JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 2761-2772

Growth and Oil Production of *Nannochloropsis salina* Cultivated under Multiple Stressors

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(Received: 30 May 2014; accepted: 14 July 2014)

The problem of the unlimited increasing need for the unrenewable fossil fuels (petroleum) and its increasing price has urged scientists to use microalgae as a source for the production of biodiesel. The eustigmatophyte microalga *Nannochloropsis salina* has been cultivated under different laboratory conditions to increase its growth and potentiality for oil production. Results demonstrated that the maximum value of algal biomass (approximately 0.3 g L^{-1}) was obtained where the alga cultivated under light intensity of 1830 lux and 16:8 hrs light/dark regime. However oil content increased gradually with increasing light intensity under continuous light to reach the maximum value (0.12 g L^{-1}). The best nitrogen source and concentration that stimulate biomass downloading and oil production is 0.5 g L^{-1} urea. In regard to nitrogen limitation, algal growth shows different responses towards the effects of nitrogen limitation. However, oil content of the starved algal cells was increased after adding urea with maximimum concentration of 0.26 g L^{-1}. Furthermore, algal cell number, biomass and oil content were increased under cultivation with sodium acetate and light conditions indicating that this organism can grow better mixotrophically rather than heterotrophically.

Key words: Biodiesel, Bio oil, Fatty acid, Cultivation conditions, *Nannochloropsis salina*.

The concept of microalgae biomass production for conversion to fuels was first suggested in the early 1950s[1]. In view of the fact that algae have high photosynthetic efficiency, high biomass productivities, a faster growth rate than higher plants, highest CO_2 fixation and O_2 production, growing in liquid medium which can be handled easily, can be grown in variable climates and non-arable land including marginal areas unsuitable for agricultural purposes (e.g. desert and seashore lands), in non-potable water or even as a waste treatment purpose, use far less water than traditional crops, and do not displace food crop cultures; their production is not seasonal and can be harvested daily[2,3]. However, not all microalgae strains are good candidates for biodiesel production purposes. The characteristics deemed necessary for an organism to be considered for biodiesel fuel production as follow: 1- Exhibit rapid growth rate. 2- Have appreciable lipid yields. 3- Demonstrate tolerance for the climatic conditions. 4- Possess a life cycle conductive to continuous culturing[4].

Nannochloropsis algae have relative high intracellular lipid content[5,6,7,8] in comparison to other microalgae. Therefore, the genus is a good source for biofuel production[8,9,10]. In addition, *Nannochloropsis* showed a high tolerance against many different environmental stresses[11-14]. However, the amount of intracellular triglycerides of *N. salina* is relatively low[15].

In the framework to optimize biotechnological aspects involved in mass

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production of *Nannochloropsis salina* to enhance biomass and oil production, the algal cultivation was optimized under laboratory conditions, using different light intensities and light regimes, different nitrogen sources and concentrations, cultivation under different growth mode and nitrogen starvation stress were also applied.

MATERIALS AND METHODS

Biological material and culture maintenance

The eustigmatophyte *Nannochloropsis salina* Hibberd was obtained from the culture collection at phycology lab, Faculty of Science, Alexandria University. *Nannochloropsis salina* was grown batch-wise in BES (Boussiba's Enriched Seawater)[16]. Four light intensities were applied through 1, 2, 3, and 4 1m cool white fluorescent lamps measuring 740, 1000, 1830 and 2650 lux, respectively. The irradiance of these lamps was measured by HI 97500 Hanna portable Luxmeters. The light regimes (12: 12 hrs light/dark; 16: 8 hrs light/dark, and 24 hrs of continuous light) were applied.

Growth measurements

The growth of the examined alga was determined daily by cell count with haemocytometer slide using light microscopy. At least five replicas were taken to get the mean number of cells per ml culture. The growth rate coefficient (r) was calculated according the equation[17]:

$$r = \ln x_1 - \ln x_0 / t_1 - t_0$$

Where:

x_0 and x_1 = the number of cells at time t_0, and t_1, respectively

r = the cell division per day

For biomass estimation, the optical density of algal suspension was determined spectrophotometrically at 625 nm, using Perkin Elmer Spectrophotometer, Lambada l. Biomass concentration was calculated accordingly[18].

Nitrogen source and concentration

Three different nitrogen sources (Potassium nitrate, Urea and Ammonium sulphate) were added in a concentration of 0.5, 1.0 and 1.5 g L^{-1} respectively.

Nitrogen limitation

The axenic cultures were grown in nitrogen limited-medium. After 7 days of nitrogen limited medium, a concentration of 0.5 g L^{-1} of above nitrogen sources used was added.

Carbon source

Two sets of axenic cultures were cultivated for 14 days with13.6 g L^{-1} of Sodium acetate, as a carbon source. The first set was grown mixotrophically under light intensity of 1830 lux and 16: 8 hrs light/dark regime. However, the second set was grown heterotrophically, in complete darkness.

Lipid and oil extraction

At the end of the logarithmic growth phase (day 14), the cells were harvested by centrifugation and the lipid content was extracted with chloroform/methanol (2:1, v/v)[19]. On the other hand, algal oil was extracted using *n*-hexane[20].The average weight of total lipid and oil was calculated.

Fatty acid composition of algal oil

The crude oil, extracted from controlled cultures, was esterified[21] and analyzed in a Shimadzu gas-liquid chromatography (Kyoto, Japan), equipped with a flame ionization detector and Hp-5 column material (Agilent, Santa Clara, CA, USA). The carrier gas was nitrogen and the flow rate was 5 mm/min. Identification of the FAMEs (fatty acid methyl esters) was carried out by comparing their retention times with those of standards. Quantification was based on the internal standard method.

Statistical analysis

The standard deviation (SD) of three replicas was calculated. In addition, T-test was also applied to data obtained.

RESULTS

Fatty acid methyl esters of algal oil

The composition of algal oil fatty acid methyl esters (biodiesel) of *Nannochloropsis salina*, grown under the basal conditions, is presented in (Table 1). Both saturated and unsaturated components were detected, besides some unknowns. The unsaturated fractions were identified as C16:1 (palmitoleic acid methyl ester, 16.7%), C18:1 (oleic acid methyl ester, 12.9%), C18:2 (linoleic acid methyl ester, 12.8%), C18:3 (linolenic acid methyl ester, 12%), C20:1 (gadoleic acid methyl ester, 9%), and C20:5 (eicosapentaenoic acid, EPA, methyl ester, 13.1%). On the other hand, the saturated ones are composed of C14:0 (myristic acid methyl ester, 4%), C16:0 (palmitic acid methyl

Table 1. Fatty acid methyl esters composition of *N. salina* grown under controlled batch conditions

FAME[a]	Content(%)[b]
C14:0 (myristic)	4
C16:0 (palmitic)	6
C16:1(palmitoleic)	16.7
C18:0 (stearic)	7.5
C18:1 (oleic)	12.9
C18:2 (linoleic)	12.8
C18:3 (linolenic)	12
C20:1(gadoleic)	9
C20:5 (EPA)	13.1
Unknowns	6

[a]Fatty acid methyl esters (biodiesel).
[b]Values obtained from three parallel measurements with SD±1 for C14:0 and C16:0, and ± 0.2 for the remaining fatty acids.

ester, 6%), and C18:0 (stearic acid methyl ester, 7.5%). The amount of unsaturated to saturated fatty acids was approximately 4.4. A detailed characterization of biodiesel from *N. salina* was described[15].

Effect of different light intensities on growth of *Nannochloropsis salina*

Data at Figure 1 showed a linearly increasing in the cell number of *N. salina* with increasing time till the day 15 from the beginning of culturing, where the organism entered its stationary growth phase. The cell number of *N. salina* increased progressively with increasing the light intensities from 740 lux to 2650 lux. The maximum increase was recorded at 1830 lux, followed by 2650 lux then 1000 lux. On the other hand the cell number recorded its maximum value under the light intensity of 1830 lux (14.17×10^6 cells/ml) after 15 days.

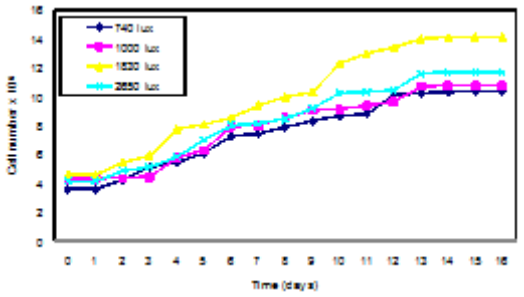

Fig. 1. Effect of different light intensities on the cell number of *N. salina* cultured under laboratory batch conditions

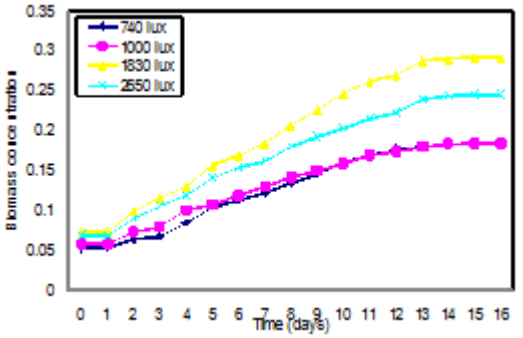

Fig. 2. Effect of different light intensities on the biomass concentration of *N. salina* cultured under laboratory batch conditions

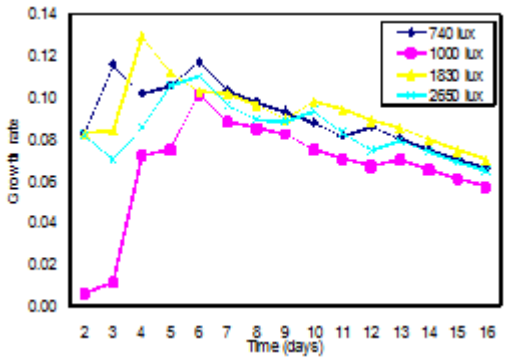

Fig. 3. Effect of different light intensities on the growth rate coefficient of *N. salina* cultured under laboratory batch conditions

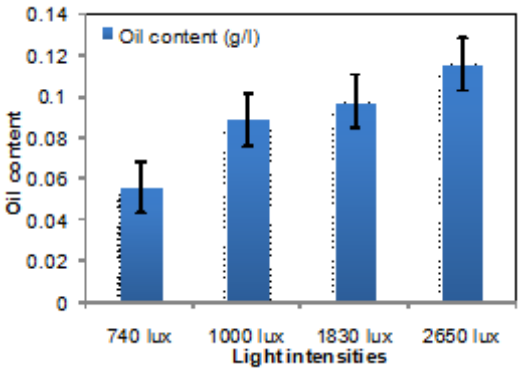

Fig. 4. Effect of different light intensities on oil content (g L^{-1}) of *N. salina* cultured for 14 days under laboratory batch conditions

Biomass based data (g L^{-1}) of *N. salina* were recorded in Figure 2. It is noticed that the biomass increased progressively with increasing the light intensities from 740 lux to 2650 lux, with maximum increase under 1830 lux followed by 2650 and 1000 lux respectively. On the other hand the biomass concentration recorded its maximum value under the light intensity of 1830 lux (0.291 g L^{-1}) after 15 days.

The influence of applied light intensities on the growth rate of the organism was shown in Figure 3. The maximum growth rate was recorded at 740, 1000 and 2650 lux after 6 days from the beginning of culturing. These values were 0.12, 0.10 and 0.11 respectively. Meanwhile, the maximum growth rate at 1830 lux (0.13) was recorded after 4 days from culturing. It is remarkable to mention that this maximum growth rate that recorded at 1830 lux was the maximum one of the four light intensities.

Effect of light intensities on oil production of *Nannochloropsis salina*

Data represent the effect of different light intensities (740, 1000, 1830 and 2650 lux) on the oil content of *N. salina* were shown in Figure 4. These data cleared that oil content increased gradually with the increase of the light intensity. It can be noticed that the maximum value of oil content (0.116 g L^{-1}) was recorded when the alga was cultured under light intensity of 2650 lux.

Effect of different light regimes on the cell number of *Nannochloropsis salina* cultured under laboratory batch conditions

The effects of different light regimes on cell number of *N. salina* cultured under light intensity of 1830 lux were recorded in Figure 5. Generally the cell number of *N. salina* increased gradually with increasing time till the day 15, followed by a decrease in cell number with increasing the light period. On the other hand the

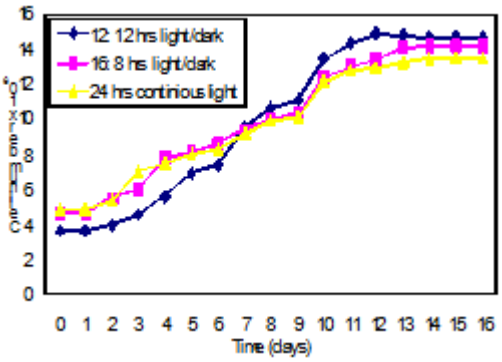

Fig. 5. Effect of different light regimes on the cell number of *N. salina* cultured under laboratory batch conditions

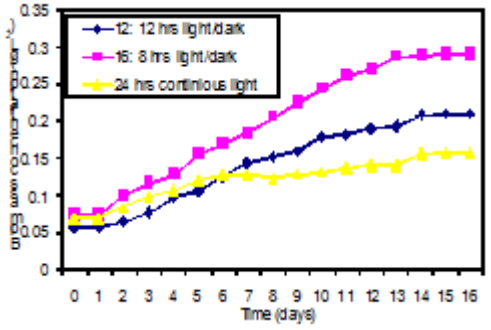

Fig. 6. Effect of different light regimes on the biomass concentration (g L^{-1}) of *N. salina* cultured under laboratory batch conditions

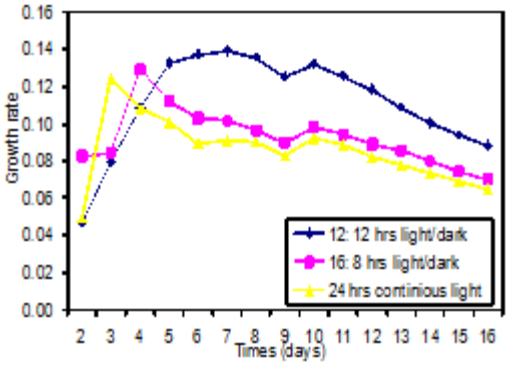

Fig. 7. Effect of different light regimes on the growth rate coefficient/day of *N. salina* cultured under laboratory batch conditions.

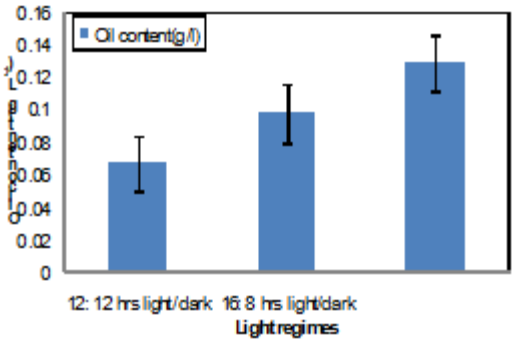

Fig. 8. Effect of different light regimes on oil content (g L^{-1}) of *N. salina* cultured for 14 days under laboratory batch conditions

cell number recorded its maximum value under the light regime of 12: 12 hrs light/dark (14.86×10^6 cells/ml) at the day 12.

Data obtained from the effect of different light regimes on the biomass concentration (g L^{-1}) of *N. salina* cultured under light intensity of 1830 lux were shown in Figure 6. The biomass concentration of *N. salina* increased gradually with increasing time under all used light regimes tested till the day 15, and then no increase was noticed. Data obviously showed that *N. salina* preferred light regime of 16: 8 hrs light/dark, where the biomass concentration recorded its maximum value (0.291 g L^{-1}).

Effect of different light regimes on the growth rate coefficient/day of *Nannochloropsis salina* cultured under laboratory batch conditions

Results (Figure 7) showed that under 12: 12 hrs light/dark regime the growth rate recorded its maximum value (0.14) after 7 days. Under 16: 8 hrs light/dark regime, the growth rate recorded its maximum value (0.13) after 4 days from culturing. Meanwhile, less than 24 hrs of continuous light, the growth rate recorded its maximum value (0.12) after 3 days. It is noteworthy to mention that the maximum growth rate that recorded at 12: 12 hrs light/dark regime was the maximum one of the three light regimes.

The effect of different light regimes on the oil content of *N. salina* was recorded in Figure 8. It is clear that oil content increased gradually with increasing the light period. Maximum oil content (0.129 g L^{-1}) was obtained at the continuous light.

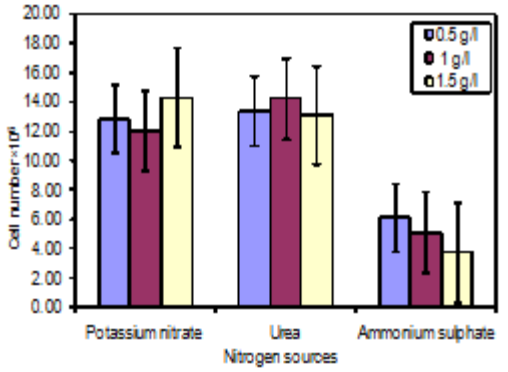

Fig. 9. Effect of different nitrogen sources on the cell number of *N. salina* cultured for 14 days under light intensity of 1830 lux and light regime of 16: 8 hrs light/dark

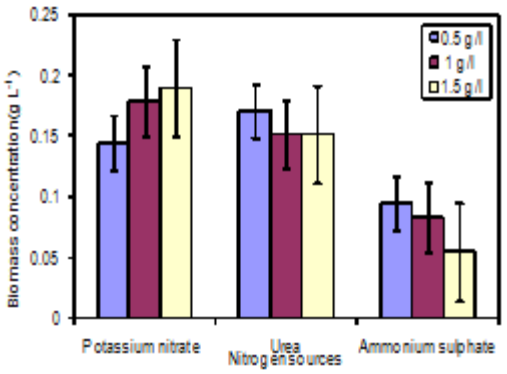

Fig. 10. Effect of different nitrogen sources on the biomass concentration of *N. salina* cultured for 14 days under light intensity of 1830 lux and light regime of 16: 8 hrs light/dark

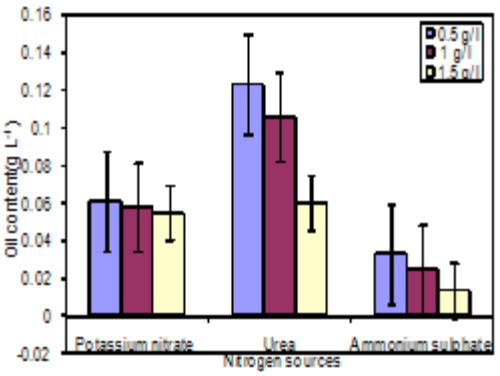

Fig. 11. Effect of different nitrogen sources on oil content of *N. salina* cultured for 14 days under light intensity of 1830 lux and light regime of 16: 8 hrs light/dark.

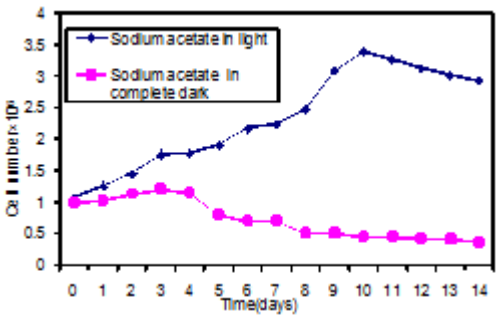

Fig. 12. Effect of sodium acetate on the cell number of *N. salina* grown under light regime of 16: 8 hrs light/dark and light intensity of 1830 lux and under a complete darkness

Effect of different nitrogen sources on the cell number of *Nannochloropsis salina* cultured for 14 days under light intensity of 1830 lux and light regime of 16: 8 hrs light/ dark

Results obtained for cell number of *N. salina* were recorded in Figure 9. Data obtained showed that increase of potassium nitrate concentration from 0.5 to 1.0 g L^{-1} resulted in a decrease in the cell number (12.89 to 12.04×10^6cells/ml). On the other hand, further increase of potassium nitrate concentration to 1.5 g L^{-1} led to the increase in the cell number (14.32×10^6cells/ml). Regarding the effect of different concentrations of urea on the growth of *N. salina*, both 0.5 and 1.5 g L^{-1} showed lower cell numbers than 1.0 g L^{-1}. However, the cell number decreased gradually with increasing the concentration of ammonium sulphate from 0.5 to 1.0 and 1.5 g L^{-1}. On the other hand, the cell number recorded its maximum value (14.32×10^6 cells) in the culture fed with 1.5 g L^{-1} of potassium nitrate.

Effect of different nitrogen sources on biomass of *Nannochloropsis salina*

Data represent the effect of different nitrogen sources and concentrations on the biomass concentration of *N. salina* were recorded in Figure 10. These experimental data showed that the increase in potassium nitrate concentration from 0.5 to 1.5 g L^{-1} led to gradual increase in the biomass concentration. However, the biomass concentration decreased gradually by increasing the concentration of both urea and ammonium sulphate from 0.5 to 1.5 g L^{-1}. The results also showed that the biomass concentration recorded its minimum value (0.055 g L^{-1}) when the culture was fed with 1.5 g L^{-1} of ammonium sulphate. On the other hand, the maximum biomass concentration (0.190 g L^{-1}) was noticed in the culture fed with 1.5 g L^{-1} of potassium nitrate.

Data obtained from the effect of different nitrogen sources with different concentrations on oil content (g L^{-1}) of *N. salina* were recorded in Figure 11. It can be noticed that the oil content decreased gradually with the increase of the concentrations of the three used nitrogen sources, potassium nitrate, urea and ammonium sulphate, from 0.5 to 1.5 g L^{-1}. However the oil content recorded its maximum value (0.123 g L^{-1}) in the culture fed with 0.5 g L^{-1} of urea.

In the absence of nitrogen the cell number increased gradually till the 6th day (144 hrs), where it reached 1.43×10^6cells/ml, and then it decreased at the 7th day (168 hrs) to be 1.4×10^6 cells/ml (Table 2). With addition of potassium nitrate, urea and ammonium sulphate to the culture of nitrogen-free medium after the 168 hrs the cell number increased progressively till 194 hrs in all used nitrogen sources. It is also clear that the cell number recorded its maximum value (4.38×10^6 cells/ml) after 24 hrs of addition of potassium nitrate. After 28 hrs from addition of the three nitrogen sources, the cell number showed a large drop to reach its minimum value (2.23×10^6 cells/ml) in the case of potassium nitrate.

However, biomass concentration (Table 3) of *N. salina* increased gradually with time when it was cultured in nitrogen-free medium until the 7th day (168 hrs). After 2 hours from the addition of both potassium nitrate and urea at the 7th day, the biomass decreased by 0.005 g L^{-1} (Table 3), this decrease was continued for the next 12 hrs and 24 hrs compared to the last day of nitrogen limitation (7th day). No change in the biomass concentration value of the alga was noticed after 2 hours in the case of addition of ammonium sulphate. However, when the alga was allowed to grow for longer time in the presence of this nitrogen source, its biomass increased gradually for the next 48 hrs to reach its maximum value (0.043 g L^{-1}).

The results obtained for the effect of nitrogen limitation on the oil content (Table 4) showed that oil content increased with the addition of urea to reach its maximum value (0.261 g L^{-1}). Meanwhile, the addition of either potassium nitrate or ammonium sulphate led to the decrease in the oil content of the alga. The maximum decrement (0.068 g L^{-1}) was recorded after 2 hrs from the addition of ammonium sulphate.

Effect of sodium acetate on cell number

Nannochloropsis salina cultivated with sodium acetate was exposed to light and complete darkness conditions. The cell number, under light (16: 8 hrs light/dark and light intensity of 1830 lux) and complete darkness were recorded in Figure 12. The cell concentration increased gradually with increasing time till the 10th day then decreased in culture fed with sodium acetate under 16: 8hrs light/ dark and light intensity of 1830 lux. On the other hand, the cell number of *N. salina* increased gradually with the increase of time till the 4th day

then decreased in culture fed with sodium acetate grown in complete darkness.

Effect of sodium acetate on biomass

Concerning biomass concentration (Figure 13), the results increased gradually with time till the 10th day then decreased under both light and complete dark conditions. On the other hand, the biomass concentration showed higher

Table 2. Effect of nitrogen starvation on the cell number of *N. salina* cultured for 7 days (168 hrs) with nitrogen free-medium, then adding 0.5 g L^{-1} of three nitrogen sources for four times intervals

Treatments	Time (hours)	Cell number ×10^6/ml		
		Potassium nitrate	Urea	Ammonium sulphate
Nitrogen starvation	0	0.63	0.63	0.63
	24	0.63	0.63	0.63
	48	0.74	0.74	0.74
	72	1.30	1.30	1.30
	96	1.43	1.43	1.43
	120	1.43	1.43	1.43
	144	1.43	1.43	1.43
	168	1.40	1.40	1.40
0.5 g L^{-1}	170	1.58	1.86	2.06
	182	2.49	2.73	3.13
	194	4.38	3.75	4.36
	218	2.23	2.91	2.93
P*.value		0.295	0.045*	
			0.053*	

P*.value was considered significant at p≤0.05 probability level according to paired t-test

Table 3. Effect of nitrogen starvation on the biomass concentration of *N. salina* cultured for 7 days (168 hrs) with nitrogen free-medium, then adding 0.5 g L^{-1} of three nitrogen sources for four times intervals

Treatments	Time (hours)	Biomass concentration (g L^{-1})		
		Potassium nitrate	Urea	Ammonium sulphate
Nitrogen starvation	0	0.030	0.020	0.017
	24	0.030	0.020	0.017
	48	0.042	0.022	0.018
	72	0.037	0.033	0.021
	96	0.055	0.048	0.042
	120	0.059	0.067	0.047
	144	0.064	0.085	0.052
	168	0.090	0.094	0.076
0.5 g L^{-1}	170	-0.005	-0.005	0.000
	182	-0.004	-0.010	0.009
	194	-0.006	-0.009	0.028
	218	0.029	0.076	0.043
P*.value		0.375	0.202	
			0.165	

P*.value was considered significant at p≤0.05 probability level according to paired t-test.

- : means decrease from the value of the 7th day (168 hrs).

Table 4. Effect of nitrogen starvation on the oil contents (g L^{-1}) of *N. salina* cultured for 7 days (168 hrs) with nitrogen free-medium, then adding 0.5 g L^{-1} of three nitrogen sources for four times intervals

Treatments	Time	Oil content (g L^{-1})		
	(hours)	Potassium nitrate	Urea	Ammonium sulphate
Nitrogen starvation	168	0.04	0.007	0.08
0.5 g L^{-1}	170	-0.01	0.026	-0.068
	182	-0.02	0.082	-0.005
	194	-0.02	0.261	-0.004
	218	-0.02	0.251	-0.004
P*.value		0.023*	0.372	
			0.034*	

P*.value was considered significant at p≤0.05 probability level according to paired t-test

- : means decrease from the value of the 7th day (168 h).

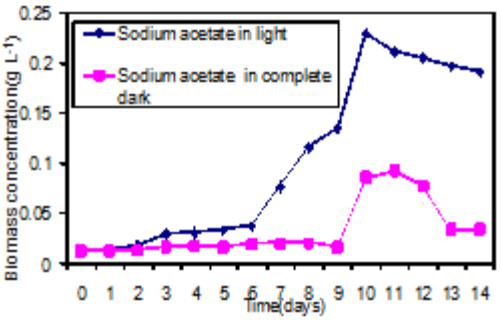

Fig. 13. Effect of sodium acetate on the biomass concentration of *N. salina* grown under light regime of 16: 8 hrs light/dark and light intensity of 1830 lux and under a complete darkness

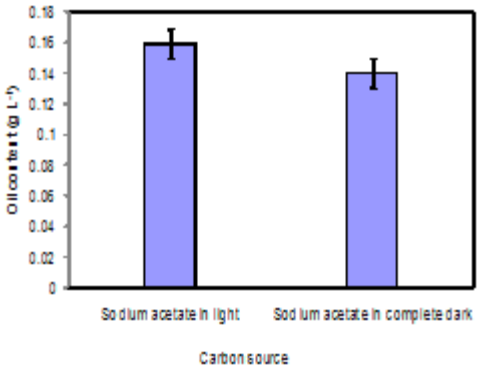

Fig. 14. Effect of sodium acetate on oil content of *N. salina* grown under light regime of 16: 8 hrs light/dark and light intensity of 1830 lux and under a complete darkness

values under mixotrophic conditions than under heterotrophic conditions. Also the biomass concentration reached maximum value (0.213 g L^{-1}) in the culture fed with sodium acetate under 16: 8 hrs light/dark and light intensity of 1830 lux.

Effect of sodium acetate on oil content

Oil content recorded its maximum value (0.159 g L^{-1}) in culture fed with sodium acetate under 16: 8hrs light/dark and light intensity of 1830 lux (Figure 14).

DISCUSSION

In the present work, fatty acid composition of *Nannochloropsis salina* was found to be in agreement with the general distribution pattern of fatty acids in other microalgae[22]. *N. salina* is described by many authors as a single cell lipid producer, the content of its TAG is relatively low[15]. For this reason, the study here looks to manipulate the cultivation conditions of *N. salina* to enhance the amount of oil production. The results here showed relatively lower values of *N. salina*'s biomass. The slowly growth of the tested strain may be lower than expected results due to not enough agitation or the carbon dioxide being bubbled through the culture, which was the case in previous[4,23,24]. However, there is an increasing response of biomass of *N. salina* towards different light intensities. A result goes in agreement with that obtained previously on *Nannochloropsis oculata*[25].

Results show a gradual increase of *N salina*'s oil content with increasing light intensity. Low light intensity causes the formation of polar lipids, particularly the membrane polar lipids that are associated with the chloroplast. However, high light intensity causes a decrease in the total polar lipid content with a concomitant increase in the amount of neutral storage lipids, mainly TAGs[26].

Exposure time of algal cell to light intensity, which is known as photoperiod, could affect its growth[23]. Adaptation of microalgae to fixed light regimes has received considerable attention and has provided detailed knowledge on the structure and functioning of their photosynthetic apparatus[27]. The optimal light/dark regimes have been found to vary from 12: 12 to 16: 8 hrs light/dark cycles for most algae cultures[23]. In this connection, growth of *N. salina*, expressed as cell number, shows a positive response under 12: 12 hrs light/dark regime cultivation, while the highest biomass was produced in cells grown under 16: 8 hrs light/dark regime. A result indicating that exposure of *N. salina* to 16: 8 hrs light/dark regime with intensity of 1830 lux may stimulate the synthesis of some cellular biochemical compounds. As mentioned above, TAG is the mainly storage material associated with downloaded biomass. Therefore, in spite of a slowing of cell division at 12: 12 hrs light/dark regime, biomass could still increase with light prolong time, due to net production of TAG[28]. On the other hand, the highest growth rate was found for cells grown under 12: 12 hrs light/dark regime at the first 7 days, a result goes parallel with data of cell population density, since the growth rate in this study was calculated according to the cell number of the organism. Responses of algal growth rate to illumination are species-specific and depend on both the average light intensity and the period of light exposure[29]. In this study, 1830 lux was found to be the best light intensity that accelerates cell division, biomass downloading and the growth rate of *N. salina*. However, the continuous light showed a negative effect on cell number, biomass and growth rate of *N. salina*. An observation which was previously explained[30], since over illumination can cause photo-inhibition by photo-oxidative stress on the algae. Therefore, we can postulate that the preferred light cycle for the growth of *N. salina* is16: 8 hrs light/dark regime, although the maximum growth rate was obtained at 12: 12 hrs light/dark cycle.

Although data in this study support the growth of the tested micralga under light/dark cycle, the production of the crude oil was stimulated in cells grown under continuous light. A result found in agreement with previous studies on other strains[31-34].

Reduction of biomass in cells growing under nitrogen limitation was previously explained[35]; it's a result of decreasing the intracellular chlorophyll content during the stationary growth phase. Decreasing in chlorophyll content in algal cell leads to minimize the size of the chloroplast[36]. Therefore, biomass yield is often inhibited in nitrogen-lacking situations[37].

Previous studies have long-established that lipid content in some micro algae strains could be increased by various cultivation conditions[38]. Among all the factors, nitrogen is known to have the strong influence on metabolism of lipids and fatty acids in various microalgae. In addition, nitrogen is easy to manipulate and is less expensive when compared to other factors. So it is critical to enhancing the lipid productivity for bio-fuel production[39]. Cells of *N. salina* fed with 0.5 g L^{-1} of urea gave approximately two and four folds of oil yield by the cells fed with the same concentration of nitrate and ammonium respectively. Therefore, we recommend urea as a nitrogen source for growing *N. salina* for biofuel purposes.

The lowermost growth values (cell density and biomass) of *N. salina* were detected in cells fed with ammonium sulphate. Declining of the growth in cells fed with ammonium sulphate was previously explained[40]. High concentration of ammonium may cause a drastic pH drop in the growth medium of microalgae. Such conditions can have detrimental effect on algal photosynthesis[40]. In addition, the salt in the medium may play role in creating osmotic pressure that could affect cell viability[41]. However, the highest cell number was detected in cultures fed with 1g L^{-1} urea and 1.5 g L^{-1} potassium nitrate, both gave approximately the same population density. Based on biomass, the maximum growth values were in cultures fed with 0.5 g L^{-1} urea and 1.5 g L^{-1} potassium nitrate. Therefore, we recommend using of 0.5 g L^{-1} urea, as the best nitrogen concentration for *N. salina*, for its low price. Urea can be used as a very efficient

nitrogen source for microalgae cultivation. Urea is very cheap compared to other nitrogenous nutrients available for microalgae which ultimately make it economically suitable for industrial production of microalgal fuel[42].

Nutrient limitation usually causes a decrease in cell division[43,44]. In this study, no correlation between the effect of nitrogen limitation on cell number and biomass as a function of the growth was noticed. Perhaps, as a result of limitation conditions for 7 days, dead and non-valid cells were taken into consideration during cell counting. Therefore, biomass data were considered as a growth determinant. The maximum biomass of *N. salina* was achieved in cells fed with urea after 4 days of starvation conditions. Again, urea is the preferred nitrogen substrate for the growth of *N. salina*.

In this study, the maximum oil amount (0.261 g L^{-1}) was extracted from the N-starved cells after 24 hrs of feeding with urea. A result agrees with other studies[45,46] and on microalgae *Phaeodactylum tricornutum, Chaetoceros sp., Isochrysis galbana, Nannochloris atomus*, *Tetraselmis sp. Gymnodinum sp*. and *C. vulgaris*.

Further studies reported that lipid content can be enhanced by imposing nitrogen starvation leading to decreased cell division, if division was blocked, the rate of neutral lipid utilization would be slower than the rate of synthesis, so triglycerides would accumulate in the cells[43].

The algal cells specific growth rates, biomass concentrations and biochemical compositions were significantly influenced by the nutritional modes[47]. Most microalgal strains are well grown under autotrophic growth conditions; however, some spp have the ability to increase its biomass and lipid production under heterotrophic conditions[48]. Our results showed that supplementation of exogenous carbon sources such as sodium acetate could result in an increase in cell number and biomass yield. Under such conditions, carbon fixation through photosynthesis is greatly reduced and intercellular carbon will be mainly derived from acetate assimilation via the glyoxylate cycle. In the meantime, starch will be degraded to glucose through the glycolytic pathway to produce pyruvate, and thus triacylglycerols[49].

Our data showed a significant increase of oil production by *N. salina* grown under photoautotrophic and mixotrophic with sodium acetate, compared with heterotrophic conditions. *Chlorella protothecoides* can grow under photoautotrophic, mixotrophic and heterotrophic conditions[50]. The highest lipid accumulation was obtained under heterotrophy[48]. In mixotrophic and heterotrophic culture, the lipid content was much higher than that in the autotrophic culture. The same result was concluded[47] previsouly on *Chlorella vulgaris*.

CONCLUSION

What was done previously at our lab demonstrates that *Nannochloropsis salina* is not a superior source for biodiesel manufacturing purposes; the amount of algal oil is relatively low, and the quality of biodiesel needs to be improved. To achieve our goal for enhancing the quantity of oil production, some of biochemical engineering work was carried out on *N. salina*. The response of algal cell showed positive results towards the cultivation under different stresses. Continuous high light intensity, feeding with urea as a nitrogen source, nitrogen limitation, mixotrophic nutritional mode; are all collectively enhancing the amount of oil produced by the examined strain. However, further applications of many other circumstances, e.g. mutation and other omics engineering, may positively contribute to our research. A compromise between high oil production while maintaining high cell division of *N. salina* is also an important target. In addition, we still need to correlate between the stress conditions applied in this study and the quality of biodiesel manufactured.

ACKNOWLEDGMENTS

This work was financially supported by the European Union, ENPI Program, grant number I-B/2.2/099.

REFERENCES

1. Meier, R.L. Biological cycles in the transformation of solar energy into useful fuels. In: *Solar Energy Research* (Daniels F and Duffie JA eds.). Wisconsin: Madison University, 1955;

pp 179–183.
2. Campbell, C.J. The coming oil crisis Multi-science Publishing Company and petro consultants S.A, Essex, England, 1997; pp 210.
3. Chisti, Y. Biodiesel from microalgae beats bioethanol. Trends in Biotechnology, 2008; 26:126-131.
4. Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. A look back at the U.S. Department of Energy's Aquatic Species Program: biodiesel from algae. In: Closeout Report NREL/TP-580-24190, U.S.Department of Energy's Office of Fuels Development, National Renewable Energy Laboratory, Golden, CO, 1998.
5. Suen, Y., Hubbard, J.S., Holzer, G., Tornabene, T.G. Total lipid production of the green-alga *Nannochloropsis* sp. QII under different nitrogen regimes. J. Phycol., 1987; **23**: 289-296.
6. Zou, N., Zhang, C.W., Cohen, Z., Richmond, A. Production of cell mass and eicosapentaenoic acid (EPA) in ultrahigh cell density cultures of *Nannochloropsis* sp. (Eustigmatophyceae). Eur. J. Phycol., 2000; **35**: 127-133.
7. Chiu, S.Y., Kao, C.Y., Tsai, M.T., Ong, S.C., Chen, C.H., Lin, C.S. Lipid accumulation and CO2 utilization of *Nannochloropsis oculata* in response to CO2 aeration. *Bioresour. Technol.*, 2009; **100**: 833-838.
8. Gouveia, L., Gouveia, A.C. Microalgae as a raw material for biofuels production. *J. Ind. Microbiol. Biotechnol.*, 2009; **36**: 269-274.
9. Rosenberg, J.N., Oyler, G.A., Wilkinson, L., Betenbaugh, M.J. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Curr. Opin. Biotechnol.*, 2008; **19**: 430-436.
10. Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.*, 2009; **102**: 100-112.
11. Lubián, L.M. Ultraestructura y pigmentos de algunas Chlorophyceae y Eustigmatophyceae planctonicas de morfologia similar. *Collectanea Botanica.*, 1982; **13**: 873-880.
12. Gonen-Zurgil, Y., Carmeli-Schwartz, Y., Sukenik, A. Selective effect of the herbicide DCMU on unicellular algae-a potential tool to maintain monoalgal mass culture of *Nannochloropsis*. *J. Appl. Phycol.*, 1996; **8**: 415-419.
13. Moreno-Garrido, I., Blasco, J., Gonzalez-Delvalle, M., Lubian, L.M. Differences in copper accumulation by the marine microalga *Nannochloropsis gaditana*, submitted to two different thermal treatments. *Ecotox. Environ. Restoration*, 1998; **1**: 43-47.
14. Mohammady, N.G., Chen, Y.C., El-Mahdy, A.A., Mohammad, R.F. Temporal alterations of *Nannochloropsis salina* (Eustigmatophyceae) grown under aqueous diesel fuel stress. *J. Appl. Phycol.*, 2005; **17**: 161-170.
15. Mohammady, N.G. Characterization of Fatty acid composition of *Nannocloropsis salina* as a determinant of biodiesel properties. Z. *Naturforsch.*, 2011; **66**: 328-332.
16. Boussiba, S., Vonshak, A., Cholen, Z., Avissar, Y., Richmond, A. Lipid and biomass production by halotolerant microalga *Nannochloropsis salina*. *Biomass.*, 1987; **12**: 37-47.
17. Wood, A., Everroad, Wingrad, L.M. Measuring growth rates in microalgae cultures. In: *Algal culturing techniques* (Andersen RA, ed). Amsterdam: Elsevier, 2005; pp 429-538.
18. Mirón, A.S., Garcia, C., Camacho, F.G., Grima, E.M., Chisti, Y. Growth and biochemical characterization of microalgal biomass produced in bubble column and airlift photobioreactors: studies in fed-batch culture. *Enzyme Microb Technol.*, 2002; **31**: 1015-1023.
19. Bligh, E.G., Dyer, W.M. Rapid method for lipid extraction. *Can. J. Biochem. Physiol.*, 1959; **35**: 911-915.
20. Miao, X., Wu, Q. Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol.*, 2006; **97**: 841-846.
21. Radwan, S.S. Sources of C20-polyunsaturated fatty acids for biotechnological use. *Appl. Microbiol. Biotechnol.*, 1991; **35**: 421-430.
22. Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., Xian, M. Biodiesel production from oleaginous microorganisms. *Renew. Energ.*, 2009; **34**: 1-5.
23. Andersen, R.A. Algal culturing techniques. San Diego: Elseveier Academic Press, 2005.
24. Chisti, Y. Biodiesel from microalgae. Biotechnol. Adv., 2007; 25: 294-306.
25. Banerjee, S., Hew, W., Khatoon, H., Shariff, M., Yusoff, F. Growth and proximate composition of tropical marine *Chaetoceros calcitrans* and *Nannochloropsis oculata* cultured outdoors and under laboratory conditions. *Afr. J. Biotechnol.*, 2011; **10**: 1375-1383.
26. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.*, 2008; 54: 621-639.
27. Falkowski, P.G., Laroche, J. Acclimation to spectral irradiance in algae. *J. Phycol*., 1991; **27**: 8-14.
28. Khozin-Goldberg, I., Bigogno, C., Shreshta, P., Cohen, Z. Nitrogen starvation induces the

accumulation of arachidonic acid in the freshwater green alga *Parietochloris incisa* (Trebouxiophyceae). *J. Phycol.*, 2002; **38**: 991-994.

29. Litchman, E. Growth rates of phytoplankton under fluctuating light. *Freshwater Biol.*, 2000; **44**: 223-235.
30. Leon, R., Galvan, D. Interaction between saline stress and photo inhibition of photosynthesis in the freshwater green algae *Chlamydomonas reinhardtii*. Implication for glycerol photoproduction. *Plant Physiol. Biochem.*, 1999; **37**: 623-628.
31. Sukenik, A., Carmeli, Y., Berner, T. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. *J. Phycol.*, 1989; **25**: 686-692.
32. Napolitano, G.E. The relationship of lipids with light and chlorophyll measurement in freshwater algae and periphyton. *J. Phycol.*, 1994; **30**: 943-950.
33. Brown, M.R., Dunstan, G.A., Norwood, S.J., Miller, K.A. Effects of harvest stage and light on the biochemical composition of the diatom *T. pseudonana*. *J. Phycol.*, 1996; **32**: 64-73.
34. Khotimchenko, S.V., Yakovleva, I.M. Lipid composition of the red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance. *Phytochemistry.*, 2005; **66**: 73-79.
35. Yamaberi, K., Takagi, M., Yoshida, T. Nitrogen depletion for intracellular triglyceride accumulation to enhance liquefaction yield of marine microalgal cells into a fuel oil. *J. Mar. Biotechnol.*, 1998; **6**: 44-48.
36. Vesk, M., Jeffre, S.W. Effect of blue-green light on photo synthetic pigments and chloroplast structure in unicellular marine algae from six classes. *J. Phycol.*, 1977; **13**: 280-288.
37. Shen, Y., Pei, Z., Yuan, W., Mao, E. Effect of nitrogen and extraction method on algae lipid yield. *Int. J. Agric. and Biol. Eng.*, 2009; **2**: 51-57.
38. Illman, A.M., Scragg, A.H., Shales, S.W. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme Microb. Technol.*, 2000; **27**: 631-635.
39. Takagi, M., Watanbe, K., Yamaberi, Yoshida, T. Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris* sp. UTEX LB 1999. *Appl. Microbiol. Biotechnol*., 2000; **54**: 112-117.
40. Tarn, N.F.Y., Wong, Y.S. Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. *Bioresour. Technol.*, 1996; **57**: 45-50.
41. Schuldiner, S., Rotenberg, H., Avron, M. Determination of change in pH in chloroplasts. *Eur. J. Biochem.*, 1972; **25**: 64-70.
42. Goswami, R., Kalita, M. *Scenedesmus dimorphus* and *Scenedesmus quadricauda*: two potent indigenous microalgae strains for biomass production and CO_2 mitigation - A study on their growth behavior and lipid productivity under different concentration of urea as nitrogen source. *J. Algal Biomass Utln*., 2011; **2**: 42-49.
43. Takagi, M., Karseno, Yoshida, T. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* Cells. *J. Biosci. Bioeng.*, 2006; **101**: 223-226.
44. Mohammady, N.G., Rieken, C.W., Lindell, S.R., Reddy, C.M., Taha, H.M., Ling Lau, C.P., Carmichael, C.A. Age of nitrogen deficient microalgal cells is a key factor for maximizing lipid content. *Res. J. Phytochem.*, 2012; **6**: 42-53.
45. Reitan, K.I., Rainuzzo, J.R., Olsen, Y. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. *J. Phycol.*, 1994; **30**: 972-979.
46. Widjaja, A., Chien, C.C., Yi-Shu. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *J. Tai Inst. Chem. Eng*., 2009; **40**: 13-20.
47. Kong, W., Song, H., Cao, Y., Yang, H., Hua, S., Xia, C. The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation. *Afr. J. Biotechnol.*, 2011; **10**: 11620-11630.
48. Miao, X., Wu, Q. High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. *J. Biotechnol*., 2004; **110**: 85-93.
49. Lin, L.P., Chen, T. Factors affecting the mixotrophic maximum growth of *Chlorella pyrenoidosa*. *J. Chin. Agric. Chem. Soc.*, 1994; **32**: 91-102.
50. Jiménez-Ruiz, N., Cerón-García, M.D.C., Sanchez-Mirón, A., Belarbi-Haftalaui, E.H., García-Camacho, F., Molina-Grima, E. Lipids accumulation in *Chlorella protothecoides* through mixotrophic and heterotrophic cultures for biodiesel production. *New Biotechnol.* 2009; **25**: S266.