Biol Res 36: 185-192, 2003

Effect of salinity on the quantity and quality of carotenoids accumulated by *Dunaliella salina* (strain CONC-007) and *Dunaliella bardawil* (strain ATCC 30861) Chlorophyta

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ABSTRACT

Dunaliella salina and *D. bardawil* are well-known microalgae accumulating high levels of β-carotene under growth-limiting conditions. In both taxa, this pigment is primarily composed of the isomers 9-cis and all-trans. The 9-cis β-carotene occurs only in natural sources and is the most attractive from a commercial point of view. The conditions that enhance the preferred accumulation of 9-cis β-carotene in *D. salina* are controversial and they have not been well established yet. This study examined the effect of salinity on the quantity and quality of total carotenoids and β-carotene isomers accumulated by *D. salina* (strain CONC-007) and *D. bardawil* (strain ATCC 30861) grown in two media with different nutritional compositions (PES and ART) and at salt concentrations of 1M, 2M and 3M NaCl. Total carotenoids were determined by spectrophotometry and β-carotene isomers, by HPLC. The highest carotenoid contents per cell were obtained at 2M NaCl in both taxa. In both media, an increase of the 9-cis/all-trans β-carotene ratio was observed in *D. bardawil* when the salt concentration increased, with a maximum value of 2.6 (in ART medium at 3M NaCl). In *D. salina* this ratio did not exhibit the same pattern, and the salt concentrations for maximal ratios were different in both media. The highest ratio obtained for this strain was 4.3 (in ART medium at 2M NaCl).

Key terms: *D. salina, D. bardawil,* ß-carotene isomers, salinity, culture medium.

INTRODUCTION

Dunaliella salina Teodoresco and *Dunaliella bardawil* Ben-Amotz & Avron (*nomen nudum* of *D. salina*, according to Borowitzka and Borowitzka, 1988) have the exceptional ability of synthesizing and accumulating high amounts of β-carotene when maintained under growth limiting conditions (Ben-Amotz *et al*., 1982; Ben-Amotz *et al*., 1988; Borowitzka *et al*., 1984).

The β-carotene is a terpenoid pigment that is highly valuable due to its nutritional benefit as a precursor of vitamin A and for its antioxidant properties. In *D. salina*, the β-carotene is accumulated in electrodense globules located within the inter-thylakoid spaces in the chloroplast. The β-carotene occurs as a number of isomers, two of which, 9-cis and all-trans, make up approximately 80% of the total β-carotene in *Dunaliella* (Ben-Amotz *et al*., 1988; Borowitzka and Borowitzka 1988; Jiménez and Pick, 1994). The 9-cis isomer is synthesized only by natural sources and it is a highly valuable metabolite because of its interesting physicochemical properties i.e. it has a high liposolubility, being effectively preserved into animal tissues; furthermore, this isomer has proved to have a better antioxidant capacity than the all-trans isomer for which, at present, there have also been reports concerning its application in diseases related to the production of free radicals (Ben-Amotz *et al*., 1988; Jiménez and Pick, 1993; Becker, 1994).

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Received: June 5, 2002. Accepted: November 4, 2002

The extent of carotenoid accumulation in *D. salina* and *D. bardawil* has been extensively studied, and it is well established that it is triggered by high salinity (Borowitzka *et al.*, 1990; Cifuentes *et al.*, 1996a), high temperature and high irradiance (Ben-Amotz *et al.*, 1988; Araneda *et al.*, 1992) and also, under growth limiting nutritional conditions (Araneda *et al.*, 1992; Markovits *et al.*, 1993; Cifuentes *et al.*, 1996b).

On the other hand, the conditions that favor the accumulation of 9-cis β-carotene have received much less attention and the information available is controversial. Regarding light, Ben-Amotz and co-workers (1988), working with *D. bardawil* ATCC 30861, detected an increase of the 9-cis/all-trans β-carotene ratio when increasing the photon flux density from 50 to 2000 μmol $m^{-2}s^{-1}$. On the contrary, Jiménez and Pick (1994), and more recently, Orset and Young (2000), found just the opposite, i.e., an increase of this ratio at decreasing photon flux densities (1500 to 50 μmol $m^{-2}s^{-1}$) in two different strains of *D. salina*. Temperature is another parameter involved in the 9-cis β-carotene accumulation, since low temperature exposition (10-15ºC) has been reported to encourage specifically 9-cis β-carotene production in *D. bardawil* (Ben-Amotz, 1996).

No reports have been published to date with respect to the effect of salinity on the 9-cis/all-trans β-carotene ratio. Considering that salinity is a parameter easy to control in outdoor cultures of *Dunaliella* for commercial purposes, the aim of this work was to study the effect of this parameter on the total carotenoids content and, specifically, on the 9-cis/all-trans β-carotene ratio in the Chilean strain *D. salina* CONC-007 and in *D. bardawil* strain ATCC 30861, cultured in two media with different nutritional composition.

MATERIALS AND METHODS

Algae: Two carotenogenic strains of *Dunaliella* were used for the experiments: *D. salina* CONC-007, isolated from the Atacama Desert, Chile (23º30´S; 68º15´W) and *D. bardawil* strain ATCC 30801, isolated from the Bardawil Lake, Egypt. Both strains are maintained in unialgal cultures in the Microalgae Culture Collection, University of Concepción, Chile.

Culture conditions: The strains were grown in two media i) Provasoli-enriched sea water medium (PES) (McLachlan, 1973), and ii) artificial medium (ART) (Ben-Amotz, 1988) (Table I) with different salinities: 1M, 2M and 3M of NaCl. The experiments were carried out in 300-ml Erlenmeyer flasks containing 150 ml of the respective medium. The flasks were maintained under a continuous photon flux density of 40 μmol $m^{-2}s^{-1}$ (provided from fluorescent day light tubes), at 25 ± 1 ºC, without aeration and manually shaken twice a day. All the experiments were started by inoculating the flasks with exponentially growing cells acclimatized to the experimental conditions for 30 days. The initial cell density was approximately 5000 cells ml^{-1}. Three replicates of each treatment were established.

Pigments analysis: The pigments were estimated in 28 day-old cultures (stationary phase). The total carotenoids and the chlorophyll were extracted from algal pellets using a 90% (v/v) acetone/water mixture and determined by spectrophotometry (Wegmann and Metzner, 1971). For carotenoids per cell determination, counting was done in 1 ml Utermöhl chambers.

Carotenoid composition was analyzed in dicloromethane 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 mm particle size was used. Elution was performed with isocratic solvent of 1 ml min^{-1} methanol: dicloromethane 90:10 v/v and a wavelength of 450 nm was utilized for detection. All solvents were filtered and degassed prior to use. Pigment identification was done by using synthetic β-carotene as 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

TABLE I

Composition of media used in the cultures of two carotenogenic taxa of *Dunaliella*

Chemical compound	Provasoli (PES)(*)	Artificial (ART)(**)
NaCl	1.0 M- 2.0 M- 3.0 M	1.0 M- 2.0 M- 3.0 M
$NaNO_3$	0.65 mM	9.0 µM
Na_2glycerophosphatex$5H_2O$	0.026 mM	5.0 µM
$FeCl_3$ x $6H_2O$	0.7 µM	
Na_2EDTA x $2H_2O$	7.8 µM	
Vitamin B-12	1.6 µg l^{-1}	
Thiamine	8.0 µg l^{-1}	
Biotin	0.8 µg l^{-1}	
Buffer TRIS	0.66 µM	
H_3BO_3	72.0 µM	10.0 µM
$CoSO_4$ x $7H_2O$	0.07 µM	
$CuSO_4$ x 5 H_2O		
$CoCl_2$ x 6 H_2O		
$ZnCl_2$		
$ZnSO_4$ x $7H_2O$	0.3 µM	
$MgSO_4$		5 mM
$MnCl_2$ x $4H_2O$		0.2 µM
$(NH_4)_6$ Mo_7O_{24} x $4H_2O$		0.3 µM
$CaCl_2$		0.3 mM
KNO_3		0.75 µM
KH_2PO_4		0.2 µM
$NaHCO_3$		50 µM
	Complete volume with sea water	Complete volume with distilled water

(*) Natural enriched medium (**) Artificial medium.

estimating each peak's area respect to the integral of the areas.

RESULTS

Even though both strains exhibited their maximum growth rates at the lowest salinity (1M NaCl), *D. salina* showed its maximum cell density at 2M NaCl ($1.1x10^6$ cells ml^{-1}) while *D. bardawil* reached it at 1M NaCl ($2.5x10^6$ cells ml^{-1}) (data not shown).

Total carotenoids accumulated by the two strains of *Dunaliella* were affected by the medium composition. When production of total carotenoids per culture volume was compared in the two media at the same salinity, *D. salina* showed the highest values in ART medium, while *D. bardawil* showed it in PES medium (Table II). When the content of carotenoids per cell was compared, no clear tendency was observed: *Dunaliella salina* grown in PES medium showed the highest values at 1M and 2M NaCl, whereas in ART medium the highest production was obtained at 3M NaCl. In *D. bardawil*, on the other hand, the highest values in this parameter were registered in PES medium at 1M and 3M NaCl, whereas in ART medium the greatest value was obtained at 2M NaCl (Table II).

Only *D. salina* showed a clear tendency in accumulating more carotenoids per volume when increasing the salinity, being this response independent of the medium composition (Table II).

Carotenoids to chlorophyll ratio increased with salinity in *D. salina*, whereas *D. bardawil* showed the opposite tendency (Figure 1).

Under the culture conditions employed, α-carotene was only detected in *D. salina*. The salinity did not affect the accumulation of α and β-carotene in this strain (Figure 2).

Dunaliella salina exhibited higher 9-cis/ all-trans β-carotene ratios than *D. bardawil* in both media and the three salinities used (Figure 3).

TABLE II

Carotenogenesis parameters in *D. salina* and *D. bardawil* cultivated in two media (PES and ART) with different salinities (1M, 2M and 3M NaCl) after 28 days of incubation at 25ºC and 40 μmol $m^{-2}s^{-1}$, n=3 ±SD.

	D. salina					
	1M		2M		3M	
	PES	ART	PES	ART	PES	ART
Total carotenoids (mg l^{-1})	6.9±1.3	8.0±0.1	10.8±0.6	12.9±0.2	12.9±0.5	29.5±0.7
Total carotenoids (pg cell $^{-1}$)	33.4±6.2	14.0±0.1	47.7±2.5	14.2±0.1	32.2±0.8	36.3±0.8
	D. bardawil					
	1M		2M		3M	
	PES	ART	PES	ART	PES	ART
Total carotenoids (mg l^{-1})	23.3±0.7	18.0±0.4	24.7±0.6	17.8±0.3	23.0±0.9	14.8±0.4
Total carotenoids (pg cell $^{-1}$)	9.8±0.3	7.2±0.2	13.1±0.6	26.9±0.4	14.3±0.5	7.5±0.2

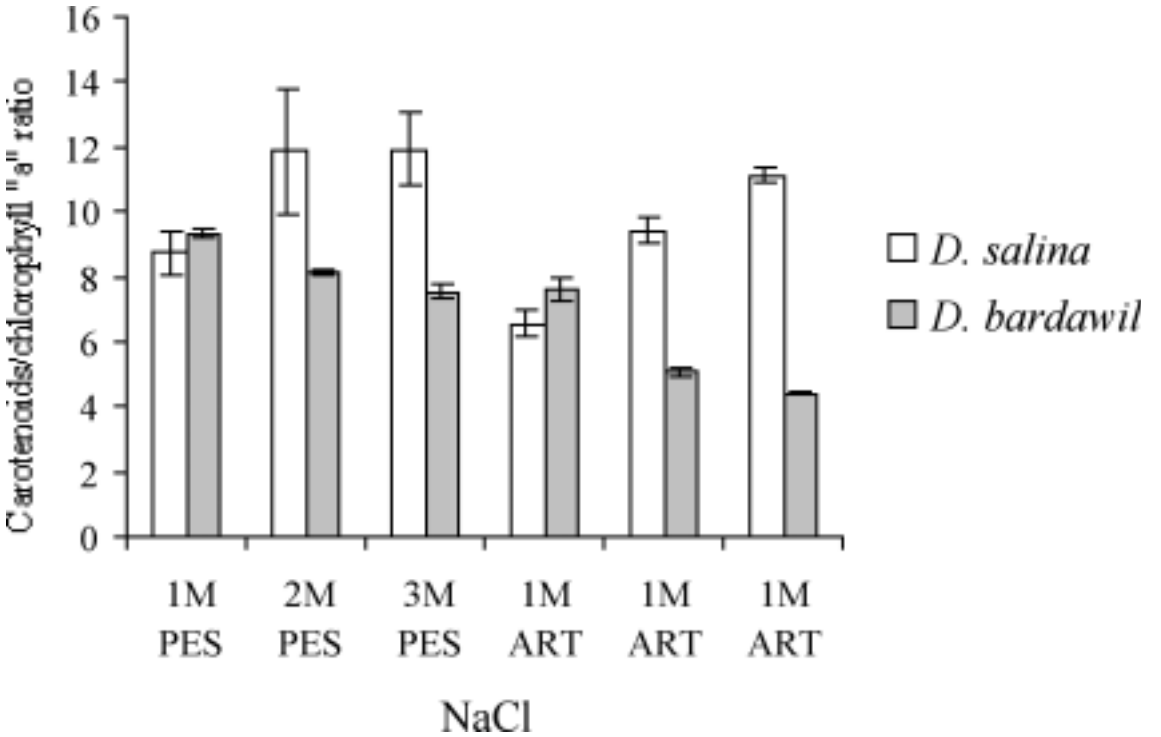

Figure 1
The effect of the medium composition and salinity on the carotenoid to chlorophyll “a” ratio in 28 day-old cultures of *D. salina* and *D. bardawil*, maintained at 25ºC and 40 μmol $m^{-2}s^{-1}$ (n=3 ±SD).

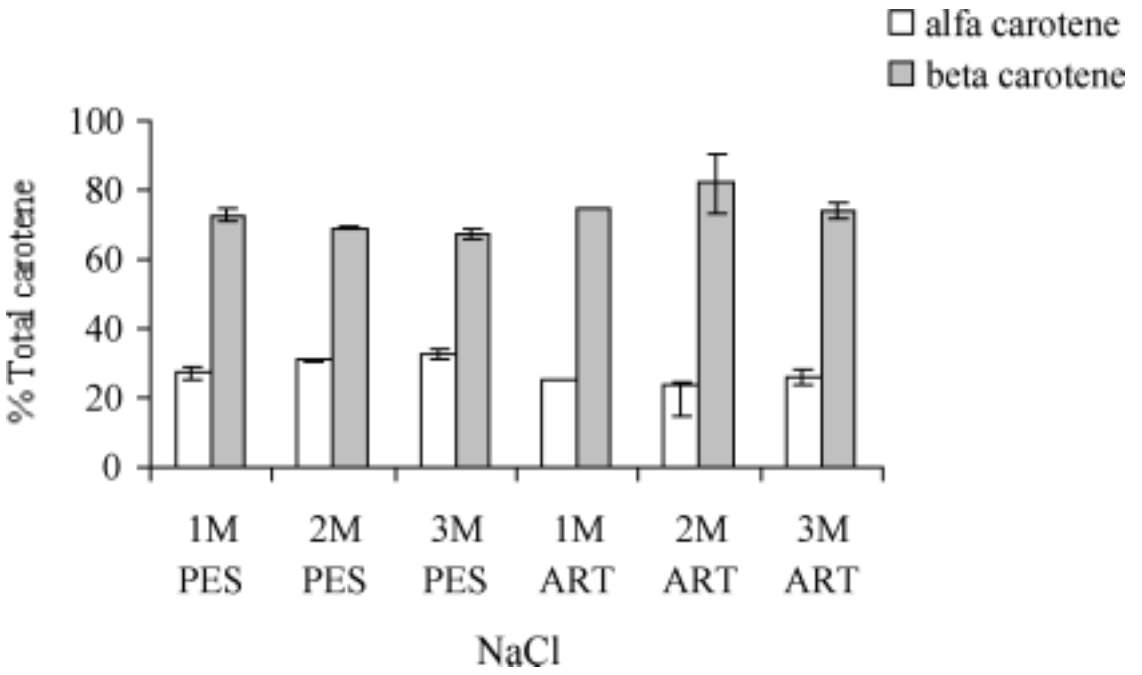

Figure 2
The effect of the medium composition and salinity on the α- and β-carotene percentage in 28 day-old cultures of *D. salina* strain CONC-007, maintained at 25ºC and 40 µmol $m^{-2}s^{-1}$ (n=3 ±SD).

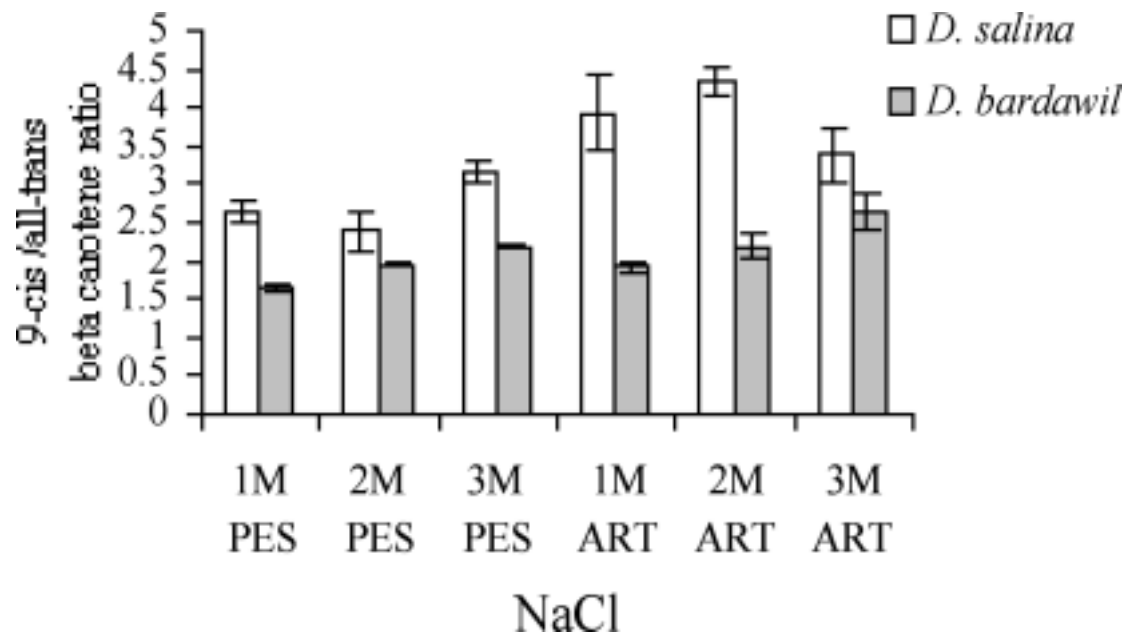

Figure 3
The effect of the medium composition and salinity on the isomeric composition of β-carotene in 28 day-old cultures of *D. salina* and *D. bardawil*, maintained at 25ºC and 40 µmol $m^{-2}s^{-1}$ (n=3 ±SD).

In *D. bardawil*, the 9-cis/all-trans β-carotene ratio increased as the salinity of the medium increased. The maximum value was obtained in ART medium at 3M NaCl (2.6). In *D. salina*, on the contrary, there was no a clear pattern (Fig. 3).

DISCUSSION

Both culture media used in this research have proven to be effective in inducing carotenogenesis in taxa of *Dunaliella*. The PES medium is an enriched natural medium, with a low supplement of nitrate and phosphate ($NaNO_3$ 0.65 mM; Na_2glycerophosphate 0.026 mM). Cifuentes and co-workers (1996a, b) have demonstrated the effectiveness of this medium to induce carotenogenesis in several Chilean strains of *D. salina*. On the other hand, ART medium is an artificial medium already used by Ben-Amotz and co-workers (1988) in *D. bardawil*. Both media have similar nitrate concentrations (see Table I), but ART medium contains inorganic phosphate (KH_2PO_4) at a concentration at least 10 times greater (0.026 versus 0.2) than the organic phosphate of PES medium (Table I).

It is interesting to note that despite the phosphate difference (source and concentration) in the two media, both strains of *Dunaliella* accumulated their maximum total carotenoids in either media (Table II). Since both media contain a limiting nitrogen contribution, this response can be explained by the well-known effect of limitation in this nutrient as an inductive factor of carotenogenesis in *Dunaliella* (Ben-Amotz *et al*., 1982)

Cifuentes and co-workers (1996a) analyzed the effect of the salinity on the accumulation of carotenoids per cell and on the carotenoids/ clorofila "*a*" ratio in *D. salina* strain CONC-007 at stationary phase (3 months) when grew in PES medium. They found that a significant increase in these parameters is observed only above 15% NaCl (2.6 M). On the other hand, Orset and Young (1999) commented that the influence of salinity on carotenoid biosynthesis in *D. salina* does not show clear tendencies between 0.4 and 3.5 M NaCl. In accordance to that, we did not observed a clear pattern on the accumulation of carotenoids per cell along the range 1M to 3M NaCl in the two strains studied (Table II).

While in *D. salina* the carotenoids/ clorophyll "*a*" ratio tended to increase with the salinity, in *D. bardawil* the opposite response was observed (Fig. 1). This result demonstrates the physiological differences exhibited by both strains cultivated in identical conditions, which have been previously observed even between strains of *D. salina* from very close geographic origins (Cifuentes *et al*., 1992; 1996b).

Neither the composition of the medium nor the salinity (in the tried range) significantly affected the accumulation of α and β-carotene of *D. salina* strain CONC-007 (Fig. 2). That means that nitrogen limitation, *per se*, would induce the carotenogenesis in CONC-007 without affecting the relative amount of α and β-carotene. Similar to our results, Orset and Young (1999) did not find a clear effect of the salinity on the levels of α-and β-carotene between 0.5M and 3.5M NaCl, obtaining a practically constant α-carotene/ β-carotene ratio along this range.

The chemical composition of the culture medium, significantly affected the quality of β-carotene in both taxa. At the same salinity, the 9-cis β-carotene isomer was preferentially accumulated in ART medium, a result that enhances the complexity of the carotenogenic response of *Dunaliella*, since the same variable significantly affects a parameter without affecting other closely related one (Figure 3). Unlike *D. bardawil*, *D. salina*, CONC-007 did not increase the 9-cis/all-trans β-carotene ratio when the salinity increased (Fig. 3), this response can be explained by the intrinsic differences between the two strains, which could require different size stimuli for the preferential accumulation of 9-cis β-carotene.

Ben-Amotz and Avron (1983) and Ben-Amotz and co-workers (1988) reported that β-carotene accumulation, as well as 9-cis/ all-trans β-carotene ratio, increased in *D. bardawil* as a function of the amount of light that the alga received during a division cycle. If we consider that the photon flux

density used in the present study was extremely low, the high values of the 9-cis/all-trans β-carotene ratio obtained for both strains is surprising. Nevertheless, Jiménez and Pick (1994), and more recently Orset and Young (2000), found that the synthesis of 9-cis β-carotene is promoted by low (50 μmol $m^{-2}s^{-1}$), rather than by high irradiances in some carotenogenic strains of *Dunaliella*. This finding could contribute to explaining the high proportion of 9-cis β-carotene accumulated by both taxa at 40 μmol $m^{-2}s^{-1}$ (Fig. 3).

Orset and Young (2000) also found that in cultures at its logarithmic phase of growth, the 9-cis/all-trans β-carotene ratio increased from 0.2 to more than 2.1 at the stationary phase, demonstrating the importance of the harvesting time for obtaining high proportions of the 9-cis isomer. Even more, these results allow us to find an explanation for the high values of the 9-cis/all-trans β-carotene ratio obtained here for *D. bardawil* (2.2, Figure 3) under the same culture conditions used previously by Ben-Amotz and co-workers (Ben-Amotz *et al.*, 1988, pp:1287). The latter group of authors do not clearly explain the period of growth at which the cultures were analyzed but, according to the data obtained from our study, it can be assumed that they were in the exponential phase of growth.

Considering the increasing biotechnological interest in searching for highly carotenogenic taxa of *Dunaliella*, showing high 9-cis/all-trans β-carotene ratios, the high values of this ratio obtained for the Chilean strain CONC-007 in ART medium at 1M and 2M NaCl (3.9 and 4.3 respectively, see Fig. 3) give new argument to considering it as a very promising strain for biotechnological applications.

The carotenogenic strains of *Dunaliella* studied showed great physiological plasticity in response to both culture media and salinity. The combination of both factors proved to have a complex effect on the amount and quality of the carotenes accumulated. The results demonstrated that there is no set of unique conditions that favor the carotenogenesis in these microalgae, and that there is a great relevance in the intrinsic capacity of each strain to respond to inductive factors of this ability. In this sense, recent investigations have demonstrated an interesting correlation between the carotenogenic capacity of *D. salina* strains and polymorphisms at level of their genome (Gómez and González, 2001).

ACKNOWLEDGEMENTS

This work was supported by FONDECYT Grant Nº 2990047 to Patricia Gómez.

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