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The effect of temperature and irradiance on the growth and carotenogenic capacity of seven strains of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions

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ABSTRACT

The carotenogenic microalga *Dunaliella salina* is cultivated as a natural source of β-carotene. The 9-cis isomer of β-carotene is found only in natural sources having commercial advantages over the all-trans isomer due to its high liposolubility and antioxidant power. High irradiance appears to stimulate specifically all-trans β-carotene accumulation in *D. salina*, whereas low temperature apparently elicits α-carotene and 9-cis β-carotene production. We studied the effect of temperature and irradiance on the growth and the carotenogenesis of three Chilean (CONC-001, CONC-006 and CONC-007) and four non-Chilean (from Mexico, China, Australia and Israel) strains of *D. salina* cultivated under two photon flux densities (40 and 110 μmol photons·m^{-2}·s^{-1}) and two temperatures (15 and 26ºC). The Chilean strain CONC-001 and all of the non-Chilean strains exhibited the highest growth rates and the maximum cell densities, whereas the Chilean strains CONC-006 and CONC-007 showed the lowest values in both parameters. The Australian strain showed the highest accumulation of total carotenoids per unit volume (40.7 mg·L^{-1}), whereas the Chilean strains CONC-006 and CONC-007, the only ones isolated from Andean environments, yielded the highest amounts of carotenoids per cell (61.1 and 92.4 pg·$cell^{-1}$, respectively). Temperature was found to be more effective than irradiance in changing the qualitative and quantitative carotenoids composition. The Chilean strains accumulated 3.5-fold more α-carotene than the non-Chilean strains when exposed to 15ºC and, unlike the non-Chilean strains, also accumulated this pigment at 26ºC. The 9-cis/all-trans β-carotene ratio was > 1.0 in all treatments for all strains, and the values were not greatly influenced by either temperature or photon flux density. Physiological and biotechnological implications of these results are discussed.

Key terms: *Dunaliella salina* strains, growth, α-carotene, β-carotene, temperature, irradiance.

INTRODUCTION

Dunaliella salina Teodoresco is a green microalga recognized at present as the most salt-tolerant eukaryote known. Under growth-limiting conditions, some strains of *D. salina* can accumulate more than 10% of their dry weight in β-carotene (Ben-Amotz et al., 1982; Ben-Amotz & Avron, 1983b; Borowitzka & Borowitzka, 1988), giving rise to the richest natural source of this pigment known so far.

β-carotene is marketed as a food coloring agent, as pro-vitamin A in food and animal feed, as an additive to cosmetics, for multivitamin preparation and, in the past decade, for its purported anti-oxidant properties. At present, most of the commercially available β-carotene is produced synthetically. Unlike synthetic β-carotene, which is composed solely of the all-trans isomer, the β-carotene of *D. salina* also includes the 9-cis isomer (Ben-Amotz et al., 1982; 1988). The higher liposolubility and antioxidant activity of the latter explains the increasing demand for the pigment derived from this microalga (Borowitzka & Borowitzka, 1989; Ben-

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Amotz & Avron, 1989; 1990; Palozza & Krinsky, 1992; Jiménez & Pick, 1993; Becker, 1994; Levin & Mokady, 1994; Ben-Amotz & Levy, 1996). Furthermore, some strains of *D. salina* also accumulate α-carotene, which is a pigment that only recently is receiving attention due to its anti-oxidant and anti-carcinogenic properties (Challem, 1997). It has been stated that α-carotene is more effective than β-carotene in suppressing experimental carcinogenesis (Murakoshi et al., 1992) and also that the intake of this pigment in the human diet helps to reduce the incidence of lung cancer in smokers (Heber, 2000) and ovarian cancer in postmenopausal women (Cramer et al., 2001).

It is already known that the massive accumulation of β-carotene in *D. salina* is triggered by environmental stresses such as intense irradiance, high salinity, nutrient starvation and extreme temperatures (Ben-Amotz et al., 1982; Ben-Amotz & Avron, 1983a; Borowitzka et al., 1984). Less information exists on the influence of these parameters on α- and β-carotene cell proportion and in the β-carotene isomers ratio. In this respect, Ben-Amotz (1996) found in *D. bardawil* (*nomen nudum* of *D. salina* according to Borowitzka and Borowitzka, 1988) that decreasing the culture temperature from 30ºC to 10ºC caused a four-fold increase in the 9-cis/all-trans β-carotene ratio (0.5 to 2.0), with no significant changes in the other cell pigments. Given the superior liposolubility of 9-cis β-carotene, this author explains the preferential accumulation of this isomer as a protective mechanism against the crystallization of β-carotene at low temperatures.

Although α-carotene has been detected in *D. salina* (in CONC-001 and CONC-006, Markovits et al., 1993; in *D. bardawil,* Ben-Amotz, 1996), conditions that stimulate its accumulation are poorly understood. Orset and Young (1999) analyzed the effect of temperature on the accumulation of α-carotene in a strain of *D. salina* (CCAP 19/30 = *D. bardawil*), detecting a 7.5-fold increase in the levels of α-carotene when the temperature was decreased from 34 to 17ºC, whereas the levels of β-carotene were unaltered. These results are in conflict with those obtained by Ben-Amotz (1996) for the same strain that did not detect any change in α-carotene content in cells growing either at 30ºC or at 10ºC.

Furthermore, it has been demonstrated that the accumulation level of β-carotene as well as its 9-cis/all-trans isomers ratio depend on the integral light intensity to which this microalga is exposed during a division cycle (Ben-Amotz & Avron, 1983a; Ben-Amotz et al., 1988). There is little doubt regarding the photostimulation of β-carotene biosynthesis in *D. salina*; however, it is not clear as to whether high or low irradiance is more effective in stimulating synthesis of 9-cis β-carotene (Ben-Amotz et al., 1988; Jiménez & Pick, 1994; Orset & Young, 2000).

Since Parra and colleagues (1990) reported the presence of *D. salina* in Chile, several Chilean research groups have performed studies that have shown that strains of *D. salina* differ greatly in their physiological (Cifuentes et al., 1992; 1996a; 1996b; Markovits et al., 1993; Gómez et al., 1999) and genetic attributes (González et al., 1999; Gómez & González, 2001; González et al. 2001), even when collected from geographically proximate locations. Among the eight Chilean strains of *D. salina* studied to date, three strains demonstrate physiological attributes with industrial potential: CONC-001 from the coastal saline lagoon, La Rinconada, shows high growth rate; CONC-006 and CONC-007 from the Andean saline ponds, Salar de Atacama, demonstrate high total carotenoids/cell (Cifuentes et al., 1992; 1996b; Gómez et al., 1999).

Considering that the natural 9-cis isomer produced by *D. salina* is a more potent antioxidant than the all-trans (Palozza & Krinsky, 1992; Jiménez & Pick, 1993; Becker, 1994; Levin & Mokady, 1994; Ben-Amotz & Levy, 1996) and α-carotene is a more effective antioxidant and anti-carcinogenic agent than β-carotene (Murakoshi et al., 1992; Challem, 1997; Heber 2000; Cramer et al., 2001), it makes sense to concentrate on identifying the strains that naturally produce more of these pigments as well as on studying the

environmental factors that trigger their accumulation.

In this study, we compared the physiological response of seven strains (three from Chile and one each from Australia, Mexico, China and Israel) of *D. salina* already used or with potential applicability in commercial mass cultures, grown under four different treatment combinations of irradiance (40 and 110 μmol m^{-2} s^{-1}) and temperature (15 and 26ºC). The aim of this research was to discover any differences in the total carotenoids amount, α- and β-carotene proportion, and β-carotene isomeric ratio between the strains grown under these different irradiance and temperature regimes.

MATERIALS AND METHODS

Algal strains

Unialgal cultures of three Chilean (CONC-001, CONC-006 and CONC-007), and four non-Chilean (from Mexico, China, Australia and Israel) strains of *Dunaliella* were used (Table I). All of the strains are maintained at the Microalgae Culture Collection, Universidad de Concepción, Chile.

Culture conditions

The strains were grown in 250 ml Erlenmeyer flasks containing 150 ml of Provasoli-enriched seawater medium (PES) (McLachlan, 1973; Gómez et al., 2003) supplemented with 12.5% w/v NaCl. The flasks were maintained in culture chambers at constant temperature with continuous light provided from fluorescent day-light tubes, without aeration, and manually shaken twice a day. The strains were grown under the following combined conditions of temperature and photon flux density: 26ºC and 40 μmol photons·m^{-2}·s^{-1}; 26ºC and 110 μmol photons·m^{-2}·s^{-1}; 15ºC and 40 μmol photons·m^{-2}·s^{-1}; 15ºC and 110 μmol photons·m^{-2}·s^{-1}. All experiments were initiated by the inoculation of each flask with exponentially growing cells (acclimated to the experimental conditions for 30 days) at a density of $5.0x10^3$ cells ml^{-1}. Three replicates of each treatment were established.

TABLE I

Strains of *Dunaliella salina* included in this study

Strain	Geographic origin	Source[1]
D. salina CONC-001	La Rinconada Lagoon, Antofagasta, Chile	CONC-001
D. salina CONC-006	Sector Burro Muerto, Salar de Atacama, Chile	CONC-006
D. salina CONC-007	Sector Puilar, Salar de Atacama, Chile	CONC-007
D. salina Mexican	Yucatán, Mexico	Provided by Ernesto Retamales, U. Antofagasta, Chile
D. salina Chinese	Tanggu, China	Provided by Dr. Ralph Lewin, Scripps Institution, USA
D. salina Australian	Hutt Lagoon, Australia	CCAP-19/18
D. bardawil	Bardawil Lake, Israel/Egypt	ATCC-30861

CONC: Culture Collection of Microalgae, Universidad de Concepción, Concepción, Chile.
CCAP: Culture Collection of Algae and Protozoa, United Kingdom.
ATCC: American Type Culture Collection, VA, USA.
[1] All the strains are currently available in CONC.

Growth parameters

Growth was monitored over a 30-day period by cell counts of the replicate whose A_{750nm} was the nearest to the replicates' treatment average. Cell counts were performed every four days in 1 ml Utermöhl counting chambers. The growth rate, k (divisions·day^{-1}) was evaluated during the logarithmic growth phase as in Cifuentes and colleagues (1996a).

Pigments analysis

Total carotenoids, chlorophylls *a* and *b*, α-carotene, and 9-cis and all-trans β-carotene were estimated at an early stationary phase: in 25 day-old cultures for the treatments at 26ºC and in 33 day-old cultures for the treatments at 15ºC. The total carotenoids and chlorophyll content were determined spectrophotometrically from algal pellets using a 90% (v/v) acetone/water mixture (Wegmann & Metzner 1971). Carotenoid composition was analyzed in dichloromethane extracts by HPLC using a chromatograph LDC Analytical with UV-Visible detector. A stainless steel column of 25 cm x 4 mm i.d. packed with C18 reversed phase material of 5 μm particle size was used. Elution was performed with an isocratic solvent of 1 ml min^{-1} methanol: dichloromethane 90: 10 v/v, and a 450 nm wavelength was utilized for detection. All solvents were filtered and degassed prior to use. Pigment identification was performed using synthetic β-carotene (Merck, Germany) as a standard (100% all-trans isomer) and the elution order reported for these pigments in similar chromatographic systems (Ben-Amotz et al., 1988). The relative content of each carotene (α-carotene, 9-cis β-carotene and all-trans β-carotene) was determined by estimation of each peak's area with respect to the sum of all their areas.

Statistical analysis

Carotenogenesis parameters were analyzed by three-way analysis of variance (ANOVA) with the factors: strain, irradiance and temperature as independent variables. For multiple comparisons, tests of Tukey and Scheffe were used. Differences were considered to be significant at a probability of 5% ($P \leq 0.05$). The computational program used was STATISTICA version 6.0.

RESULTS

Growth

Under all the culture conditions, the non-Chilean strains and the strain CONC-001 showed the highest growth rates and maximum cell densities, whereas the strains CONC-006 and CONC-007 exhibited the lowest values in both parameters (Fig. 1, Table II).

Independent of the photon flux density, all the strains reached higher growth rates at the highest assayed temperature (26ºC). However, the strains from Mexico, China and Australia showed a slight decrease of growth rate at the highest photon flux density (Fig. 1, Table II).

In cultures maintained at the same photon flux density, the higher temperature increased the carrying capacity in most of the strains. Nevertheless, the strains from Mexico, China and CONC-006 from Chile exhibited, in some conditions, equal or lower maximum cell density at 26ºC than at 15ºC (Table II).

Total carotenoids

The three-way ANOVA revealed that strain identity was the most significant source of variation in total carotenoids accumulation (expressed as carotenoids per volume, carotenoids per cell and carotenoids/chlorophyll *a* ratio). Moreover, and even though the temperature effect alone was more significant than the irradiance effect in determining these responses, a significant interaction between both parameters was detected.

The Australian strain and the strain CONC-007 were the most carotenogenic under all culture conditions. The Australian strain exhibited the maximum carotenoid accumulation per culture volume unit (40.7

± 1.0 mg·L^{-1} at 15ºC and 110 µmol photons·m^{-2}·s^{-1}), while CONC-007 showed the maximum amount of carotenoids per cell (92.4 ± 4.7 pg·cell^{-1} at 26ºC and 40 µmol photons·m^{-2}·s^{-1}) (Table III). Even though *D. bardawil* showed a carotenogenic capacity similar to that of CONC-007 in terms of carotenoids per culture volume unit, *D. bardawil* had a four- to six-fold lower accumulating capacity in carotenoids per cell than CONC-007 (Table III).

CONC-006 was the only strain that showed a statistically significant tendency ($p<0.05$) for higher carotenoid accumulation per cell at the highest irradiance (110 µmol photons·m^{-2}·s^{-1}) at either temperature. At 26ºC, the Chinese and Australian strains as well as CONC-007 accumulated more carotenoids per cell at the lowest irradiance (Table III). The same tendency was exhibited by the last two strains in terms of carotenoids per culture volume (Table III).

The strain CONC-007 had the highest carotenoid/chlorophyll ratio in all the assayed culture conditions. At 110 µmol photons·m^{-2}·s^{-1}, the carotenoid/chlorophyll ratio was significantly higher ($p<0.05$) for all the strains at the lowest temperature (Table III). At the highest temperature, all strains except for CONC-006 exhibited higher carotenoid/chlorophyll ratio at 40 rather than at 110 µmol photons·m^{-2}·s^{-1} (Table III).

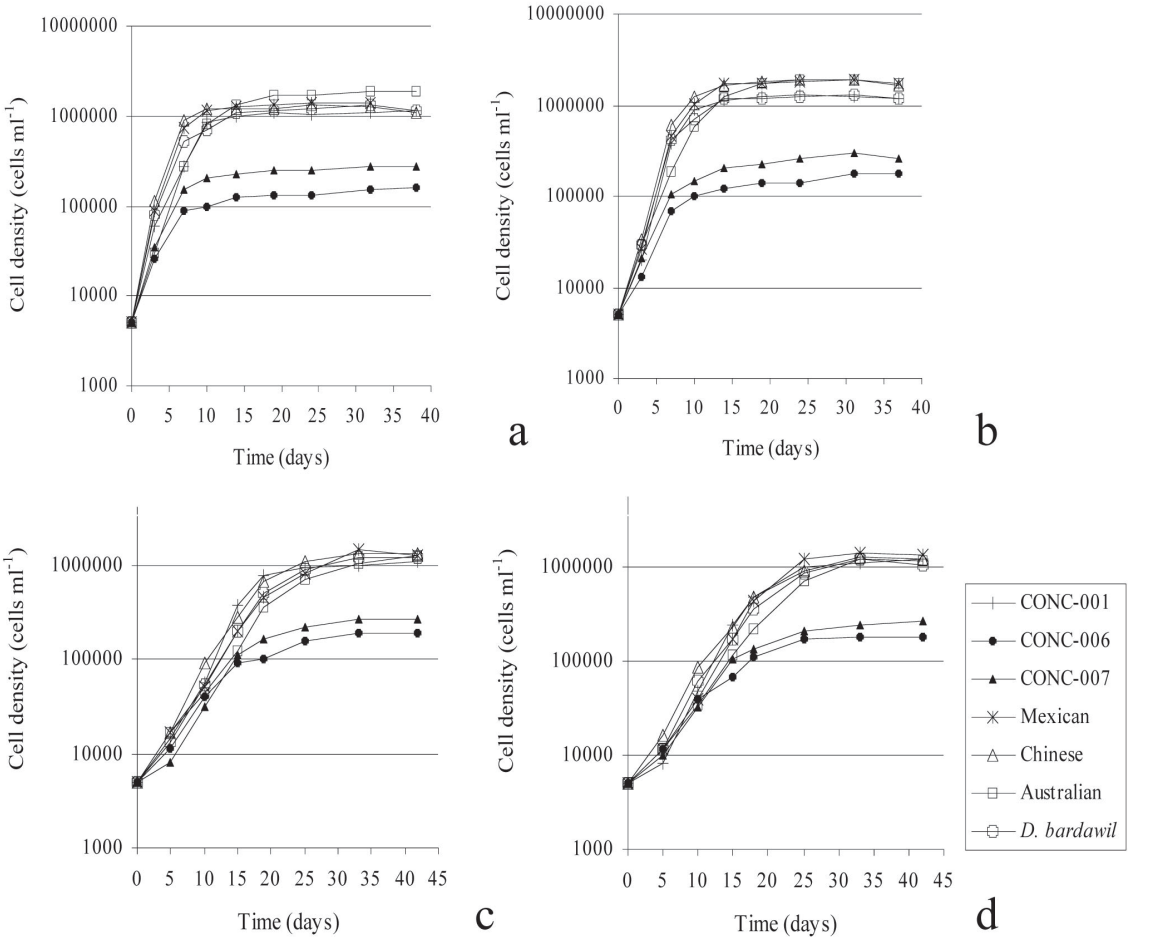

Fig. 1. Comparison of growth of three Chilean and four non-Chilean strains of *D. salina* in cultures maintained under four different treatment combinations of temperature and irradiance: a: 26ºC and 40 µmol photons·m^{-2}·s^{-1}; b: 26ºC and 110 µmol photons·m^{-2}·s^{-1}; c: 15ºC and 40 µmol photons·m^{-2}·s^{-1} and d: 15ºC and 110 µmol photons·m^{-2}·s^{-1}.

TABLE II

Division rate (k) and maximum cell density (N_{max}) reached by the seven strains of *D. salina* in each culture condition

Strain	26ºC and 40µmol photons·m^{-2}·s^{-1}		26ºC and 110µmol photons·m^{-2}·s^{-1}		15ºC and 40µmol photons·m^{-2}·s^{-1}		15ºC and 110 µmol photons·m^{-2}·s^{-1}	
	k (div day^{-1})	N_{max} (cells ml^{-1})	k (div day^{-1})	N_{max} (cells ml^{-1})	k (div day^{-1})	N_{max} (cells ml^{-1})	k (div day^{-1})	N_{max} (cells ml^{-1})
CONC-001	0.9	1.2 x 10^6	0.9	1.3 x 10^6	0.4	1.1 x 10^6	0.4	1.2 x 10^6
CONC-006	0.6	1.6 x 10^5	0.6	1.8 x 10^5	0.2	1.9 x 10^5	0.2	1.8 x 10^5
CONC-007	0.7	2.7 x 10^5	0.7	2.9 x 10^5	0.3	2.6 x 10^5	0.3	2.7 x 10^5
Mexican	1.1	1.4 x 10^6	0.9	1.9 x 10^6	0.3	1.4 x 10^6	0.4	1.4 x 10^6
Chinese	1.2	1.3 x 10^6	1.0	1.9 x 10^6	0.4	1.3 x 10^6	0.4	1.3 x 10^6
Australian	0.9	1.9 x 10^6	0.8	1.9 x 10^6	0.3	1.3 x 10^6	0.3	1.2 x 10^6
D.bardawil	1.0	1.3 x 10^6	1.0	1.3 x 10^6	0.3	1.2 x 10^6	0.3	1.2 x 10^6

TABLE III

Chlorophyll *a* (Chl *a*) and *b* (Chl *b*), total carotenoid content (Car) per volume (mg·L^{-1}) and per cell (pg·cell^{-1}), Car/Chl *a*, relative content of α and β-carotene (α-car %; β-car %), and 9-cis/all-trans β-carotene ratio (given as mean value ± standard deviation) in different strains of *D. salina* (estimated at stationary phase of growth), grown at the following conditions of temperature and irradiance: a) 26ºC/40 µmol photons·m^{-2}·s^{-1}; b) 26ºC/110 µmol photons·m^{-2}·s^{-1}; c) 15ºC/40 µmol photons·m^{-2}·s^{-1}; d) 15ºC/110 µmol photons·m^{-2}·s^{-1}.

a) 26ºC/40 µmol photons·m^{-2}·s^{-1}

Strain	Chl *a* (µg·L^{-1})	Chl *b* (µg·L^{-1})	Car (µg·L^{-1})	Car/Chl *a*	Car (pg·cell^{-1})	α-car (%)	β-car (%)	9-cis/all-trans βcar
CONC-001	950±26	240±30	7979±511	8.4±0.5	8.4±0.5	17.3±2.8	82.7±2.8	1.1±0.1
CONC-006	464±50	181±20	4560±290	9.9±0.7	35.7±2.3	27.3±2.6	72.7±2.6	2.2±0.05
CONC-007	935±79	568±96	26248±1337	28.2±3.1	92.4±4.7	21.3±1.4	78.7±1.4	2.3±0.4
Mexican	1328±56	502±59	21894±416	16.5±0.4	14.4±0.2	ND	100±0.0	1.9±0.1
Chinese	1441±148	572±28	17889±819	12.5±1.7	15.0±0.7	ND	100±0.0	2.1±0.1
Australian	1902±83	447±25	38683±2442	20.4±1.7	19.6±1.2	ND	100±0.0	1.5±0.05
D.bardawil	2389±136	667±26	16536±418	6.9±0.5	14.3±0.4	ND	100±0.0	2.2±0.2

Table III. Cont.

b) 26ºC/110 μmol photons·m^{-2}·s^{-1}

Strain	Chl *a* (μg·L^{-1})	Chl *b* (μg·L^{-1})	Car (μg·L^{-1})	Car/Chl *a*	Car (pg·cell^{-1})	α-car (%)	β-car (%)	9-cis/all-trans βcar
CONC-001	1338±32	473±32	10292±298	7.7±0.2	9.0±0.3	17.4±0.4	82.6±0.4	1.1±0.0
CONC-006	431±12	250±19	5421±48	12.6±0.2	44.3±0.4	30.6±1.7	69.4±1.7	1.8±0.2
CONC-007	694±15	375±58	16000±952	23.2±1.0	72.1±1.1	24.5±0.8	75.5±0.8	2.1±0.0
Mexican	1935±29	672±10	22765±477	11.8±0.1	17.7±0.3	ND	100±0.0	1.9±0.3
Chinese	1585±63	544±11	21092±402	13.3±0.2	11.2±0.2	ND	100±0.0	1.8±0.1
Australian	1807±13	530±5	24580±19	13.6±0.1	13.2±0.1	ND	100±0.0	1.4±0.1
D.bardawil	2702±88	924±11	20504±526	7.6±0.05	16.9±0.4	ND	100±0.0	2.1±0.2

c) 15ºC/40 μmol photons·m^{-2}·s^{-1}

Strain	Chl *a* (μg·L^{-1})	Chl *b* (μg·L^{-1})	Car (μg·L^{-1})	Car/Chl *a*	Car (pg·cell^{-1})	α-car (%)	β-car (%)	9-cis/all-trans βcar
CONC-001	1011± 14	362±36	8784±16	8.7±0.2	8.8±0.3	34.6±2.0	65.4±2.0	1.3±0.2
CONC-006	458 ± 29	262±35	8939±49	19.6±1.2	46.8±2.3	33.7±1.8	66.3±1.8	2.1±0.06
CONC-007	626 ± 7	696±16	20921±134	33.4±0.6	79.8±1.0	37.0±2.6	63.0±2.6	2.1±0.2
Mexican	1708 ± 17	649±22	25589±892	15.0±0.5	17.8±0.6	11.8±0.6	88.2±0.6	1.8±0.1
Chinese	1716 ± 21	774±12	29013±446	16.9±0.2	22.5±0.3	11.4±0.8	88.6±0.8	2.4±0.2
Australian	1833 ± 30	822±19	35851±255	19.5±0.2	34.3±1.0	8.0±0.4	92.0±0.4	2.0±0.1
D.bardawil	1967 ± 24	809±7	24453±636	12.4±0.2	21.1±0.5	11.6±0.4	88.4±1.9	2.3±0.1

d) 15ºC/110 μmol photons·m^{-2}·s^{-1}

Strain	Chl *a* (μg·L^{-1})	Chl *b* (μg·L^{-1})	Car (μg·L^{-1})	Car/Chl *a*	Car (pg·cell^{-1})	α-car (%)	β-car (%)	9-cis/all-trans βcar
CONC-001	1207±100	452±54	12355±306	10.3±0.6	11.1±0.3	32.7±0.4	67.3±0.4	1.2±0.06
CONC-006	494±24	276±23	10010±32	20.3±1.0	61.1±1.6	36.3±0.4	63.7±0.4	2.0±0.1
CONC-007	644±48	326±12	21789±207	33.9±2.8	90.7±0.8	39.9±1.6	60.1±1.6	2.2±0.1
Mexican	1924±34	1144±137	31857±1016	16.6±0.5	18.7±0.6	12.9±1.0	87.1±1.0	1.8±0.2
Chinese	1547±45	832±31	27315±1263	17.7±0.3	21.7±1.0	11.6±1.0	88.4±1.0	2.0±0.06
Australian	1729±24	599±7	40665±1051	23.3±1.2	33.9±1.3	10.4±0.2	89.6±0.2	1.2±0.1
D.bardawil	1660±38	689±49	25812±754	15.5±0.4	21.2±0.4	13.0±1.0	87.0±1.0	1.9±0.1

Relative composition of carotenes

Temperature was the principal determining factor in α- and β-carotene accumulation in the strains, whereas irradiance was the most important determining factor in the 9-cis/all-trans β-carotene ratio, as indicated by the three-way ANOVA. Temperature and irradiance did not interact: while 15ºC was the optimal temperature for the α-carotene accumulation, 26ºC was best for β-carotene accumulation, and 40 μmol photons·m^{-2}·s^{-1} the most favorable photon flux density for a maximum 9-cis/all-trans β-carotene ratio.

The Chilean strains of *D. salina* accumulated α-carotene (15% to 40%) at both assayed temperatures, while the non-Chilean strains accumulated it in detectable amounts (<15%) only during growth at 15ºC (Table III). Moreover, low temperature favored α-carotene production in the Chilean strains, this response being especially remarkable for CONC-001 and CONC-007 (Table III).

In general, irradiance did not affect the α-carotene production in the strains analyzed, except for the Australian strain, which accumulated a higher proportion of this pigment at 110 μmol photons·m^{-2}·s^{-1}.

The relative content of β-carotene in all the strains was higher at 26ºC than at 15ºC (Table III). The accumulation of this pigment by the cells was independent of the photon flux density, except for the Australian strain, which showed a significantly higher ($p<0.05$) accumulation of this pigment at 40 μmol $m^{-2}s^{-1}$ for growth at 15ºC (Table III).

All of the strains under any culture conditions exhibited 9-cis/all-trans β-carotene ratios higher than 1.0. In general, the highest values in this parameter were obtained at the lowest photon flux density. These values were significantly higher in the strain CONC-006 at 26ºC, in the Australian strain at 15ºC and in the Chinese strain at both temperatures. The maximum value of 2.4 was obtained by the Chinese strain for growth at 15ºC and 40 μmol$m^{-2}s^{-1}$ (Table III).

DISCUSSION

Dunaliella salina is a photosynthetic organism with a unique ecophysiological adaptive capacity, as has been shown by the wide range of physiological responses to different culture conditions, exhibited by the seven strains studied. These strains have been genetically characterized using random amplified polymorphic DNA (RAPD) band patterns and nuclear ribosomal DNA internal transcribed spacer (ITS-1 and ITS-2) sequences (Gómez & González, 2004). The physiological attributes revealed by the strains in this study are mostly in agreement with the genetic relationships found among them, which demonstrates the genome participation in the determination of the intraspecific physiological differences detected here.

Considering that in its natural environment the optimal growth temperatures for *D. salina* are higher than 21ºC (Borowitzka, 1981), it is not surprising that all the strains exhibited greater growth rates at 26ºC than at 15ºC (Fig. 1, Table II). Furthermore, the carrying capacity of most of the cultures was also positively affected by high temperature (Table II).

The coastal strain CONC-001 from Chile exhibited growth rates and growth curves very similar to the four non-Chilean strains (Mexican, Chinese, Australian and *D. bardawil*), which also all come from coastal hypersaline environments. In contrast, the strains (CONC-006 and CONC-007) from the Salar de Atacama (alpine hypersaline environment) exhibited lower growth rates than all of the other strains, and these two Chilean strains attained the lowest cell densities in all culture conditions (Fig. 1, Table II). On the other hand, the carotenoids content per cell was significantly higher in the strains from the Salar de Atacama under the assayed culture conditions (Table III).

Previous studies have already reported low carrying capacities accompanied by unusually high carotenoids per cell accumulation in strains CONC-006 and CONC-007 grown under very different

culture conditions (Cifuentes et al., 1992). The persisting physiological behavior of these strains might be explained as a physiological adaptation to the prevalent conditions in the habitat for which their genotypes were selected. The Salar de Atacama is a hypersaline environment of 3,000 km^2 located at an altitude of 2,340 meters in the Atacama Desert. Some of the abiotic characteristics of this salt lake are cloudless skies almost all year round and very high solar radiation (Parra et al., 1990; Alonso & Risacher, 1996; Risacher & Alonso, 1996). In this type of aquatic environment, O_2 and CO_2 solubility are strongly reduced due to altitude, salinity and high temperature (Borowitzka, 1981; Borowitzka & Borowitzka, 1988), which could explain the low carrying capacities exhibited by these strains. On the other hand, the high free radical production in a highly irradiated environment like the Salar de Atacama could explain the high carotenoid per cell accumulation shown by these strains.

Except for the extensive studies on carotenogenesis already done on *D. bardawil* from a physiological (e.g., Ben-Amotz & Avron, 1983a; Ben-Amotz et al.-1988; Ben-Amotz, 1996) and a molecular point of view (Lers et al., 1990; 1991; Levy et al., 1993), there is no published information on the carotenogenic capacity and/or the growing characteristics of most of the non-Chilean strains of *D. salina* studied in this paper. There are no previous studies on the Mexican strain, whereas the existing information for the Chinese and Australian strains is unavailable due to the commercial utilization of these strains.

According to this study's results, the strain was the main factor controlling total carotenoid accumulation and carotenoids/chlorophyll ratio. The most carotenogenic strain, expressed as carotenoids per cell, was the Chilean strain CONC-007; while when expressed as carotenoids per volume of culture, the Australian strain was the most carotenogenic (Table III). From a commercial point of view, a strain with high carotenoids per cell yield is tremendously profitable, since its culture should produce a much purer final product. In carotenoids/chlorophyll ratio – another final product purity parameter – the strain CONC-007 was, definitively, the best (see Table III). Therefore, in spite of the fact that the strain CONC-007 produces less carotenoids per volume than the Australian strain, it is better for commercial applications since its algal power is much richer in carotenoids.

Ben-Amotz and Avron (1983a) demonstrated that the β-carotene/chlorophyll ratio in *D. bardawil* increases as a function of the integral amount of light that the alga receives during a division cycle (irradiance x doubling time). Therefore, an increase of the doubling time, by a decrease of temperature at the same irradiance, should increase the β-carotene/chlorophyll ratio, as occurred in this study. Even though this tendency was reported for exponentially growing cells, our results show that it is also true during the stationary growth phase as a higher carotenoids/chlorophyll ratio demonstrates, at least at 110 μmol photons$\cdot m^{-2}\cdot s^{-1}$, for all strains at the lower culture temperature (Table III).

Ben-Amotz and colleagues (1988) found that the 9-cis/all-trans β-carotene ratio increased from 0.21 to 1.3 in *D. bardawil* when the photon flux density increased from 50 to 2000 μmol photons$\cdot m^{-2}\cdot s^{-1}$. However, Jiménez and Pick (1994) and Orset and Young (2000), revealed that 9-cis β-carotene synthesis is promoted by low (20 to 50 μmol photons$\cdot m^{-2}\cdot s^{-1}$), rather than high (200 to 1250 μmol photons$\cdot m^{-2}\cdot s^{-1}$) irradiances in the same strain. Even though the irradiance range used in this study was much narrower (40 to 110 μmol photons$\cdot m^{-2}\cdot s^{-1}$) than that used by the previous authors, we almost always found a higher proportion of 9-cis over the all-trans isomer at the lower irradiance for the same culture temperature (Table III).

Under any culture condition, the Chilean strain CONC-007 exhibited a higher 9-cis/all-trans β-carotene ratio than the Australian strain (Table III), which is another commercially advantageous attribute.

In its natural environment, when *D. salina* is exposed to extremely high

irradiances the β-carotene accumulated at the interthylakoidal globules prevents its photo-oxidative cell damage (Ben-Amotz et al., 1982; 1989). Ben-Amotz (1996) analyzed the temperature effect in the β-carotene isomers composition of *D. bardawil* globules, finding that if the culture temperature decreases from 30ºC to 10ºC, the 9-cis/all-trans β-carotene ratio increases four-fold. In the present study, a temperature decrease from 26ºC to 15ºC did not significantly affect the 9-cis/all-trans β-carotene ratio in *D. bardawil* at any photon flux density (Table III). In contrast to Ben-Amotz (1996), we determined pigment composition in the total algal extract, which includes β-carotene from the interthylakoid globules as well as that from the thylakoid light-absorbing antennas. In this context, Jiménez and Pick (1994) pointed out that photosynthetic pool of β-carotene could make an important contribution to the amount and composition of *D. salina* pigments. They established that both chloroplastic pools of pigments (globules and thylakoids) exhibit different β-carotene isomer composition and suggested that the globular and thylakoid pool of β-carotene are distinct in their biosynthesis regulation. In the present study, only the Chinese and Australian strains showed a 9-cis/all-trans β-carotene ratio significantly higher at 15ºC than at 26ºC, when cultivated at 40 μmol photons$\cdot m^{-2}\cdot s^{-1}$ (Table III). These results provide further evidence of the physiological diversity that exists among strains of *D. salina*.

Future studies should elucidate the molecular mechanisms that determine the physiological differences detected among the strains. For example, studies could evaluate if these differences are related with the differential expression of genes related to the biosynthesis and/or accumulation of carotenoids either in globules or in thylakoids.

In 1993, Markovits and colleagues reported the presence of α-carotene in two Chilean strains of *D. salina*, which at that time was considered to be an undesirable attribute since no studies existed on the effect that a high consumption of this pigment may have in animal organisms. However, in the past decade a number of papers have described the benefits that various carotenoids, including α-carotene, have in cancer prevention (Yuen, 1994; Levy et al., 1995; Challem, 1997; Michaud et al., 2000).

Since the main factor stimulating the synthesis of α-carotene in *D. salina* is the low temperature (Orset & Young, 1999), it was not surprising that all the non-Chilean strains accumulated α-carotene to some extent (8 to 13%) at 15 ºC (Table III). On the other hand, the Chilean strains also accumulated α-carotene (17 to 31%) at high temperature (26ºC) whereas the non-Chilean strains did not (Table III). Low temperature (15ºC) strongly increased α-carotene accumulation in Chilean strains, particularly in CONC-007 in which it increased to 40%.

Consequently, the Chilean strain CONC-007 appears as the most suitable for commercial purposes due to its desirable traits as a source of natural carotenoids.

Our results show that even though the growth and carotenogenic responses of any strain can be modified by the culture conditions, there is no doubt that they are also a function of the intrinsic attributes of each strain. The recent confirmation of a correlation between the above differences and the genetic polymorphism detected among the studied strains (Gómez & González, 2004) demonstrate that there exists a genetic basis supporting these physiological differences. However, it will be necessary to establish if these differences can be explained by the differential regulation of gene expression related to the biosynthesis and /or carotenoid accumulation in the two main pools of these pigments in *D. salina*.

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