3$, CH_4N_2O and a mixture of all these sources) Each treatment consisted of this same concentration of nitrogen at 23± 1°C, pH 8 for 12 days. After growth, biomass was separated from the medium by centrifugation at 5000 rpm for 15min, using a centrifuge (Sigma 3-30KS).

Mesurment of microalgae growth

Cell density was estimated daily by direct microscopic count using the haemocytometer counting, each sample was measures twice and the mean value was calculated.

The specific growth rate (μ) and division rate (k) was calculated by the equation (1) and (2) respectively as described by Mohanadoss (2013):

Where, n_2 and n_1 are the cell nember concentration at the times t_2 and t_1 respectively.

Lipid extraction and quantification

The total lipids were extracted from microalgal cells using a modified method of Zhu *et al.* (2002). Freeze-dried alga powder (0.2 g) was suspended in 3 mL solvent mixture of chloroform: methanol (2:1, v/v). After, the samples were centrifuged at 8000 rpm for 10 min. The solvent phase was transferred and evaporated in a rotary evaporator (Hahnvapor HS-2005 VN).

Determination of pigment concentration and composition

The pigment content is determined by a spectrophotometric method. After extraction with methanol (99.8%), the absorbance of the supernatant containing the pigments dissolved in methanol is measured at different wavelengths (480, 665 nm). These absorbances are used to calculate the concentrations of chlorophyll a and the protective carotenoids (PPC for Photo Protective Carotenoides) via the equations of (Ritchie, 2006) for chlorophyll a and (Strickland and Parsons, 1968) for carotenoids.

Statistical analysis

To test possible statistical differences between the lipid concentration, growth rate and pigments concentration the data were subjected to one-factor analysis of variance (ANOVA), and in case of significant differences, means of the treatments were compared by Tukey's test.

Result and discussion

Microalgalbiomasses are affected by a variety of physicochemical factors such as nutrients, light supply, temperature, pH and salinity (Bartley *et al.*, 2016). Particularly among various nutritional factors, nitrogen is considered one of the most critical nutrient for growth, since it is an essential constituent in all structural and functional proteins such as peptides, enzymes, chlorophylls, energy transfer molecules, and genetic materials in algal cells (Hu, 2013).

Table 1. Growth measurements and lipid content of *Nannochloropsis gaditana* operated at different nitrogen treatments and temperatures.

	Growth rate (μ) (d^{-1})	division rate (k)	Lipids (%)
Treatments			
$NaNO_3$	0.246	0.354	20.28
CH_4N_2O	0.262	0.378	36.63
NH_4OH	0.273	0.393	22.53
NH_4CL	0.278	0.401	15.99
Mixture	0.270	0.389	22.59
Temperatures			
20°C	0.226	0.326	16.01
25°C	0.244	0.352	28.00
30°C	0.243	0.350	22.79

The Most microalgae are able to utilize various forms of nitrogen, including nitrate, nitrite, ammonium and organic nitrogen sources such as urea (Becker, 1994); each nitrogen source is first reduced to the ammonium form and assimilated into amino acids through a variety of pathways (Cai *et al*., 2013). Typically, ammonium was known to be preferred by many microalgae, as it requires less energy for assimilating into amino acids, (Ruangsomboon, 2015). Depending on the source of nitrogen, biochemical composition can also be changed; for example the lipid content of *Chlorella sorokiniana* was over 2-times higher under ammonium than urea or nitrate supplementation (Wan *et al*., 2012).

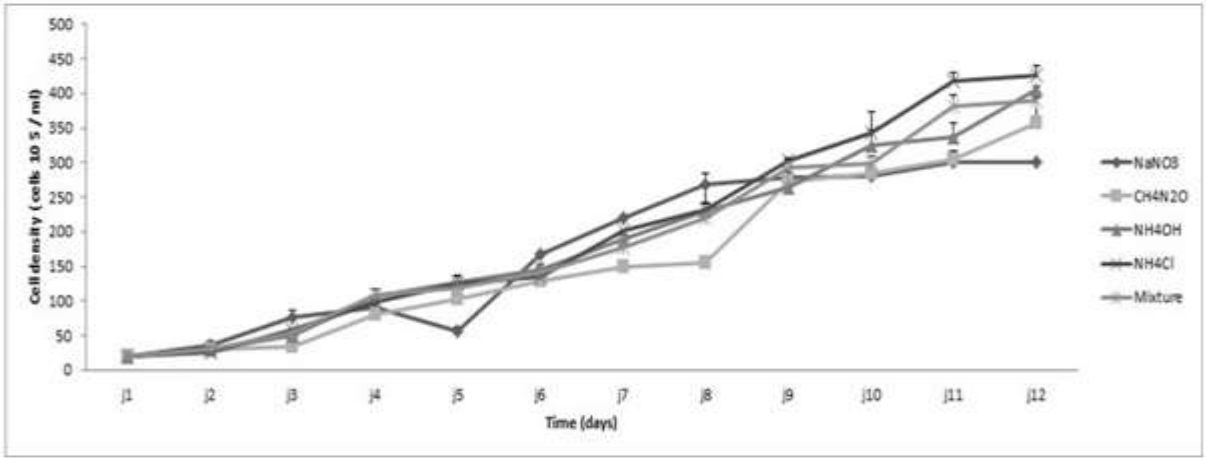

Fig. 1. Cell count of *Nannochloropsis gaditana*cultured under five nitrogene treatments (n=3) ($P < 0.05$).

Since the favorable nitrogen source for growth is different from species to species, and the biochemical composition also can be changed by the supplemented nitrogen sources, it is required to compare various nitrogen sources and select the most appropriate one for each species in order to maximize the productivity of the target product, such as lipid for biodiesel.

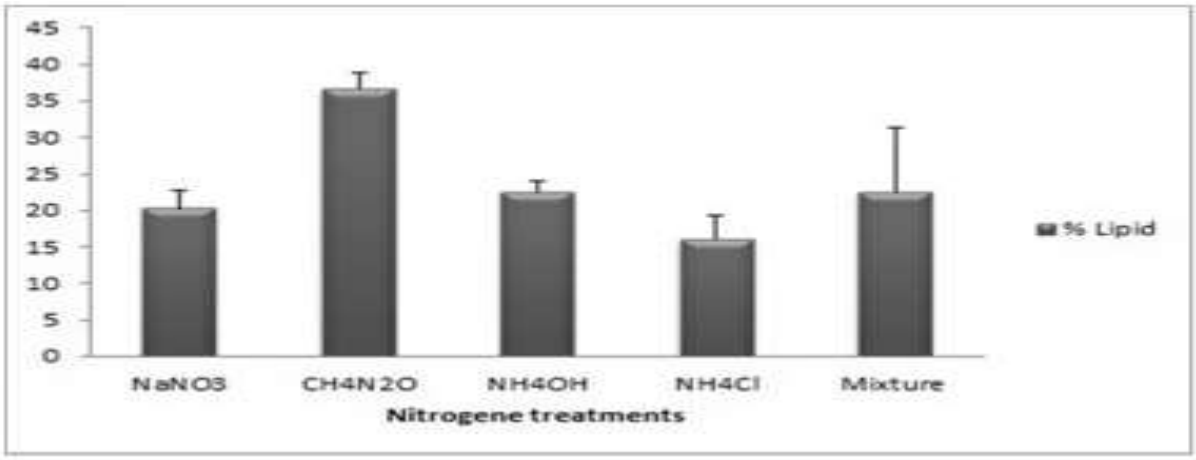

Fig. 2. Cellular lipid content of *Nannochloropsis gaditana*. The lipid contents were monitored after 12 days of incubation under optimal conditions and in response to five nitrogen treatments (n = 3) ($P < 0.05$).

In this study, a marine Microalga *Nannochloropsis gaditana*., was chosen for lipid production. The effects of different nitrogen sources including nitrate, ammonium, and organic nitrogen on the cell growth, and the biochemical composition of *Nannochloropsis gaditana* were investigated.

We found that marine microalga *N gaditana*. effciently utilize ammonium (NH_4Cl and NH_4OH) (Fig. 1). Cell concentration with NH_4Cl increased rapidly and reached the highest value of 0.278 d^{-1} after 12 days followed by NH_4OH (0.273 d^{-1}). It is well known that ammonium is generally preferred by

many microalgae rather than nitrate or nitrite. Since ammonium is the reduced form of nitrogen, it can be directly assimilated into amino acids inside the cells, whereas nitrate or nitrite must first be reduced to ammonium prior to its utilization (Podevin *et al*., 2015).

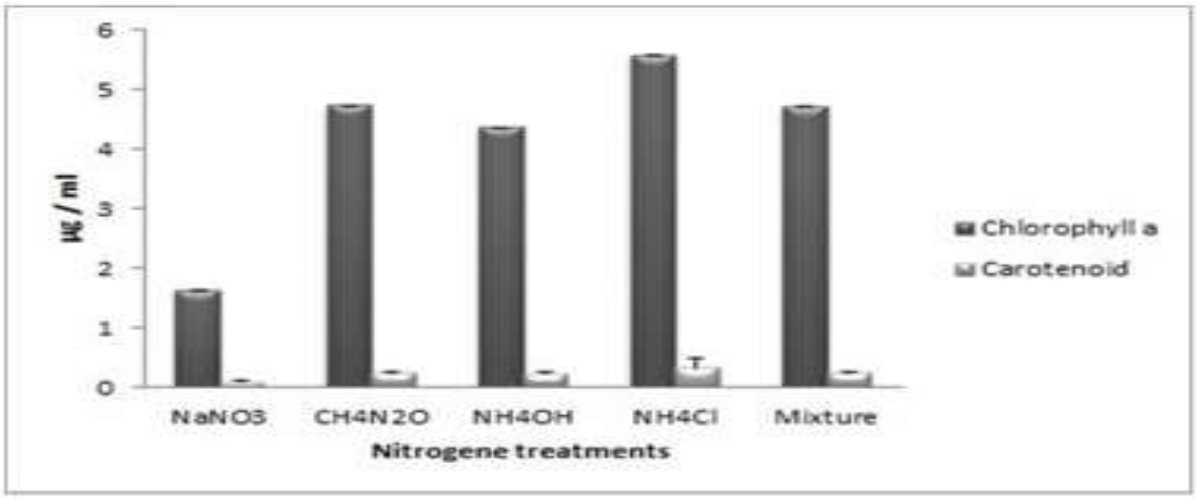

Fig. 3. Cellular pigments content of *Nannochloropsis gaditana*. after 12 days of incubation under optimal conditions and in response to five nitrogen treatments (n = 3) ($P < 0.05$).

Urea is an organic nitrogen source relatively cheaper than other nitrogen sources, and can also be easily utilized after being degraded to ammonium and bicarbonate via urease in most microalgae (Solomon and Gilbert, 2008). *Nannochloropsis gadiana*. also grew well in urea supplementation, but the final cell concentration and biomass productivity were a little bit lower than seen with NH_4CL supplementation. Conversely, a marine microalga *Nsalina*, was reported to show a clear preference to urea over nitrate for its growth (Campos *et al*., 2014).

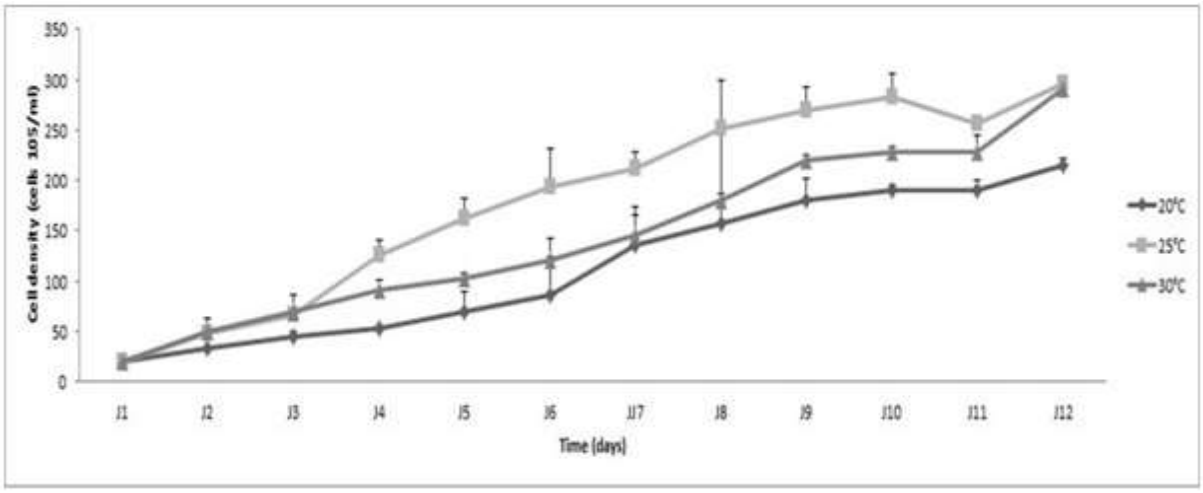

Fig. 4. Cell count of *Nannochloropsis gaditana*, cultured under three different temperatures (n=3) ($P < 0.05$).

The composition of *Nannochloropsis* cells was significantly changed depending on the supplemented nitrogen sources as well as cultivation time (Figs 2 & 3). In the cultures supplemented with urea the cells mostly showed higher lipid contents (36.63 %) than those with other nitrogene sources.

The pigments content was maximum in ammonium chloride (5.57 ± 1.39 µg ml^{-1} of chlorophyll a and 0.337 ± 0.16 µg ml^{-1} of carotenoid).

Further more, temperature is one of the key factors that affects the growth (Taoka *et al.*, 2009). It strongly affects the rate of all enzymatic electron transport and solute movement reactions within algal cells, and influences the properties of cellular components such as lipids, pigments, proteins and carbohydrates (Bojan *et al.*, 2014).

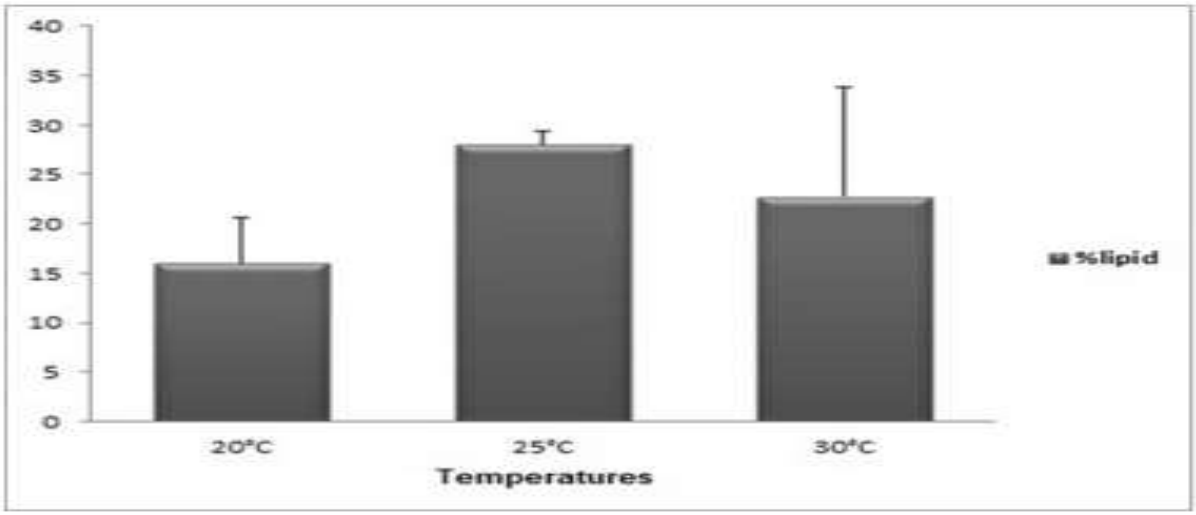

Fig. 5. Cellular lipid content of *Nannochloropsis gaditana*. The lipid contents were monitored after 12 days of incubation under optimal conditions and in response to three different temperatures (n = 3) ($P < 0.05$).

Aquatic microalgae are frequently forced to adapt to large variations in temperature owing to diel and seasonal cycles, these thermal adaptations can determine the variability in biomass and lipid productivity. In this study microalgae ware grown in variable temperatures ranges (20, 25 and 30°C), all other factors were made constant. The effect of temperature was found to be significant on microalgal growth. The results are shown in Fig 4. It was observed that *Nannochloropsis gaditana* shows higher growth rate (0.244 d^{-1}) at 25°C, followed by 30°C (0.243 d^{-1}) this result correlated with results of (Fakhry and Maghraby, 2015) and (Yongxue and Yasuyuki, 2015).

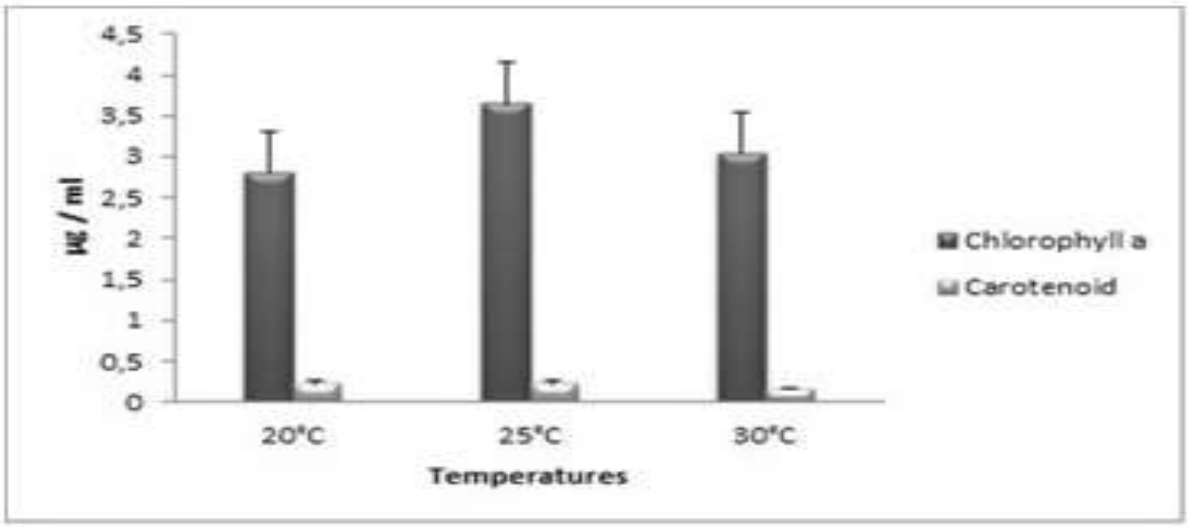

Fig. 6. Cellular pigments content of *Nannochloropsis gaditana*. after 12 days of incubation under optimal conditions and in response to three different temperatures (n = 3) ($P < 0.05$).

The specific growth, lipids and pigments productivity at stationary phase are shown in table 1. Highest lipide productivity was observed in 25°C (28%) while lowest was 16.01% in 20°C (Fig 5). Converti *et al.* (2009) have reported that microalgal lipid content is strongly influenced by temperature variation; increasing temperature from 20°C to 25°C almost doubled the lipid content of *N oculata* , from 7.90 % to 14.92 %.

The pigments of microalgae biomass was maximum in 25°C (3.64 ± 0.11µg/ml of chlorophyll a and 0.232 ± 0.03 µg ml^{-1} of carotenoid) (Fig 6), followed by 30°C (3.05 ± 0.17 µg ml^{-1} days of chlorophyll a and of 0.163 ± 0.01 µg ml^{-1} carotenoid). (Roleda *et al.*, 2013) demonstrate that growth performance and secondary metabolism vary with temperature, temperature affects nutrient uptake, cell membrane fluidity, and influences the oxygen evolving activity of photosystem II (Vonshak, 2002). In addition, temperature also affects microalgae growth by altering the activities of important enzymes vital for assimilation, for example, it has been demonstrated that temperature influences the concentration of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), as a key enzyme involved in carbon assimilation in photosynthetic organisms. Despite a low correlation between Rubisco activity and temperature, Rubisco activity declines with increasing temperature (Leggat *et al.*, 2004); Rubisco activity largely determines photosynthetic efficiency (Doubnerová, and Ryšlavá 2011).

Conclusion

Significant differences in growth and cellular components of microalgal cells have been observed depending on how the alga will act in response to variations in culture conditions. Marine microalga *Nannochloropsis gaditana* was found to efficiently utilize inorganic nitrogen sources, ammonium was clearly preferred for growth, but a higher cellular lipid and pigment content was obtained from the culture with Urea on the final day of cultivation. When applying mass cultivation for biodiesel and pigment production, urea would be more appropriate in terms of economic feasibility.

The adaptability of microalgae to a temperature regime is species dependent. *Nannochloropsis gaditana* can grow in wide temperature ranges of 20 - 30°C. The variation of parameters tested (temperature and different nitrogen treatments) strongly influenced the lipid and pigments content of microalgae.

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