Effect of Different Nutritional Stresses on Growth, Biopigments and Lipid Content of Dunaliella Salina Teod

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ABSTARCT

The need to supply mineral nutrients and other growth requirements to algae in culture has thus been known for a long time. Microalgae are autotrophic microorganisms, which utilize light energy and inorganic nutrients and synthesize valuable biomass compounds, such as pigments, lipids, proteins, carbohydrates etc. For autotrophic growth the supply of approximate 30 elements is important and the review by Kaplan et al. (1986) gives a comprehensive overview of this subject. Macronutrients are supplied at concentrations of g/l and the micronutrients in mg/l. Dunaliella require a number of macro and micro nutrients for its growth. Optimization of growth conditions requires determination of these requirements. The concentration of inorganic constituents i.e. Nitrogen (N), Phosphorus (P), Sulphur (S), Iron (Fe), Magnesium (Mg), Cobalt (Co), Zinc (Zn), Calcium (Ca), as well as Manganese (Mn), Silicon (Si) and Molybdenum (Mo) are considered to be required by microalga and not replaceable even in part by other elements (Arnon and Wessel, 1953; Wiessner, 1962 and O'kelley, 1968). Nitrogen (N), Sulphur (S), and Phosphorus (P) being essential macronutrients, have important roles in microalgae metabolism. Other elements are also required for growth of D.salina include K+, Ca2+, Mg2+, Cl-, Na+, chelated iron and trace elements. The ratios of Mg2+: Ca2+ and Cl : SO4 in the medium may also affect both growth and carotene synthesis (Ben-Amotz and Avron 1983). Dunaliella can tolerate a wide range of Mg2+: Ca2+ ratio (Borowitzka 1990). Chelated iron usually is added to the cultures in the form of FeCl3–EDTA or ferric citrate– EDTA. Borowitzka and Borowitzka (1987) showed that, compared with ferric citrate, FeCl3 increased more rapidly the initial growth rate of D. salina; however, the Fe-citrate finally gave a higher cell yield than FeCl3. Four microelements, including manganese, zinc, cobalt and copper are also necessary for optimal growth of Dunaliella; however, for most the time, there is no need to add these elements to the medium if it is composed of technical salt or seawater (Ben-Amotz and Avron 1989a).

KEYWORD: Biology,Botany, Bryology, Chimeras (Botany), Diseases and pests—Nutritional aspects, Life Sciences, Natural history Nutritional aspects, Plant and Animal Science, Plant Sciences

INTRODUCTION

Dunaliella is a photoautotroph, it can use only carbon dioxide and bicarbonate as inorganic carbon sources. The lack of an appropriate inorganic carbon source is the most common growth limiting factor under the conditions present in D. salina cultures such as the high salinity, increased pH and high temperature (Borowitzka and Borowitzka 1989). Being constituents of important primary metabolites, nitrogen, sulphur and phosphate represent for all algal cells essential macronutrients, hence their deficiency triggers a wide range of metabolic responses in microalgae. To investigate about the effects of mineral nutrients shortage in microalgal cells represent a valuable support for their rapidity of growth, simply culture system and high reproducibility of experiments. In microalgae, long- term nutrient deprivation can lead to the cell death preceded by autophagy, a self degrading process to recycle part of the cytoplasm including organelles. Although nutritional deficiencies determine in algal cells common adaptation strategies also. As a macroelement, Nitrogen (N) has a profound importance for microalgae metabolism and its limitation is compensated by radical changes in metabolic pathways. The best source of nitrogen for D.salina is nitrate. In present experiments KNO3 is added to the medium for optimal growth of the algae. On the other hand, limiting the nitrate is one of the most common ways for the reduction of growth rate leading to the induction of carotenoid production. However, prolonged nitrogen limitation in the culture can eventually lead to high mortality of cells as well as to serious reduction of carotenoids per unit culture volume. Further, Phosphorus in the forms of KH2PO4 or NaH2PO4 gives the best results (Gibor 1956). In open ponds, higher concentrations can inhibit growth because incidental presence of phosphate and calcium especially at pH higher than 8, can lead to Ca3PO4 precipitation and algal flocculation (Sukenik and Shelef 1984). Moreover, Dunaliella also

needs high concentrations (approximately 2 m.mol) of sulfate for maximal growth, but this is rarely needed to be added in commercial pond medium because natural water sources such as seawater or tapwater contain much higher contents of sulfate, around 30 m.mol) (Ben-Amotz and Avron 1989a). Changes in total N and S under Nutrient Deprivation, N- or S- deprivation causes a loss of photosynthetic capacity and a decrease in the cells chlorophyll content. As previously reported, chlorophyll content strongly decreased upon both S- and N-starvation.
All Nutrients stress conditions tested including nitrogen, phosphorus and sulfur starvation seemed to activate carotenoids production being nitrogen and sulfur starvation the conditions that drove major carotenoids accumulation. Nitrogen starvation resulted in the most effective condition to enhance carotenoids accumulation. Increasing interest on carotenoids production by microalgae is due to the important commercial applications of these natural compounds and to the market demand of carotenoids, especially for pharmaceutical and nutritional applications. Carotenoids have traditionally been commercialized as food additives including colorants, antioxidants and vitamins. Their protective ability against oxygen free radicals seems to be responsible for the therapeutic applications of carotenoids as degenerative diseases preventives, anti-cancer agents and immune-system stimulators, claimed by several studies. Indeed, the higher the stress intensity and as a result the slower the growth rate of the alga, the greater is the total amount of the light absorbed by the cell during one division cycle. This situation can lead to higher accumulation of β- carotene per cell. Further Mojaat et al. (2008 b) studied the effects of Fe2+ ions and organic carbon source on growth and carotenogenesis of Dunaliella salina. In their study, a significant increase in β-carotene contents per cell was observed, with a maximum value of 70 pg cell when the culture was supplemented with acetate and FeSO4. The approach might be a good alternative method for production of carotenoids by alga in photobioreactors after optimization. The lipid content in microalgae varies from about 1-85% of the dry weight and among other factors, it is also affected by the nutritional composition of the medium. Lipid accumulation in microalgae usually occurs during periods of environmental stress, including growth under nutrient-deficient conditions. Dunaliella species are known to respond to nitrogen starvation by increasing lipid production. Nutrient availability has a significant impact on growth and propagation of microalgae and broad effects on their lipid and Fatty acids composition. Environmental stress condition when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is sufficient light and CO2 available for photosynthesis. The supply of the limiting nutrients is eliminated under the state of nutrient starvation, where the growth of microalgae is affected, and the cells modify its physiological functions with accumulation of certain molecules to withstand in stress conditions. Nutrient starvation is one of the most widely used and applied lipid induction techniques in micro algal Triacylglycerol (TAG) production and has been reported for many species. For example, when the Diatom Stephanodiscus minutulus was grown under silicon, nitrogen or phosphorus limitation, an increase in TAG accumulation and a decrease of polar lipids were noticed in all of the nutrient- limited cultures. The major areas of interest in microalgae research were to determine which strains of algae are capable of producing high amounts of lipids, and what environmental conditions lead to the highest lipid yields. Many micro algal-species were observed to be capable of producing high amounts of lipids, it was also determined that higher lipid concentrations can be obtained in nitrogen limiting culture conditions. For instance Monallantus salina was reported to produce as much as 72% lipids in nitrogen-deficient conditions. So keeping above facts in mind the objective of this research study was to develop a procedure for optimum growth, biopigments and lipid content locally isolated D.salina, as a step towards to indicate enhancing carotenoids induction, and then for β-carotene extraction. This objective was achieved by evaluating the optimum growth, biopigments and Lipid content of D. salina under different nutritional stresses. In this study, D.salina cultures were transferred from low to high concentration of Nitrogen (nitrate), Phosphorus (phosphate) and Sulphur (sulphate) along with the control of the ASWM (ARTIFICIAL SEA WATER MEDIUM).

EFFECT OF NITROGENOUS NUTRIENTS (NITRATE) ON GROWTH, BIOPIGMENTS AND LIPID CONTENT OF DUNALIELLA SALINA

Nitrogen, which generally accounts for about 7-10% of cell dry weight, is an essential constituent of all structural and functional proteins in algal cells. In general, microalgae have a limited ability to produce nitrogen storage material when growing under nitrogen-suffient conditions (Simon, 1971).When microalgae are grown under nitrogen-limited conditions, the most striking effect is the active and specific degradation of phycobilisomes (Collier & Grossman, 1992). Nitrogen is one of the primary requirements of growth media for any microalgal cell. Nitrogen is mostly supplied as nitrate (NO3-), but often ammonia (NH4+) and urea are also used, with similar growth rates recorded (Kaplan et al., 1986). In present experiments KNO3 is added to the medium for optimal growth of the algae. But some studies shows that other nitrogen sources such as ammonium salts and urea are not appropriate because they can result in the death of the algae under certain conditions (Borowitzka 1990). It has been also shown that the use of NH4NO3 or (NH4)2CO3 as source of nitrogen has toxic effects on rapidly growing D. salina (Borowitzka and Borowitzka 1987). Absence of nitrogen or the starvation condition is considered as stress by the organisms. The algae ceased to divide when nitrogen was not supplied in the growth medium as nitrogen is the primary requirement for all the metabolic activities of the cell. Numerous studies show that the biosynthesis and accumulation of lipids is enhanced in nitrogen limited or deprived cultures of microalgae of various taxonomic groups. In contrast to the polar lipids of nitrogen-sufficient cells, neutral lipids in the form of triacylglycerols become the predominant components of lipids from nitrogen-depleted cells (Thompson, 1996). Yet, some algal species increase their carbohydrate rather than their lipid content under nitrogen depletion conditions,

e.g. many Dunaliella strains, in which large quantities of glycerol can be accumulated along with increased mono, di and polysaccharides under nitrogen deprived growth conditions (Borowitzka & Borowitzka, 1988). Whether synthesis of neutral lipids or carbohydrates under nitrogen-limited conditions is species-specific, and has physiological significance, is not clear. Within a single genus of Chlorella, for instance, some strains were found to accumulate large amounts of starch, under nitrogen starvation, whereas others accumulated neutral lipids instead (Richmond, 1986). When growing in nitrogen-limited environments, many algae also exhibit the accumulation of secondary carotenoids, which is frequently accompanied by a decline in the chlorophyll content of the cells. Dunaliella cells short of nitrogen produced more -carotene, as demonstrated by Ben-Amotz et al. (1982). According to research by Borowitzka et al. (1991), Haematococcus pluvialis is more likely to produce and accumulate astaxanthin and its acylesters when nitrogen levels are low. According to Zhekisheva et al. (2002), Haematococcus pluvialis produced five picograms of fatty acids, particularly oleic acid-rich triacylglycerols, for every picogram of astaxanthin under nitrogen depletion conditions, indicating that these two processes are connected and help the oil globules maintain the high astaxanthin esters content.

Experimental Metod

To evaluate nutritional stresses (nitrate) for the growth, biopigments and lipid content of D.salina, the microalga was grown in normal media(ASWM) further treated with excess concentration of nitrate (8 mM of KNO3), moderate concentration of nitrate (5mM of KNO3) and low concentration of nitrate /nitrogen starvation stress (2mM of KNO3). The inorganic media for the above experiment set up remain the same and it was ARTIFICIAL SEA WATER MEDIA (ASWM). Observations were performed every week over a period of five weeks. Experiment for each nitrate concentration was performed in triplicates. Growth was followed through optical density (OD), dry weight and growth rate. The biopigment of samples were estimated by the standard methods as described in the chapter “Materials and methods”. Cultures were analyzed for their growth, biopigment and lipid content every 5th day for a period of 25 days. During the process of growth, the cultures were shaken thrice in a day to avoid clumping and accelerate the growth process.

Experimental Observation

Effect of Nitrogenous nutrients (nitrate concentration) on growth of Dunaliella salina

Thus slightly high concentrations of nitrates supported the biomass, growth and chlorophyll content of the alga while reduced the carotenoids and lipid accumulation. And under nutrients deprivation negative effects exhibit on growth and total chlorophyll content while positive effects recorded on carotenoids accumulation and lipid contents. To reduce the negative influence of nitrate starvation on resulting growth, intermediate nitrate concentrations can be applied.

Table : Effect of different concentration of nitrate ongrowth of *Dunaliella salina*

Different concentration of nitrate (excess, moderate & low)				
Time	**Parameters**	**+N (8 mM)**	**N (5mM)**	**-N (2mM)**
Initial	O.D.	0.10	0.10	0.10
	D.W.(g/l)	0.120	0.120	0.120
	G.R.(div./day)	0.010	0.010	0.010
I week	O.D.	0.13	0.12	0.11
	D.W.(g/l)	0.216	0.155	0.128
	G.R.(div./day)	0.044	0.025	0.018
II week	O.D.	0.18	0.15	0.13
	D.W.(g/l)	0.373	0.252	0.130
	G.R.(div./day)	0.112	0.056	0.027
III week	O.D.	0.28	0.24	0.15
	D.W.(g/l)	0.458	0.317	0.116
	G.R.(div./day)	0.225	0.141	0.022
IV week	O.D.	0.36	0.33	0.14
	D.W.(g/l)	0.557	0.362	0.051
	G.R.(div./day)	0.266	0.175	0.015
V week	O.D.	0.42	0.38	0.10
	D.W.(g/l)	0.591	0.455	0.020
	G.R.(div./day)	0.320	0.252	0.012

O.D.- Optical density, D.W.- Dry weight(g/l), G.R.- Growth rate (divisions/day),
+N (Excess concentration of nitrate), N (Moderate conc. of nitrate) -N (Low conc. of nitr

Different concentration of Nitrate (excess +N, moderate N & low –N)			
Week	+N (8 mM)	N (5mM)	-N (2mM)
Initial	0.62±0.025	0.62±0.022	0.62±0.020
I week	1.3±0.022	1.22±0.025	0.68±0.018
II week	1.48±0.019	1.41±0.016	0.72±0.021
III week	1.52±0.014	1.46±0.024	0.44±0.015
IV week	1.65±0.026	1.54±0.018	0.22±0.022
V week	1.68±0.021	1.63±0.021	0.12±0.018

Table : Effect of different concentration of nitrate on totalCarotenoids (in %) in *D.salina*

Different concentration of Nitrate (excess +N, moderate N & low –N)			
Week	+N (8 mM)	N (5mM)	-N (2mM)
Initial	0.18±0.016	0.18±0.018	0.18±0.014
I week	0.566±0.022	0.414±0.024	0.620±0.021
II week	0.840±0.016	0.751±0.021	1.404±0.020
III week	1.535±0.014	0.971±0.023	2.266±0.018
IV week	2.266±0.027	1.427±0.030	2.941±0.024
V week	2.812±0.019	1.855±0.021	3.506±0.028

Table : Effect of different concentration of nitrate on total lipids(in %dcw) in *D.salina*

Different concentration of Nitrate (excess +N, moderate N & low–N)

Week	Excess concentration +N (8 mM)	Moderate concentration N (5mM)	Low concentration -N (2mM)
Initial	4.5±0.032	4.5±0.040	4.5±0.042
I week	6.82±0.028	5.71±0.042	8.22±0.033
II week	10.6±0.025	8.42±0.040	15.7±0.035
III week	16.3±0.033	13.4±0.028	24.2±0.036
IV week	21.7±0.041	17.5±0.035	28.6±0.028
V week	25.4±0.025	20.3±0.033	31.5±0.040

Dcw - Dry cell weight

CONCUSION

Such alterations are aimed to help balance efficiently the absorption of excitation energy and the production of reducing power (NADPH) and chemical energy (ATP) with their utilization for growth and cell maintenance. Inability to maintain this balance due to excess excitation of the photosynthetic reaction centers may result in the production of toxic oxygen species that may lead to photo- oxidative death. As implied, many of the stress responses and adaptive process are associated with the photosynthetic apparatus. Indeed, microalgae of different origins have a tendency, albeit with certain exceptions, to resemble each other in terms of cell composition, particularly in the relative amounts of crude protein, lipids, and carbohydrate that they contain when grown under more or less optimal growth conditions. For a single species, on the other hand, the variation in cell composition may differ many fold, according to the culture conditions under which it is grown. For example, Chlorella sp., Botryococcus braunii, and Dunaliella salina, which are all classified under Chlorophyceae, Volvocales, show typical biochemical composition: 30–50% proteins, 20–40% carbohydrate and 8–15% of lipids under favorable environmental conditions. These species, however, can accumulate under unfavorable environmental conditions up to 80% of fatty acids, 80% of hydrocarbons, and 40% of glycerol, respectively, on the basis of the dry weight.

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