The effect of salinity on the growth and carotenogenesis in two Chilean strains of *Dunaliella salina* Teodoresco

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We studied the effect of salt concentration on the growth and carotenogenesis in two Chilean strains of ***Dunaliella salina****, CONC-006 and CONC-007, cultivated in two media of different chemical composition, J/1 and PES, under controlled laboratory conditions. Growth rates, k (div day^{-1}), intrinsic production rates of total carotenoids per unit time, r_{car} (day^{-1}), maximum levels of total carotenoids, K (mg l^{-1}), and maximum production of total carotenoids per unit time, rK/4 (μg l^{-1} day^{-1}) were estimated from growth and carotenogenesis data. The highest maximum productivity of total carotenoids was 978 μg l^{-1} day^{-1} obtained in CONC-007 at 25% NaCl, and the lowest, 15 μg l^{-1} day^{-1}, in CONC-006 at 30% NaCl, both growing in PES medium. CONC-007 showed the highest growth rates, 0.76 and 0.65 div day^{-1}, at the lowest salt concentration (5%) in PES and J/1, respectively. On the contrary, the strain CONC-006 exhibited a different growth pattern in both media. Its maximum growth rate in J/1 was 0.37 div day^{-1} at 20% NaCl, and in PES, 0.53 div day^{-1} at 5% NaCl. According to these results, the best integration of growth and carotenogenesis in CONC-007 was obtained at 15% NaCl in J/1 and from 10 to 25% NaCl in PES and in CONC-006, from 5 to 20% NaCl in J/1 and from 5 to 10% NaCl in PES.*

Key terms: *carotenoids content, culture media, Chilean strains,* ***Dunaliella salina****, growth rates, salinity range.*

INTRODUCTION

The exceptional ability of *Dunaliella salina* to accumulate massive amounts of carotene in response to high light intensity (Ben-Amotz and Avron, 1983; Borowitzka *et al*, 1984) and growth limiting conditions, such as high salinity and restriction in mineral nutrients (Ben-Amotz *et al*, 1982), has made it an interesting and ideal subject for numerous studies on carotenogenesis. These studies have contributed to establish trial operations for commercial exploitation of this microalga as a source of ß-carotene in various parts of the world (Massyuk, 1966, 1973; Ben-Amotz and Avron, 1980; Borowitzka *et al*, 1984).

Since 1987, eight Chilean strains of *D. salina* have been maintained in our laboratory. Work has been concentrated mainly in establishing the optimal conditions for growth and carotenogenesis and on strain selection (Parra *et al*, 1990; Dellarossa and Cifuentes, 1991; Cifuentes *et al*, 1992, 1996).

Two of them, the strains CONC-006 and CONC-007, have shown a particular appeal for further studies. CONC-006, a large cell strain, shows the highest chlorophyll content when grown under non inductive caro-

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tenogenic conditions (that makes it look deep green). When induced to accumulate carotenoids with a sudden increase in salinity of the growth medium, its response was different from that of other strains. That is, it shows no changes in the kinetic of the total carotenoids accumulation following the salinity up-shock (Cifuentes *et al*, 1992). On the other hand, this strain has the ability to accumulate large amounts of total carotenoids under nutrient limitation (Cifuentes *et al*, 1996). The strain CONC-007 is smaller than CONC-006, and the most carotenogenic under all experimental inductive conditions investigated (Cifuentes *et al*, 1992, 1996).

In this study, we present the growth and carotenogenesis responses of *D. salina* CONC-006 and CONC-007 to a range of NaCl concentrations in two media of different nutritional composition: Johnson modified by Borowitzka (J/1) (Borowitzka, 1988) with a great nutrient supply and Provasoli enriched sea water (PES) (McLachlan, 1973) with a limiting supply of the major nutrients (N and P). The greater the nitrogen concentration, the less is the carotenoid content per cell, but eventually a higher carotenoid content per volume is found, due to a higher cell number. The contrary occurs at limiting nutrient concentrations (Brown and Borowitzka, 1979; Borowitzka and Borowitzka, 1987, 1988). In a previous work, we reported that the mean yields of total carotenoids obtained in eight Chilean strains grown in different culture media were just some higher in PES than in the other media assayed (Cifuentes *et al*, 1996). The objective of this study was, therefore, to find the optimal salt concentrations at which one can obtain the best integration of growth and carotenogenesis in both strains in each medium.

MATERIALS AND METHODS

Both strains of *D. salina*, CONC-006 and CONC-007, were obtained from the Algal Culture Collection (Departamento de Botánica, Universidad de Concepción) where they are maintained in unialgal cultures.

For the experiments presented here, the strains were grown in two culture media: Johnson modified by Borowitzka (J/1) (Borowitzka, 1988) and Provasoli enriched sea water (PES) (McLachlan, 1973). J/1 is an artificial culture medium with a high nutrient supply (10 mM KNO_3, 0.25 mM KH_2PO_4 and 0,14 mM Na_2HPO_4) and PES is a natural medium enriched with a limiting supply of the major nutrients (0.6 mM $NaNO_3$ and 0.026 mM Na_2 glycerophosphate x 5 H_2O). The strains were maintained in both media at a range of salinities from 5 to 30% NaCl with 5% intervals, under a continuous photon flux density of 150 µmol $m^{-2}s^{-1}$ provided from a rack of cool-white fluorescent tubes, horizontally arranged at both sides of the incubator chamber. Incubation temperature was 30 ± 2°C, without aeration. Exponentially growing cells from stock cultures acclimatized to these experimental conditions were used as inocula. The experiments were carried out in flasks containing 200 ml of the respective medium. The required volume of the inoculum was assessed by cell counting of the stock cultures in order to have an initial cell density of 5 x 10^3 cells per ml. When the volume of the inoculum exceeded 10 ml (at higher salinities), the stock culture was concentrated by centrifugation (1000 rpm for 5 minutes; appropriate to get viable and healthy cells).

Growth and carotenogenesis were monitored over a 60 day period. Growth rate **k** (divisions day^{-1}) was estimated during the phase of growth according to the formula: $k = (3{,}322/ t_2 - t_1) \times \log N_2/N_1$ (t = time, N = number of cells. Subscripts denote values at different times) (Guillard, 1973). Pigment content was estimated as in Cifuentes *et al* (1992). A final quantification of cell density, total carotenoids and chlorophyll was made at 3 months, when the cultures were at late stationary phase of growth. Due to operational reasons, only 2 flasks of each treatment were established. Therefore, the data represent averages from these two replicas.

To estimate the production rate of total carotenoids, the logistic model of growth was applied. The differential form of the logistic equation is: $dN/ dt = rN (K-N)/ K$, where N = concentration of total amount of carotenoids, r = "intrinsic" growth rate of carotenoids content per unit time (day^{-1}), K = "carrying capacity" or maximum level of carotenoids. For any value of N (expressed

as carotenoids concentration) the rate of production of carotenoids dN/dt is obtained. The maximum production of carotenoids per unit time occurs when N = K/2 and is rK/4 units of carotenoids per unit time (Moulton *et al*, 1987). According to the results, the optimal salt concentrations to obtain the best integration of growth and carotenogenesis were determined for both strains in each medium.

RESULTS

The strain CONC-007 grew better than CONC-006 with a high concentration of cells at 30 day-growth, in all assayed conditions (Fig 1). In J/1 medium, the strain CONC-007 showed high growth rates at low salt concentration (5-10% NaCl) while CONC-006 grew faster at intermediate salinities (15-20% NaCl). At 20% NaCl the rate of growth of this strain overpassed the one exhibited by CONC-007 (Fig 2A). In PES medium, on the contrary, both strains showed high growth rates at low salt concentrations (5-10% NaCl), decreasing at higher salinities (Fig 2B). The mean growth rates determined at 30 days, for either strain, were much the same under the all range of salinities in both culture media (Fig 2A, B).

The strain CONC-007 reached the highest growth rates at the lowest salt concentration assayed (5% NaCl) in both cultured media: 0.76 div day^{-1} and 0.65 div day^{-1}, in PES and J/1, respectively. They decreased gradually at higher salinities, reaching minimum values at 30% NaCl, 0.12 and 0.09 div day^{-1}, in PES and J/1, respectively (Fig 2A, B). However, the carrying capacities of the cultures at the stationary phase of growth were much higher in J/1 than in PES, where they reached 3 x 10^6 cells ml^{-1} from 5 to 20% NaCl (Fig 4A). In PES, the cell density overpassed the million of cells per ml only at 15% NaCl (Fig 5A). Even though CONC-007 exhibited the best growth rate at 5% NaCl, the production of cells at the end of the experiment was low because the growth ceased at the 8th day of culture.

The strain CONC-006 showed a different response pattern in both media. In J/1, the highest growth rate (0.37 div day^{-1}) occurred

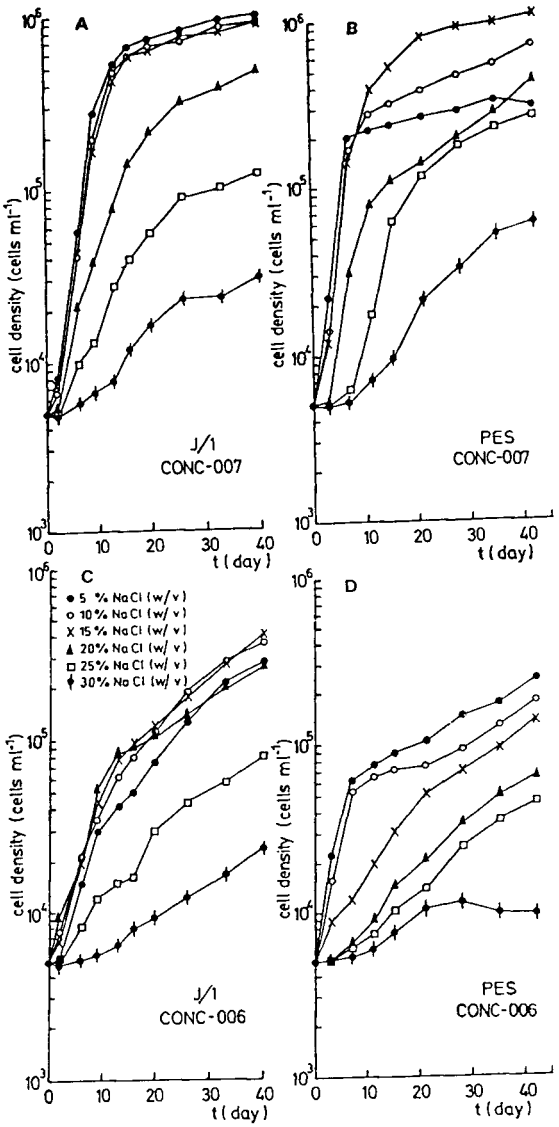

Fig 1. Comparison of growth (as cell density) of *D. salina*, strains CONC-006 and CONC-007, in J/1 and PES media at different salt concentrations, under a continuous photon flux density of 150 µmol m^{-2} s^{-1}, 30 ± 2° C, without aeration.

at 20% NaCl, being slightly lower at reduced salinities (5,10 and 15% NaCl), while in PES the highest growth rate was reached at 5% NaCl (0.53 div day^{-1}), and decreased abruptly at salinities of 15% NaCl and higher (Fig 2A, B).

The mean productivity of total carotenoids during the growth period was always higher in both strains when growing in PES at any salinity (Fig 3A-D; Tables I and II), except for CONC-007 at 5% NaCl and CONC-006 at 30% NaCl, conditions at which the production of cells of either strain was deficient.

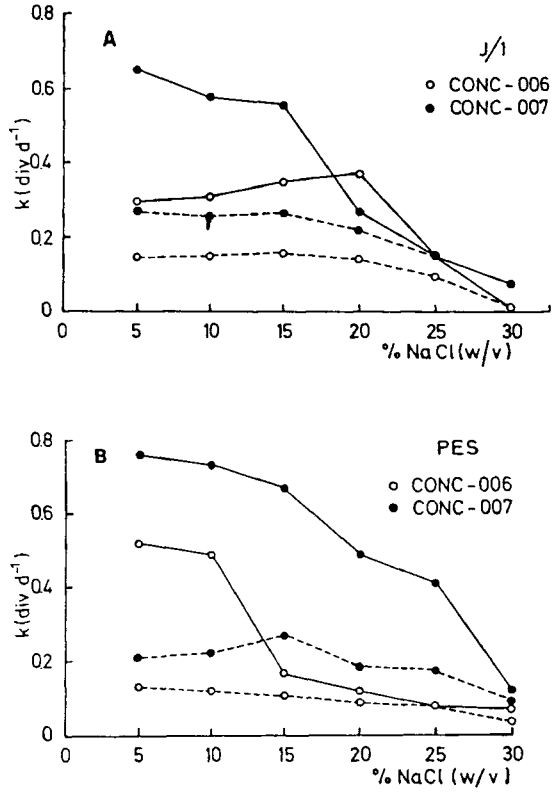

Fig 2. Growth rate (div day^{-1}) of *D. salina*, strains CONC-006 and CONC-007, during the exponential phase of growth (——) and mean growth rate after 30 days (- -), in J/1 and PES at different salt concentrations. Culture conditions as in Figure 1.

The total carotenoids content of the strains growing in PES varied from 1.2 mg l^{-1} (30% NaCl; CONC-006) to 23.9 mg l^{-1} (25% NaCl; CONC-007) (Table I). The strain CONC-006 exhibited the highest total carotenoids content in a range from 5 to 20% NaCl, in both media. CONC-007, on the contrary, presented the highest total carotenoids contents from 5 to 25% NaCl in J/1 and from 10 to 25% NaCl in PES (Tables I and II). The highest maximum productivity of total carotenoids was 978 µg l^{-1} day^{-1} in CONC-007 at 25% NaCl in PES. A much lower value, 390 µg l^{-1} day^{-1}, was obtained in CONC-006 at a salinity range of 5 to 15% NaCl, in the same medium (Table II).

In both strains, the total carotenoid content per cell, determined at late stationary phase of growth, was always higher in PES than in J/1 medium, when compared at the same salinity (Figs 4B, 5B). The highest value was 120 pg cell^{-1} in CONC-006 cultured in PES at 25% NaCl (Figs 4B, 5B). In general, there

TABLE I

Production of total carotenoids by *D. salina* (strains CONC-006 and CONC-007) grown in J/1 medium at different salt concentrations. (r_{car} = total carotenoids production rate; K = maximum level of total carotenoids; rK/4 = maximum production of total carotenoids; k_{car} = mean production (30 days for 5, 10, 15, 20% NaCl; 60 days for 25, 30% NaCl)

NaCl (%)	r_{car} (day^{-1})	K (mg l^{-1})	rK/4 (µg l^{-1} day^{-1})	k_{car} (µg l^{-1} day^{-1})
		CONC-006		
5	0.1108	6.5	180.0	190.3
10	0.1047	6.1	159.7	164.5
15	0.1013	7.0	177.3	151.6
20	0.1009	7.4	186.7	200.0
25	0.0592	4.5	66.6	58.4
30	0.0567	3.3	46.8	49.2
		CONC-007		
5	0.1761	8.8	387.4	277.4
10	0.2003	8.0	400.6	239.0
15	0.1946	14.3	695.6	411.6
20	0.1782	14.6	650.4	435.4
25	0.1115	11.2	312.2	182.0
30	0.0920	4.9	112.7	78.7

TABLE II

Production of total carotenoids by *D. salina* (strains CONC-006 and CONC-007) grown in PES medium at different salt concentrations. (r_{car} = total carotenoids production rate; K = maximum level of total carotenoids; rK/4 = maximum production of total carotenoids; k_{car} = mean production (30 days for 5, 10, 15, 20% NaCl; 60 days for 25, 30% NaCl)

NaCl (%)	r_{car} (day^{-1})	K (mg l^{-1})	rK/4 (µg l^{-1} day^{-1})	k_{car} (µg l^{-1} day^{-1})
		CONC-006		
5	0.088	17.7	389.4	382.0
10	0.086	18.3	393.5	410.3
15	0.100	15.4	385.0	323.1
20	0.079	15.6	308.1	261.4
25	0.085	6.6	66.6	140.2
30	0.050	1.2	15.0	28.0
		CONC-007		
5	0.144	4.9	176.4	146.9
10	0.142	16.0	568.0	348.7
15	0.139	18.9	656.8	435.9
20	0.164	22.0	902.0	425.6
25	0.163	23.9	978.0	423.1
30	0.103	5.6	142.2	117.9

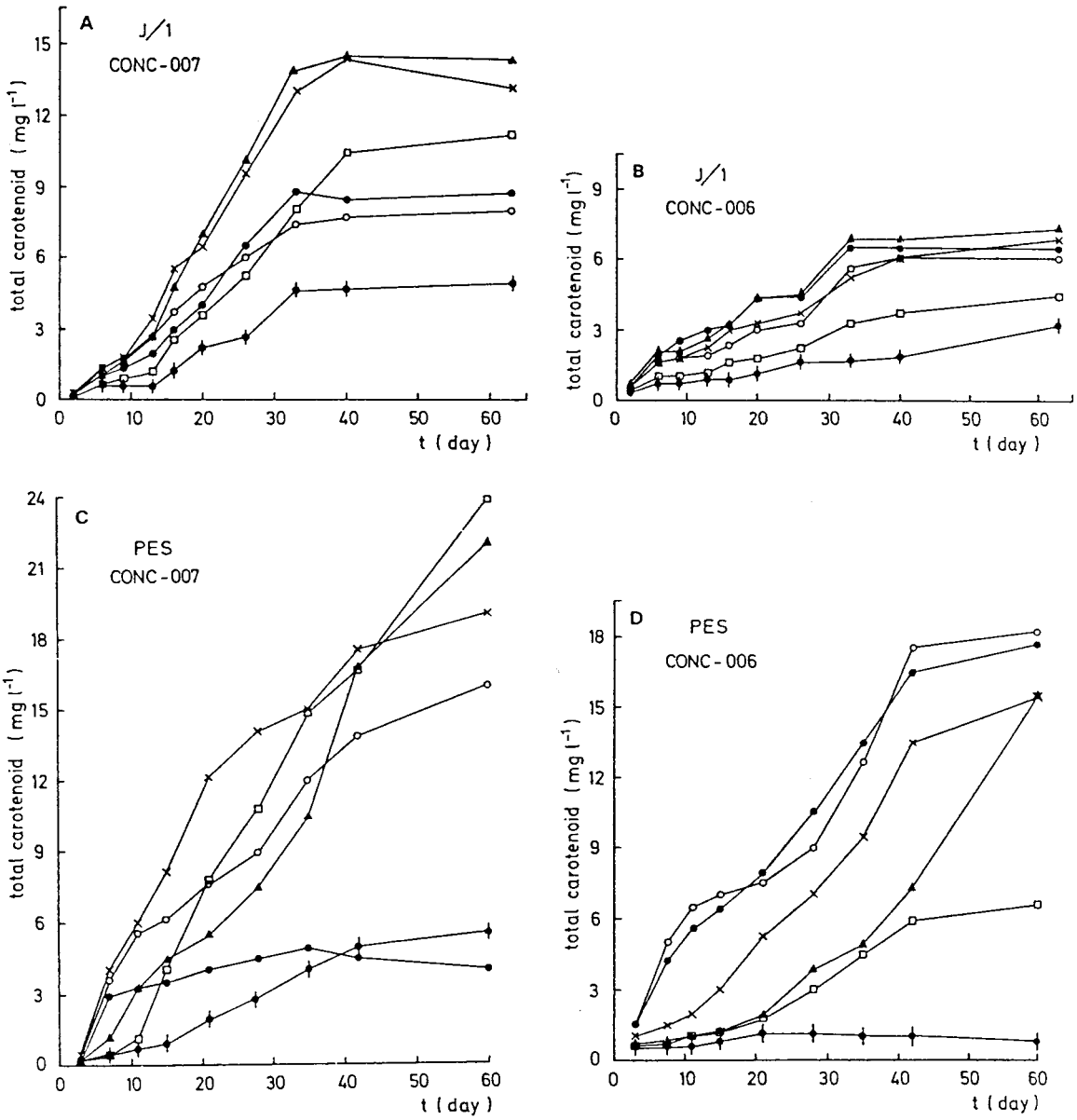

Fig 3. Changes in total carotenoids content of *D. salina*, strains CONC-006 and CONC-007, during growth in J/1 and PES at different salt concentrations. Culture conditions as in Figure 1.

was an abrupt increase of this parameter at salinities higher than 15 or 20 % NaCl, with the exception of CONC-006 at 30% NaCl. The total carotenoid to chlorophyll ratio increased concomitantly with the increase in salt concentration and CONC-007 showed the widest variation of this ratio: 2 to 70 g g^{-1} in PES. The strain CONC-006, on the contrary, exhibited in this medium carotenoid to chlorophyll ratios lower than 30 g g^{-1} because of its higher chlorophyll content at the highest salinities (Fig 5B, D).

The best integration of growth and carotenogenesis was obtained at 15% NaCl in CONC-007 and from 5 to 20% NaCl in CONC-006, both in J/1 medium; in PES, from 10 to 25% NaCl in CONC-007 and from 5 to 10% NaCl in CONC-006 (Fig 5A, B).

DISCUSSION

The media J/1 and PES are different in terms of nutrients. According to Cifuentes *et al*

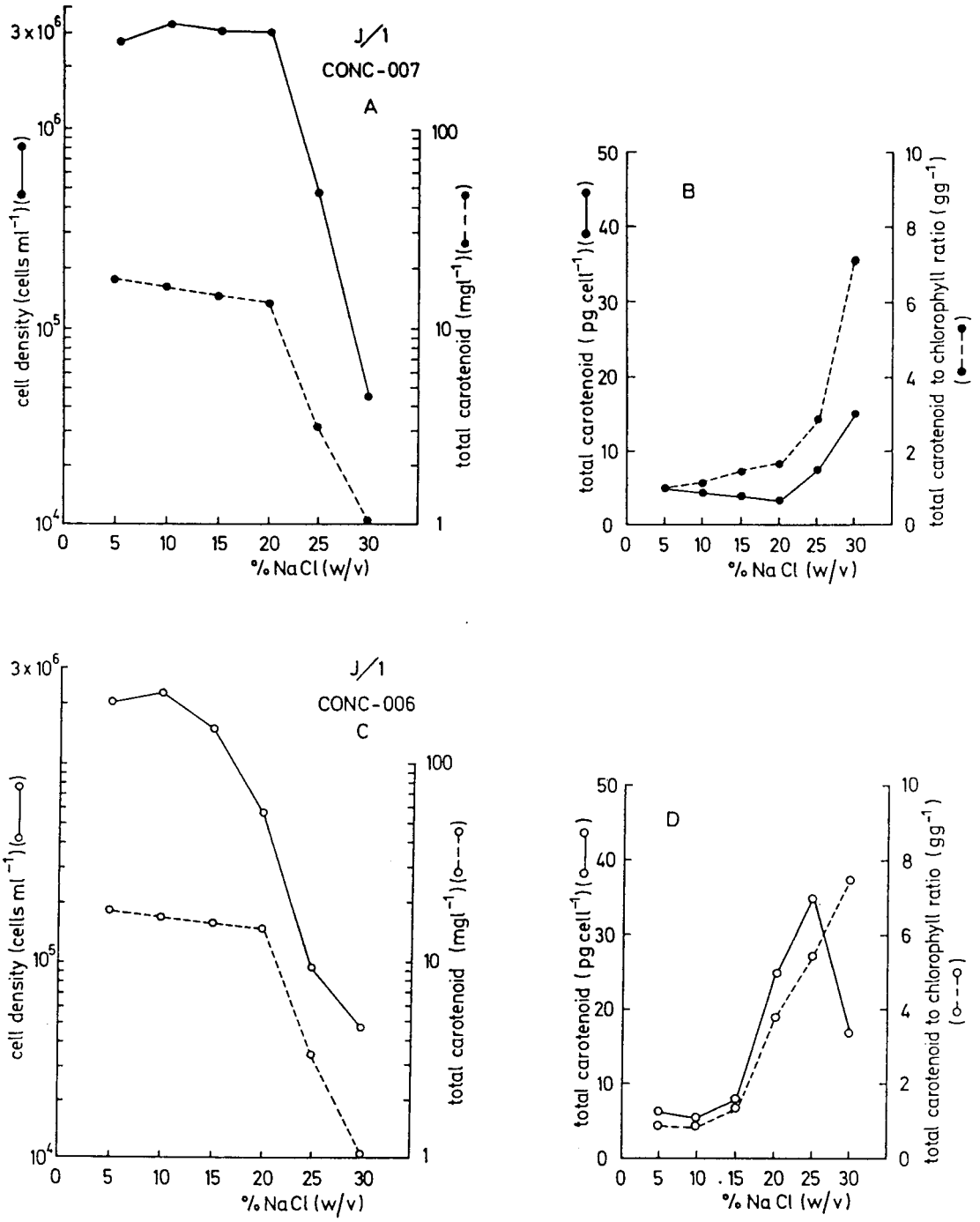

Fig 4. *D. salina*, strains CONC-007 (A,B) and CONC-006 (C, D), at stationary phase of growth (3 months), in J/1 at different salt concentrations. **A,C.** Cell density (——-) and total carotenoids content per volume (- -). **B,D.** Total carotenoids content per cell (——-) and total carotenoids to chlorophyll ratio (- -). Culture conditions as in Figure 1.

(1996), *D. salina* exhibited the highest concentration of cells in J/1, and the highest total carotenoids content per cell in PES, when these parameters were estimated in batch cultures of small volume, and at late stationary phase of growth. The maximal concentrations of cells (Nmax) and total carotenoids (K, mg l^{-1}) estimated in this study were lower than those reported by Cifuentes *et al* (1996) under similar

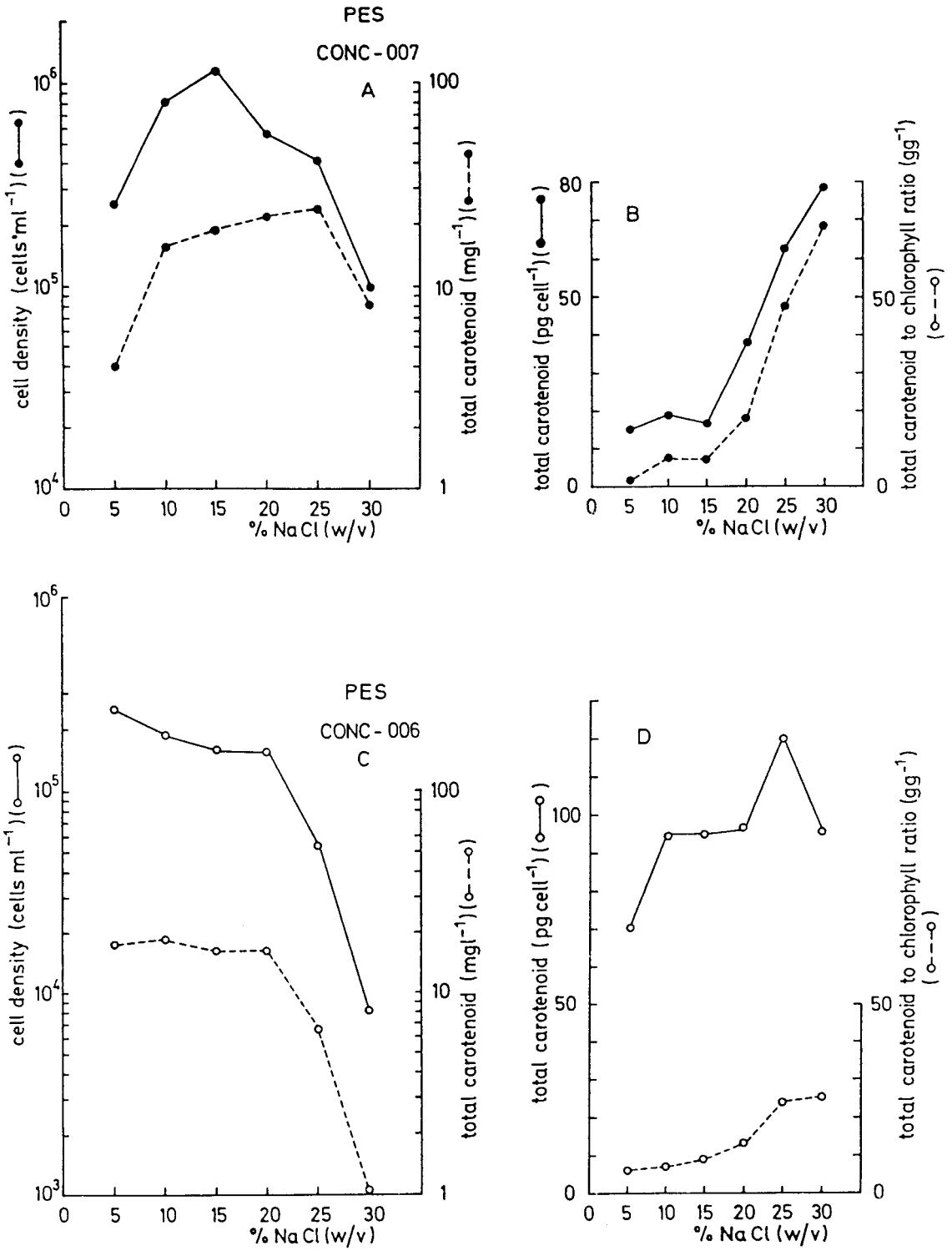

Fig 5. *D. salina*, strains CONC-007 (A,B) and CONC-006 (C,D), at stationary phase (3 months), in PES at different salt concentrations. **A,C.** Cell density (——) and total carotenoids content per volume (- -). **B,D.** Total carotenoids content per cell (——) and total carotenoids to chlorophyll ratio (- -). Culture conditions as in Figure 1.

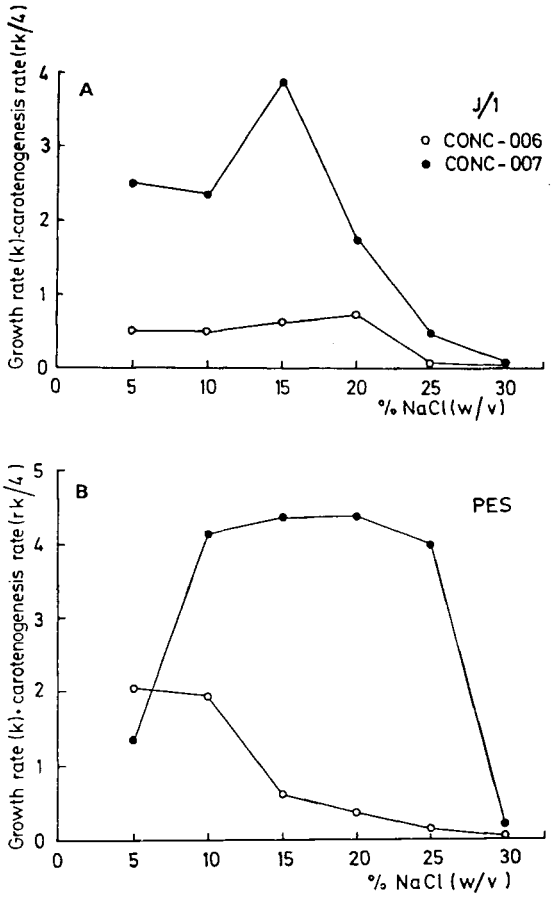

Fig 6. Effect of salt concentration on the integral growth rate per carotenogenesis rate when *D. salina* is cultured in J/1 (A) and PES (B), under conditions given in Figure 1. Peak values evidence optimal salt concentrations for optimal compromise between both parameters.

conditions of salinity and culture medium. This is probably due to a reduced effective photon flux density reaching each cell and poor gas exchange because the cultures in this study were larger in volume (10 ml versus 200 ml). This reduction in productivity has been observed in our laboratory when scaling up in volume of the culture, and has been also reported by various authors as a phenomenon of common occurrence in some large-scale microalgal operations (Goldman, 1979) and/or after the transition from indoor to outdoor cultures (Moulton *et al*, 1987; Moulton and Burford, 1990).

In relation to the accumulation of carotenoids, our experience, as well as many other studies, reveal the existence of two stages in this process. The first stage starts shortly after exposure to an inductive factor (with lag phases depending on the strains), and the second, with the onset of the stationary phase of growth (Ben-Amotz and Avron, 1983; Borowitzka *et al*, 1990; Lers *et al*, 1990; Cifuentes *et al*, 1992). The strains used in the trials were previously acclimatized to the culture media, light, temperature and salinity; thus, sudden induction of carotenogenesis did not occur. The increase in total carotenoids content fits quite well with the increase in number of cells along the period of growth. Therefore, and as Moulton *et al* (1987) already stated, the data of carotenoids content adjusted very nearly to the logistic model of growth.

Despite the different nutritional composition of the two media used, the extent of variation of the growth rates estimated in each medium and in the range of salinities assayed was very similar: 0.018 (30 % NaCl; CONC-006) to 0.65 div day^{-1} (5% NaCl; CONC-007) in J/1 and 0.07 (30% NaCl; CONC-006) to 0.76 div day^{-1} (5% NaCl; CONC-007) in PES. As it was discussed by Cifuentes *et al* (1996), the higher or lower supply of nutrients does not affect the growth rate of the strains within certain limits, but determines the carrying capacity of the cultures. In PES, the transition from the exponential to the stationary phase of growth is due probably to a sudden exhaustion of nutrients. In J/1, on the contrary, the exponential phase of growth is followed by a prolonged phase of linear growth, characteristic of cultures in which light or other factors become limiting after a determined cell density (Fogg, 1966). Accordingly, in this culture medium, the maximum cell densities and total carotenoids content estimated at late stationary phase (3 months) were higher and the carotenoids per cell much lower than those estimated at the end of the exponential phase of growth. The light-limited condition, caused by self shading at high cell density, produces cells with a low carotenoid content and a high content of chlorophyll (Loeblich, 1982; Ben-Amotz, 1986; Ben-Amotz and Avron, 1983; Lers *et al*, 1990).

The comparison among growth rates, carotenoids content and carotenoid to chlorophyll ratios reported by other authors and

those previously determined in Chilean strains, are well discussed in Cifuentes *et al* (1992, 1996). Moulton and Burford (1990) found similar values of carotenoids content per cell (50-150 pg ß-carotene cell^{-1} at *ca.* 20% NaCl) to those reported here for *D. salina*. More recently, Araneda *et al* (1992) working with three Chilean strains (CONC-001, CONC-003 and CONC-007), found that CONC-007 accumulated the highest carotenoid content (90-100 pg cell^{-1}) under all the conditions assayed by them (two different irradiances and two culture media with two different nitrate concentrations).

The relationship among salinity, growth and carotenogenesis is complex. The greater the growth, the lesser is the accumulation of carotenoids. This is the basic dilemma in building the model to optimize the production of carotenoids at a commercial level. But a high productivity of cells can compensate a low production of carotenoids at cellular level, as it occurs in *D. viridis* cultures (Moulton and Burford, 1990). The results revealed that in either strain, CONC-006 and/or CONC-007, a wide range of salinities was found appropriated to obtain the best integration between growth and carotenogenesis. If a culture at higher scale is planned, the highest salt concentration should be preferred, because at higher salinities there is a minor danger of predation and/or presence of competitor organisms. Besides, it should be considered that variable periods of time are required to obtain a culture with a high cell density. The duration of the exponential growth phase, and the occurrence of slower growth phases at the beginning or at the end of the exponential phase, depend on the strain, on the culture medium, on the salinity and probably, on other factors such as the size and quality of the inoculum, the initial cell density, acclimation time (Borowitzka *et al*, 1977) and, on the temperature as demonstrated by Ginzburg and Ginzburg (1985).

The strain CONC-006 is large (22.5 µm length, 18.0 µm wide) and has a high chlorophyll content, which under certain culture conditions, can mask the actual carotenoids content (Cifuentes *et al*, 1992). In media with no limitation of nutrients (J/1), this strain presents the most common pattern of growth exhibited by halophilic species (Ginzburg and Ginzburg, 1985): a higher growth rate at intermediate salinities (15-20% NaCl). The strain CONC-007, on the contrary, exhibits higher rates in the lower range of salinities (5-15% NaCl), with a significant decrease starting at 20% NaCl. These results add physiological differences to those found in previous studies (Cifuentes *et al*, 1992, 1996) and they suggest a higher halophilism of strain CONC-006, under no-limiting nutrient condition (J/1), compared with strain CONC-007. This would explain, in part, the lack of change in the kinetics of carotenogenesis that this strain shows when it is exposed to abrupt increase in salt concentration (Cifuentes *et al*, 1992). More research at the molecular level (DNA) should help to elucidate the physiological differences found between both strains.

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