Turk. J. Fish.& Aquat. Sci. 19(5), 399-407
http://doi.org/10.4194/1303-2712-v19_5_05

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Isolation, Molecular Identification and the Carotenogenesis Process of the Microalgae *Dunaliella salina* Strain *Duna*DZ1 Isolated from an Algerian Salt Lake

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Article History
Received 11 January 2018
Accepted 07 May 2018
First Online 15 May 2018

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Keywords
Dunaliella
Taxonomy
ITS
rbcL
Carotenoids

Abstract

Algeria has a high algal biodiversity, which is of great interest and should be exploited. Isolation of *Dunaliella* strain DunaDz1 from Sidi Ameur Lake in Algeria was carried out in this study. The aim of this research was to identify the isolated strain by morphological and molecular taxonomy using ITS and rbcL genes. The effect of two stress factors, salinity and nitrate starvation on the carotenoids accumulation in *Dunaliella* strain was investigated on f/2 and Johnson media. The results for both genes showed that strain DunaDz1 is part of a clade containing several strains of *D. salina*. The strain grew well in Johnson medium, with a cell number of 56.09 10^5 cell/ml and a chlorophyll *a* amount of 63.63 µg/ml. Salt stress lead to a noticeable increase in the total carotenoids levels of strain DunaDz1, 23.68 ± 0.12 and 23.15 ± 0.23 pg/cell, for f/2 and Johnson media, respectively. In nitrate starved media a lesser amount was recorded comparing with salt stress: 11.42 ± 0.22 and 16.2 ± 0.24 pg/cell, for f/2 and Johnson media, respectively. The combination of these two factors was more effective on f/2 medium than Johnson medium.

Introduction

Dunaliella is a unicellular, eukaryotic and halo-tolerant microalgae. This genus is grouped in the class *Chlorophyceae*, the order *Dunaliellales* and the family *Dunaliellaceae*. Cells are motile with two flagella. The main morphological characteristic of the *Dunaliella* genus is the lack of a rigid wall (Shariati & Hadi, 2011).

Molecular taxonomy is an advanced and reliable method for the characterization and differentiation of morphological plastic organisms (Preetha, John, Subin, & Vijayan, 2012; Olmos Soto, 2015). Identification of *Dunaliella* species/strains has already been the subject of morphological, physiological and molecular studies (Hexin *et al*., 2016; Kim, Ahn, Jeon, & Jin, 2017; Shaker, Morowvat, & Ghasemi, 2017). Internal Transcribed Spacer (ITS) regions have been frequently utilized for discrimination of genetic variation in green algae and to study the phylogeny and taxonomy of *Dunaliella*, as well as the ribulose-bisphosphate carboxylase (rbcL) gene (Coleman, Suarez, & Goff, 1994; González, Coleman, Gómez, & Montoya, 2001; Buchheim, Kirkwood, Buchheim, Verghese, & Henley, 2010). The ITS region is one of the more frequently utilized regions at the genus and species levels (Hejazi, Barzegari, Gharajeh, & Hejazi, 2010; Preetha *et al*., 2012). However, the main difficulty of performing phylogenetic analyses of the genus *Dunaliella* is the misinformation available at culture collections and GenBank regarding the identification of strains and sequences. The taxonomy of the whole genus *Dunaliella* should be re-examined (Assuncao *et al*., 2012).

Dunaliella salina (Dunal) Teodor. is known as a

famous β-carotene producer, and could accumulate the later up to 10% of its dry cell weights. β-carotene is one of the most precious nutraceuticals of both preventive and therapeutics importance in medicine and pharmacy. The pigment β-carotene is in high demand as an antioxidant and as a food coloring agent (Shaker *et al*., 2017). Other species belonging to the genus *Dunaliella* can also accumulate β-carotene as *D*. *parva* W. Lerche. and *D*. *pseudosalina* Massjuk & Radcz., but the amount is less than *D*. *salina* (Borowitzka & Siva, 2007).

Under stress conditions, such as following an osmotic shock, *Dunaliella* alter their cell diameter and become spherical by involving glycerol synthesis, and the cellular glycerol content change (Xu, Ibrahim, & Harvey, 2016). *D*. *salina* accumulates a large amount of β-carotene when subjected to adverse conditions, such as high salinity and nitrate deprivation, as well as other factors (e.g., high light intensity and temperature) (Hexin *et al*., 2016). In the carotenogenic species, numerous oily droplets of carotenoids accumulate in the inner thylakoid spaces, in the chloroplast, and/or in the cytoplasm. These usually form initially at the chloroplast periphery and then spread throughout the chloroplast as the carotenoid content increases (Borowitzka & Siva, 2007). The optimal conditions for carotenogenesis in *D*. *salina* are those that limit growth and include exposure to high salinity and nitrate depletion (Subramaniyan, Munuswamy, Chinnasamy, Sailendra, & Ramasamy, 2013). To deal with the problem of growth inhibition, a two-phase method is proposed and developed. This method consists of biomass accumulation in the first stage with low β-carotene to chlorophyll ratio; after which the culture is then transferred to the second stage, diluted to about one third and induced for carotenogenesis (Borowitzka & Borowitzka, 1990; Ben-Amotz, 1995).

However, microalgae, especially *Dunaliella*, are not investigated and characterized taxonomically from Algerian salt lakes. This research was conducted to isolate a strain of *Dunaliella*, to study the morphological characteristics, to identify the isolated strain by means of molecular tools, as well as studying the optimal conditions for carotenogenesis by high salinity, nitrate starvation, and the combination of these two factors on the isolated strain of *Dunaliella*.

Materials and Methods

Site Description and *Dunaliella* Isolation

Water sample was collected in 250 ml sterile flasks from Sidi Ameur sebkha (shallow salt lake), which is located between M'Sila and Djelfa provinces (latitude 35.27 °N, longitude 3.68 °E), in Algeria.

Multiple physical parameters of the water were analyzed: pH using Hanna pH meter, electrical conductivity (EC) with multi-parameters WTW Multi 340i, and salinity with a refractometer Hanna instruments RS100. Calcium, magnesium, sodium, potassium and chloride were analyzed with an autoanalyser SAN-system by reference to calibration curve. The method ISO 22743 (2006) was used to estimate the level of sulfate in water.

Samples were plated on f/2 medium (Guillard & Ryther, 1962) supplemented by 2% agar. The plates were incubated at 22±2°C and illuminated with white fluorescent light (120 µmol photons m^{-2} s^{-1}), with a 24 h photoperiod. The isolated colonies were streaked on a new plate until monoalgal culture was obtained. To eliminate bacterial contamination, a mixture of antibiotics (Sigma) was used: penicillin, streptomycin and neomycin, in the proportion of 50 mg, 50 mg and 100 mg per liter, respectively.

Morphological and Molecular Identification of *Dunaliella* Strain

The morphology of the isolated *Dunaliella* strain was examined under a light microscope (Leica) by using the software LAS EZ (Leica DM500). The morphological characteristics were studied referring to Browitzka & Siva (2007).

Genomic DNA from *Dunaliella* culture was isolated according to Chelex-100 (Biorad, USA) method (Richlen & Barber, 2005). The Chelex was used at 10%. The DNA extract was purified using Real Clean Spin Kit, RBMCS02, from Real laboratory. Purification was performed according to the manufacturer's instructions.

ITS and rbcL regions were amplified using primers ITS-AB28, ITS-TW81, rbcL-17 and rbcL-18 (Table 1), respectively (Goff, Moon, & Coleman, 1994; Nozaki *et*

Table 1. Primers used for PCR amplification

Amplified genes	Primer	Primer sequences	Annealing temperature (°C)
ITS	ITS-AB28 (F)*	GGGATCCGTTTCCGTAGGTGAACCTGC	69.5
	ITS-TW81(R)*	GGGATCCATATGCTTAAGTTCAGCGGGT	66.6
rbcL	rbcL-17 (F)	ATGGTTCCACCAACAGAAAAC	55.3
	rbcL-18 (R)	TGTGCTTTGTAAATAGCTTCAG	54.7

*(F) - forward; (R) - reverse.

al., 1995; Díez, Pedrós-Alió, Marsh, & Massana, 2001). Amplification reactions were performed on a Bio-Rad thermocycler. PCR amplification was carried out in a total volume of 25 µl, containing 19.5 µl H_2O, 1.87 µl PCR buffer, 2 µl dNTPs (2.5 mM of each), 0.125 µl *Taq* polymerase (Takara Extraq Hot Start Version, in 4.5 mM of $MgCl_2$), 1 µl DNA and 0.25 µl of each primer (25 pM). The PCR conditions were as follows for ITS: 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and 1 cycle of final extension at 72°C for 5 min. For rbcL, the PCR conditions were: 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s, and 1 cycle of final extension at 72°C for 5 min.

All PCR products were then checked by 2% agarose gel electrophoresis in 1X TAE buffer (Tris; acetic acid; EDTA), at 100 V, 400 mA, 250 W for 30 min, and the PCR products were checked with a UV illuminator, referring to molecular weight marker (100-2642 bp). An enzymatic method was used to purify the products using Illustra ExoProStar 1-Step from GE Healthcare Life Sciences. Sequencing was performed by Macrogen services, using Sanger's Dideoxy method. The sequences obtained were aligned with others sequences available from GenBank BLAST in the NCBI database, using ClustalW algorithm in MEGA version 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). A phylogenetic tree was constructed using neighbor-joining with the Tamura-Nei model. The trees topologies were evaluated by bootstrap analysis using 1000 resamplings. The trees were established using *Chlamydomonas reinhardtii* P.A. Dang. as an outgroup.

Growth Conditions

Two culture media were tested for the growth of *Dunaliella* strain: Johnson medium (Johnson, Johnson, Mac Elroy, Speer, & Bruff, 1968) and f/2 medium (Guillard & Ryther, 1962). These media contain 1 M NaCl. The pH was adjusted at 7.5, and the media were autoclaved at 121°C for 20 min.

Carotenogenesis Process

The carotenogenesis process was carried out in two phases. The first phase (growth phase) consisted of the accumulation of a high level of biomass, with low β-carotenes amounts by culturing the *Dunaliella* strain at optimal conditions. Tests were performed by inoculation of 20 ml of stock culture into 250 ml of f/2 and Johnson's media (1 M NaCl). They were incubated at 22 ± 2°C with continuous illumination given by white lamps (120 µmol photons m^{-2} s^{-1}), with injection of a filtrate air through a solid glass tube. When the culture was in a high level of biomass, it was used for carotenogenesis tests.

In the second phase (stress phase for carotenoids production), cultures at the end of the exponential phase were transferred into other media to induce cells for carotenogenesis as follows: media containing 2 M NaCl (salt stress), media without nitrate source (nitrate starvation) and media with 2 M NaCl and without nitrate source (combination of the two stress factors).

Growth and Pigments Measurements

Cells growth was estimated by cells number measured by direct counting using a light microscope with Malassez cell. Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and total carotenoids (car T) were estimated according to the Wellburn method (Wellburn, 1994), using methanol (100%) as a solvent, according to the equation cited below:

$$\text{Chl } a = 15.65 \text{ X (A666)} - 7.34 \text{ X (A653)}$$

$$\text{Chl } b = 27.05 \text{ X (A653)} - 11.21 \text{ X (A666)}$$

$$\text{Car T} = (1000) \text{ X A470} - 2.86 \text{ X Chl } a - 129.2 \text{ X Chl } b)/221$$

A: absorbance

All the data are presented graphically as mean ± standard deviation (n = 3).

Results

Physical and Chemical Characteristics of the Sampled Water

To evaluate the composition of the natural habitat of the genus *Dunaliella*, the physical and chemical characteristics of the water sampled from the studied lake were established (Table 2). pH ranged from 7.2 to 7.5, which is the optimum for the growth of microalgae belonging to the genus *Dunaliella*. This pH range is very close to that obtained by Boutaiba, Hacene, Bidle & Maupin-Furlow (2011), who found the pH of the same lake to be around 7.1 to 7.4. Electrical conductivity was 135.6 mS/cm. The lake has salinity ranging from 300 to

Table 2. Chemical and physical properties of water from Sidi Ameur lake

pH	EC (mS/cm)	Salinity	Ca^{+}	Mg^{2+}	Na^{+}	K^{+}	Cl^{-}	SO_4^{2-}
				Represented as g per liter (g/L)				
7.2-7.5	135.6	300- 350	0.85	2.75	40.25	0.125	158	24.7

350 g/l. The highest minerals in the water were sodium and chloride, 40.25 and 158.00 g/l, respectively. Sulfate level was 24.7 g/l. Potassium and calcium were the lowest minerals present in the water (0.125 and 0.850 g/l, respectively).

Morphological Characteristics of the Isolated Strain

The isolated microalgal strain was named DunaDz1. Cells exhibited a high degree of variability in size and shape, changing from ovoid to spherical shape with changing growth conditions, as well as different sizes. The strain showed a mobility due to the presence of two flagella. The cell size, length as well as width, were measured from the isolation medium (f/2 medium) containing 1 M NaCl, by averaging different repetitions. Average length and width were 17.2 ± 0.1 and 11.5 ± 0.3 µm, respectively. The strain DunaDz1 was assigned morphologically to *Dunaliella* genus referring to Borowitzka & Siva (2007).

Amplification, Sequences Analysis and Phylogenetic Study

ITS and rbcL genes used for DunaDZ1 strain were successfully amplified and gave a unique band on the electropherogram (Figure 1).

The obtained sequences were analyzed by BLAST and aligned with other *Dunaliella* sequences from GenBank. Great attention has to be given to the choice of this sequences, since the taxonomy of the green algal *Dunaliella* is often seen as confusing and the names associated with species in culture collections are sometimes suspect (Borowitzka & Siva, 2007).

Phylogenetic analysis of the ITS region (730 bp) showed that the strain DunaDZ1 was related to members of the genus *Dunaliella* and showed 98.9% identity (the highest percentage) with *D. salina* CCAP 19/12. Molecular investigation using ITS marker clustered strain DunaDZ1 with different strains of *D. salina* (Figure 2).

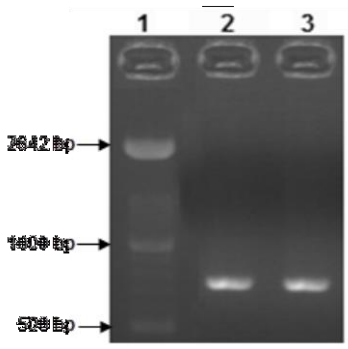

Figure 1. Agarose gel electrophoresis of PCR products amplified from the DNA of DunaDz1 strain. (1) Marker; (2) ITS gene and (3) rbcL gene.

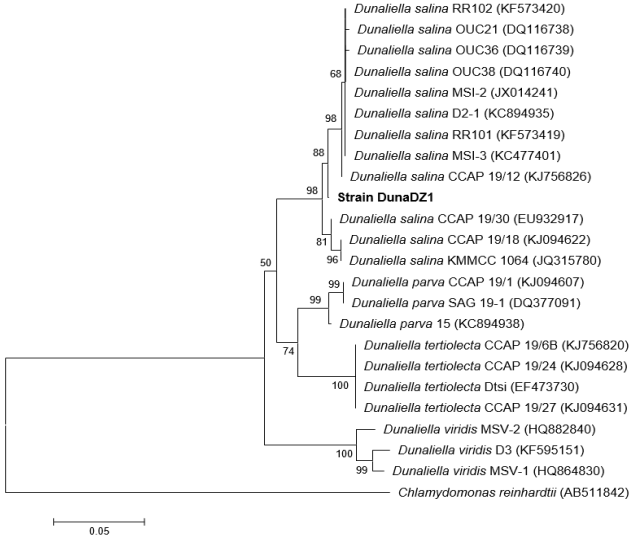

Figure 2. Neighbor-joining tree based on ITS gene sequences showing the position of strain DunaDZ1 and its related species of the genus *Dunaliella*. The numbers at the nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. *Chlamydomonas reinhardtii* was used as an outgroup. *Bar*, 0.05 substitutions per nucleotide position.

Primers used for rbcL produced a sequence of 700 bp. Strain *D. salina* DunaDZ1 was clustered for rbcL, as it was for ITS, with other strains of *D. salina* (97.9 to 100% of similarity) (Figure 3).

Carotenogenesis Process Results

Carotenogenesis testing in this study consisted of the investigation of the most appropriate stress factor for carotenoids production by the microalgae *D. salina* strain DunaDZ1. Two stress factors were tested: high salinity 2 M NaCl and nitrate starvation, as well as the combination of these two factors. These tests were conducted as it was described previously, in two phase culture strategy and two cultures media, Johnson and f/2 media.

Growth Phase: Cell Number, Chlorophyll (*a, b*) and Total Carotenoids

In Johnson medium, the maximum amounts of chlorophylls were 63.63 ± 0.01 µg/ml for chlorophyll *a* and 24.76 ± 0.13 µg/ml for chlorophyll *b*. The maximum cell number of *D. salina* strain DunaDZ1 was found to be 56.09 10^5 ± 0.8 cell/ml. This level was obtained between 12 and 15 days from the beginning of the tests (Figure 4). However, in f/2 medium, lower levels were found: chlorophyll *a* 21.97 ± 0.03 µg/ml, chlorophyll *b* 7.35 ± 0.8 µg/ml and maximum cell number 32.86 10^5 ± 0.9 cell/ml.

Carotenogenesis process in f/2 medium was accelerated, increasing from 13.44 ± 0.10 µg/ml on the 6th day of incubation to 28.58 ± 0.08 µg/ml on the 8th day, to achieve the high amount at the end of the exponential phase. This fact could be explained by nutrients depletion in the medium, since the initial nutrients concentration in f/2 medium was lower compared with Johnson medium. However, in Johnson medium, the carotenoids amount was 22.43 ± 0.07 µg/ml at the end of the growth phase, which corresponds to 4.54 ± 0.15 pg/cell. The ratio of total carotenoids to chlorophyll remained constant in Johnson medium (~0.30), but the ratio increased quickly in f/2 medium from 0.46 at the beginning to 2.11 at the end of the exponential phase (Figure 5). This is due to the high level of total carotenoids in the f/2 medium, because of nutrient depletion.

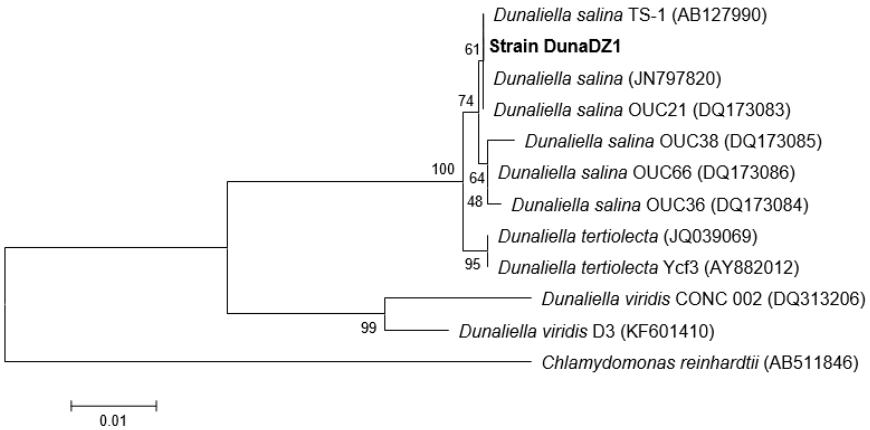

Figure 3. Neighbor-joining tree based on rbcL gene sequences showing the position of strain DunaDZ1 and its related species of the genus *Dunaliella*. The numbers at the nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. *Chlamydomonas reinhardtii* was used as an outgroup. *Bar*, 0.01 substitutions per nucleotide position.

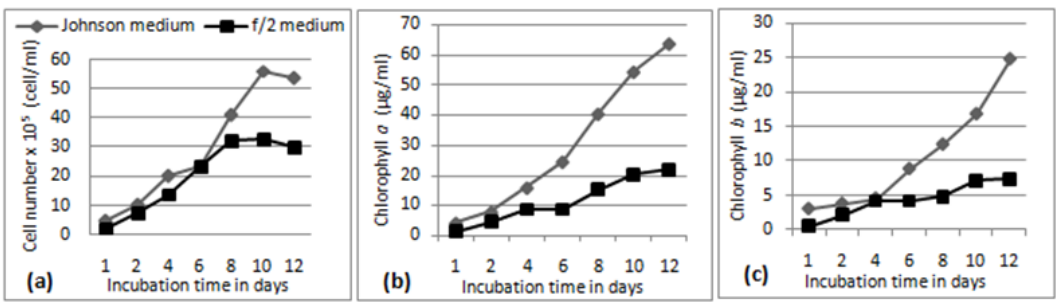

Figure 4. Growth phase and production of chlorophyll by the strain DunaDz1. (a) cell number. (b) chlorophyll *a*. (c) chlorophyll *b*.

Response to High Level of Salinity (NaCl 2 M) during Stress Phase

All stress factors tested led to an increase in carotenoids levels in *D. salina* culture. Induction of carotenoids production in one phase system retards growth and the level of carotenoids is low, that is why the use of two-phase growth strategy lead to a better accumulation of carotenoids (Borowitzka & Borowitzka, 1990).

If we want to induce the production of carotenoids directly by using the stress phase (without going through the growth phase), the growth of *D. salina* will be minimal and the total amount of carotenoids obtained will be low. This shows the need to use the two phases separately (Borowitzka & Borowitzka, 1990).

Cultures in Johnson and f/2 media were incubated at 2 M NaCl. The level of total carotenoids increased reaching 20.12 ± 0.44 and 23.68 ± 0.12 pg/cell, at the 8th day of incubation, for Johnson and f/2 media, respectively (Figure 6). These values were higher than those calculated at the end of the growth phase, which were 4.54 and 12.64 pg/cell, for Johnson and f/2 media, respectively. The effect of high salinity on the carotenoids accumulation is thus confirmed. Several authors found that high salinity was the best strategy to achieve optimal carotenoids production in the cultures of *D. salina*, with variable carotenoids concentrations: 8.9, 12.21, 12.47 and 15 pg/cell (Kleinegris, Janssen, Brandenburg, & Wijffels, 2010; Wu, Duangmanee, Zhao, Juntawong, & Ma, 2016). This difference might be due to culture conditions and to different *D. salina* strains studied.

Response to Nitrate Starvation during Stress Phase

Accumulation of carotenoids during the stress phase with nitrate starvation was also studied. Cultures about 12 days old were transferred to nitrate-free media. This strategy resulted in an increase in carotenoids amount in f/2 and Johnson media. Within 8 days of incubation in Johnson medium, the level of carotenoids was 16.2 ± 0.24 pg/cell (3-fold increase from the initial value before stress phase). Otherwise, for f/2 medium, carotenoids level increase slightly from 12.64 ± 0.14 pg/cell at the end of the growth phase to 13.21 ± 0.05 pg/cell on the 2nd day of stress, and then decreased until 10.92 ± 0.14 pg/cell (end of test) (Figure 6). This is due to the fack that the cell was

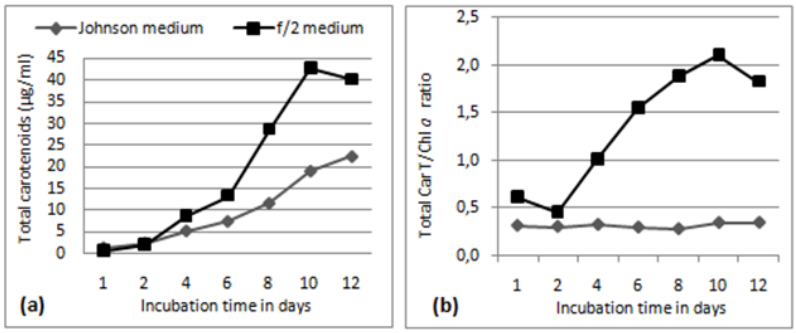

Figure 5. Carotenoids production during growth phase (without stress) by the strain DunaDZ1. (a) total carotenoids. (b) total carotenoids/chlorophyll *a* Ratio.

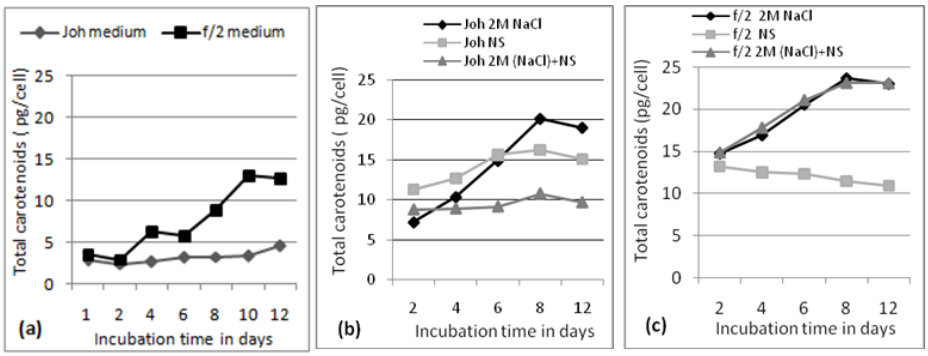

Figure 6. Total carotenoids expressed as pg per cell in the microalgae *D. salina* (DunaDZ1) during growth phase and stress phase. (a) Growth phase (without stress). (b) and (c) effect of various cultivation conditions [2M NaCl; nitrogen starved media (NS) and 2M NaCl + NS media], in Johnson (joh) and f/2 media, respectively. The values are mean of three replicates ± SD. The stress provoked by 2M NaCl and by nitrogen starvation was applied on the 12th day of growth of the strain DunaDZ1.

already stressed in the first phase by nutrient depletion, and it was subjected to nitrate starvation in the second phase.

According to some authors, a prolonged nutrient starvation leads to a considerable decrease in the growth of the microalgae, and also to a reduction in the production of carotenoids (Borowitzka & Borowitzka, 1988; Hosseini Tafreshi & Shariati, 2009).

Based upon it, the medium composition plays a great role in microalgal growth (Morowvat & Ghasemi, 2016), and subsequently affects carotenoids production. In this present study, the starvation strategy is more effective in Johnson than f/2 medium.

Response to High Level of Salinity Combined with Nitrate Starvation during the Stress Phase

High salinity (2 M) in combination with nitrate starvation also led to an increase in carotenoids concentration in Johnson medium, with lower values compared with each stress factor separately (10.76 ± 0.25 pg/cell after 8 days of incubation). The concentration increased 2 folds in Johnson medium. However, for f/2 medium the combination of salt and nitrate starvation stress gave very close carotenoids values (23.15 ± 0.23 pg/cell) compared with *D. salina* in culture with only salt stress (23.68 ± 0.12 pg/cell, on the 8th day) (Figure 6).

Discussion

The strain DunaDZ1 has different cell shapes, varies from ovoid to spherical, depending on culture conditions. This is in agreement with previous studies, which demonstrated that cells shape of *D. salina* turn to spherical in extreme conditions (Hosseinzadeh Gharajeh, Hejazi, Nazeri, & Barzegari, 2012; Preetha *et al*., 2012).

The amplification of the ITS and rbcL genes confirmed that the strain DunaDZ1 belongs to the species *D. salina*, which present a great interest regarding β-carotenes production, pigments highly sought by industry. Carotenoids content increased in strain DunaDz1 cells upon salt stress, nitrate starvation treatment and high salinity in combination with nitrogen starvation. In comparison, salt stress was the more effective stress factor on carotenoids level in f/2 and Johnson media.

Several authors tested different stress factors to maximize the production of carotenoids in *D. salina*. Hexin *et al*. (2016) found that nutrient deprivation was a mild stress in comparison to high salt concentration. This finding is confirmed in this study. Some authors recommend that adjusting light and salinity is likely one of the best strategies to achieve optimal carotenoids production in mass cultures of *D. salina* (Marín, Morales, Lodeiros, & Tamigneaux, 1998). In contrast, other authors have found that salinity does not have a clear effect on β-carotene accumulation per cell (Gomez, Barriga, Cifuentes, & Gonzalez, 2003).

The combination of two stress factors (nitrate starvation and high salinity) leads to results very close to those obtained by salt stress in f/2 medium. Otherwise, for Johnson medium, results obtained by the combination of the two stress factors were lower than those obtained by each factor separately. This fact could be explained by osmotic shock resulting from the accumulation of the two stress factors, which can lead to cell mortality, as well as the carotenoids level decrease.

The highest carotenoid amount (23.68 ± 0.12 pg/cell) was obtained in f/2 medium with high salinity. The values obtained in this study are higher than those reported by (Nikookar, Moradshahi, & Kharati, 2004) and (Fazeli, Tofighi, Samadi, Jamalifar, & Fazeli, 2006) who found amounts of 6.9 and 5.50 pg/cell of carotenoids, respectively, when *D. salina* was cultivated at the salinity of 2M. Otherwise, for the same stress conditions and for different *D. salina* strains, Cifuentes, González, Conejeros, Dellarossa & Parra, 1992 found amount of carotenoids ranging from 4.1 to 27.6 pg/cell.

These results indicated clearly that the amount of carotenoids produced by *D. salina* is highly dependent on the stress factor applied, as well as on the strain studied. It remains difficult to compare results of different studies, mainly due to the fact that various stress factors have been applied and different *D. salina* strains were used, and also, on whether the stress was applied at the beginning of test (lead to low carotenoids amount) or if the two phases strategy was utilized. All these factors should be considered when comparing results.

The intrinsic response of each strain to each inductive factor and to the complex interactions among various environmental conditions demonstrated that there is no predictable unique condition for reaching the maximum carotenoids (Hosseini Tafreshi & Shariati, 2009). Carotenogenesis varied not only according to culture media composition and environmental conditions such as light, but also varied with *D. salina* strains.

As a conclusion, the strain DunaDZ1 isolated from Sidi Ameur lake in Algeria, was assigned to the species *D. salina,* based on morphological and the molecular characteristics. The carotenogenesis tests performed demonstrated that culture of *D. salina* DunaDZ1, under a two stage strategy and under salt stress, produced elevated levels of total carotenoids in Johnson and f/2 media. The combination of two stress factors (nitrate depravation and high salinity) did not lead to the higher total carotenoids level for Johnson medium. The carotenogenesis process is highly depends on medium composition. f/2 medium can be selected for the culture of *D. salina* as it has low cost compared with Johnson medium since it is made with sea water.

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