Physiological characterization of *Dunaliella* sp. (Chlorophyta, Volvocales) from Yucatan, Mexico

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Received 7 March 2006; received in revised form 20 May 2006; accepted 20 May 2006

Available online 1 September 2006

Abstract

Physiological responses of *Dunaliella salina* and *Dunaliella viridis*, isolated from solar saltworks on the Yucatan Peninsula, were studied. Optimal growth temperature for *D. salina* was 22 °C (3.06 × 10⁶ cells mL⁻¹) and 26 °C for *D. viridis* (4.04 × 10⁶ cells mL⁻¹). Total carotenoid content in *D. salina* increased with temperature to a maximum of 35.14 pg cell⁻¹ at 38 °C. *Dunaliella salina* α-carotene and β-carotene content was 0.083 ± 0.003 and 0.598 ± 0.020 mg 100 g dry wt⁻¹ respectively, whereas lower values were found in *D. viridis* cultured under same experimental conditions (0.018 ± 0.002 and 0.136 ± 0.012 mg 100 g dry wt⁻¹ respectively). The highest specific growth rate in *D. salina* was obtained at 10% NaCl (0.28 d⁻¹), while its cell volume increased from 524 to 2066.93 l m⁻³ when cultured from 10% to 35% NaCl. Maximum photosynthetic rates were attained when increasing from optimal growing temperature to 30 °C for *D. viridis* (108 nmol O₂ l⁻¹ g chl a h⁻¹) and *D. salina* (139 nmol O₂ l⁻¹ g chl a h⁻¹). Photosynthetic responses to temperature variations indicated physiological adjustments in both species, with higher acclimation in *D. salina*. Evaluation of physiological attributes of these species will be used for to carry out mass cultivation.

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Keywords: *Dunaliella salina*; *Dunaliella viridis*; Photosynthesis; Salinity; Temperature

1. Introduction

The 23 species of the genus *Dunaliella* are wall-less flagellate eukaryotic algae found in saline environments and exhibiting optimal growth at different salt concentrations with varying abilities to turn orange–red under particular culture conditions (Massyuk, 1973). Recognition that members of the genus *Dunaliella* produce abundant carotenoid pigments under certain conditions was the scientific finding enabling the commercial application. *Dunaliella salina* (Dunal) Teodoresco and *D. viridis* Teodoresco are the predominant phytoplankton species in intermediate and high salinity ponds in commercial, solar saltworks (Davis, 1990). To date, *D. salina* strains are probably the most successful microalgae in mass cultivation, partially due to their high salinity requirements, which minimize the number of competitors and predators (Ben-Amotz et al., 1991). This species forms red blooms in the water because it accumulates about 14% of its dry weight as β-carotene, a valuable ingredient in the food and feed industries (Jin and Melis, 2003). *Dunaliella viridis*, in contrast, is not a β-carotene accumulative, and has been used as live feed in marine aquaculture.

Presently commercial cultivation of microalgae is targeted to direct human consumption for the health food market, and for extractable compounds such as nutritional supplements or food additives (e.g. β-carotene colouring, xanthophyll pigments like astaxanthin, and the fatty acids DHA and EPA) (Wikfors and Ohno, 2001). Commercial activity in the microalgae extractable chemical sector is currently limited to two main products: *Dunaliella*-derived carotenoid pigments as human nutritional supplements; and *Haematococcus*-derived pigment astaxanthin as a colouring agent (Jin and Melis, 2003). Microalgae culture has followed two main paths: one focused on isolation...
and physiological characterization of species or strains to evaluate their desirable characteristics for mass cultivation (e.g. rapid growth rate, broad tolerance of environmental conditions, high product content in cells); and the other on engineering of culture systems.

One of the main biological constraints on outdoor algal biotechnology is limited availability of a wide variety of algae species and improved strains that respond favourably to varying environmental conditions. This study provides growth and photosynthetic characterization of two Dunaliella species isolated from commercial solar saltworks at the Yucatan Peninsula, Mexico. Evaluation of physiological attributes will play an important role in further development of biotechnology for mass cultivation of these species.

2. Methods

2.1. Strain isolation and cultivation

Dunaliella salina and D. viridis were isolated from evaporating salt-ponds at Las Coloradas (21°36'N–87°59'W) and San Crisanto (21°22'N–89°08'W), in the state of Yucatan, Mexico. Identification of the isolates was established based on morphological and ultrastructural characters following Massyuk (1973). After isolation, stock cultures were established for both species under controlled laboratory conditions (29 ± 1°C, 80 μmol photons m⁻²s⁻¹, 12 L:12 D) in a modified Johnson medium (J/1) for Dunaliella sp. (Johnson et al., 1968) at 20% NaCl (w/v).

2.2. Experimental culture conditions

Both species were cultivated at six NaCl concentrations (10%, 15%, 20%, 25%, 30% and 35%) in 1 L flasks at 29 ± 1°C and 80 μmol photons m⁻²s⁻¹. Five replicate cultures per NaCl concentration were done. All experiments began by inoculating 10% of the flask volume with algae in the logarithmic growth phase. Cells were grown in J/1 medium and NaCl added as needed to obtain the target salinity. Specific growth rate and cellular volume were calculated for both species at each NaCl concentration. Cell growth was determined via cell counts using a modified Neubauer hematocytometer. Specific growth rate μ (d⁻¹) was calculated during the exponential growth phase according to

\[ \mu = (\ln x_2 - \ln x_1)/(t_2 - t_1) \]  

where \( x_1 \) and \( x_2 \) is the cell number at times \( t_1 \) and \( t_2 \), respectively, time in days (d).

For each salinity treatments 60 cell measurements were taken using an inverted microscope. Cell volume was calculated for both Dunaliella species assuming a prolate ellipsoid form (Hillebrand et al., 1999) with the formula

\[ V = \pi/6 \cdot d^2 \cdot h \]  

where, \( d \) is diameter and \( h \) the transverse radii of the cell.

Dunaliella salina and D. viridis were incubated in 1 L flasks in a temperature controlled culture chamber at six different temperatures (18, 22, 26, 30, 34 and 38 °C) over a 17-day period. The J/1 medium was used at 20% NaCl (w/v) under a continuous irradiance of 180 μmol photons m⁻²s⁻¹ and continuous air bubbling. These experimental conditions were selected to determine the increase in cell density and pigment composition (chlorophyll \( a \) and total carotenoids) over time. Cell densities were followed by daily counts and are presented as the means ± SD of the five replicates.

2.3. Pigment extraction

Pigments were extracted from algae collected after 5, 10 and 15 days cultivation. Cells were pelleted by centrifugation at 3000 rpm of 10 mL of culture medium. Chlorophyll \( a \) was extracted with 80% (v/v) acetone/water, measured at 665 nm and calculated according to Mackinney (1941). Total carotenoids were extracted with absolute methanol. Absorbance was measured at 480, 663 and 645 nm with a UV/Vis spectrophotometer (Shimadzu UV–1601, Japan) and calculated according to Davies (1976). Pigment composition is presented as the means ± SD of five replicates. In addition, \( \alpha \)-carotene and \( \beta \)-carotene were analyzed by HPLC coupled with a UV Detector (450 nm). A Luna C-18 reverse phase column (4.6 × 250 mm, 10 μm particle size) with a mobile phase of CH₃CN:CH₂Cl₂:CH₃OH (70:20:10) at a flow rate of 1.0 mL min⁻¹ was used. Concentrations (mg 100 g dry wt⁻¹) were calculated using a five-point calibration curve for pigment standards of \( \beta \)-, \( \beta \)-, and \( \beta \)-carotene (\( \alpha \)-carotene) obtained from Sigma corporation. D. salina and D. viridis \( \alpha \)- and \( \beta \)-carotene contents were compared with D. salina isolated from an hypersaline pond at the east coast of the Island of Gran Canaria, Spain (kindly provided by Gómez-Pinchetti et al., 1992) grown under same experimental culture conditions.

2.4. Photosynthesis measurements

Net photosynthesis of D. salina and D. viridis cells grown at optimum growth temperatures determined in a previous experiment, (22 and 26 °C respectively) were measured using a Clark-type oxygen electrode (Oxygraph, Hansatech Instruments, Norfolk, England). Temperatures were controlled during photosynthetic measurements with an external recirculation water bath (Hakke, Berlin, Germany). Cells in the exponential growth phase were incubated in a 2 mL cuvette for liquid-phase measurements (DW2/2). The samples were pre-incubated in the dark for 3 min, and then exposed for another 54 min to a series of eighteen increasing Photon Flux densities (PFD) ranging from 25 to 1475 μmol photons m⁻²s⁻¹. The Photosynthesis versus Irradiance curves (P–E) were used to calculate photosynthetic parameters for each species. P–E curves are the means of ten replicates ± SD.
To evaluate the short term effect of suboptimal temperature on photosynthesis, *D. salina* cultured at 22 °C and *D. viridis* at 26 °C, were exposed to one of six temperatures (18, 22, 26, 30, 34 and 38 °C) during photosynthesis measurements. The short term effect of temperature on photosynthesis was evaluated by measuring light-saturated photosynthesis during a 1 h exposure to each temperature. Maximum photosynthetic rate (*P*<sub>max</sub>) is expressed as nmol O<sub>2</sub> · h<sup>-1</sup> normalized to chlorophyll *a* content (µg) for each temperature.

2.5. Statistical analysis

Data were tested for normality (Kolmogorov-Smirnoff) and homocedasticity (Cochran-Bartlett). An analysis of variance (ANOVA) was used to determine differences between treatments. A Tukey HSD multiple comparisons of means test was used when significant differences were found.

3. Results

*Dunaliella salina* exhibited higher specific growth rates than *D. viridis* in all NaCl treatments (*p* < 0.05) (Fig. 1A). Maximum growth for *D. salina* was obtained at 10% NaCl (0.28 ± 0.01 d<sup>-1</sup>), and decreased as salinity increased. *D. viridis* exhibited maximum growth at 15% NaCl (0.179 ± 0.006 d<sup>-1</sup>) with no significant differences between 20% and 25% NaCl concentrations. Cell volume in *D. salina* increased linearly from 524 to more than 2000 µm<sup>3</sup> in response to increasing NaCl concentrations (Fig. 1B). Maximum cell volume in *D. salina* was obtained at 35% NaCl (2067 ± 305 µm<sup>3</sup>). Significant differences in cell volume were found at different NaCl concentrations (*p* < 0.05). Cell volume in *D. viridis* did not change significantly in response to NaCl concentration. Mean cell volume in this species was 114 ± 31 µm<sup>3</sup>, with a maximum recorded value of 220 µm<sup>3</sup>.

Temperature clearly affected cell density in both *D. salina* and *D. viridis* (Fig. 2). *D. salina* attained maximum cell density at 22 °C (3.06 ± 0.30 · 10<sup>6</sup> cell mL<sup>-1</sup>) and the lowest concentrations at 38 °C (0.43 ± 0.06 · 10<sup>6</sup> cell mL<sup>-1</sup>). Significant differences in cell density were found at the end of the cultivation period for all tested temperatures (*p* < 0.05), except 18 and 22 °C (Fig. 2A). Maximum cell density for *D. viridis* (4.04 ± 0.14 · 10<sup>6</sup> cells mL<sup>-1</sup>) was obtained at 26 °C and lowest densities (0.84 ± 0.11 · 10<sup>6</sup> cell mL<sup>-1</sup>) at 38 °C (Fig. 2B). No significant differences in...
cell density were found between 22 and 26 °C at the end of the cultivation period ($p > 0.05$).

Dunaliella salina exhibited two times higher chlorophyll a content than D. viridis (Fig. 3A). Chlorophyll a content in D. salina ranged from 1.96 to 4.29 pg cell$^{-1}$ and decreased with time at 18 and 22 °C. No significant differences in chlorophyll a content per cell were found at higher temperatures ($\geq 26 ^\circ C$) in relation to culture time (5, 10, 15 days) ($p > 0.05$). In D. viridis no significant differences on chlorophyll a content were found in relation to culture time (5, 10, 15 days) for the experimental temperatures, except for 18 °C at day 5, and 30 °C at days 10 and 15 (Fig. 3B). Chlorophyll a content expressed per cell at the end of the cultivation period ranged from 1.34 to 4.09 pg cell$^{-1}$.

Dunaliella salina exhibited ten times higher carotenoid content than D. viridis at all temperatures (Fig. 3C). Carotenoid content in D. salina increased with time for temperatures $\geq 26 ^\circ C$, though the contrary was observed at 18 and 22 °C. Carotenoid content ranged from a minimum of 4.7 pg cell$^{-1}$ (22 °C) to a maximum of 35.14 pg cell$^{-1}$ at 38 °C. Significant differences in carotenoid content were found for 18–22 °C and 38 °C ($p < 0.05$). Concentrations of $\alpha$-carotene and $\beta$-carotene were two times higher in D. salina from Yucatan when compared to the strain from Spain, and four times higher when compared with D. viridis (Table 1). In D. viridis carotenoid content decreased with time at 18 °C, but exhibited no significant differences at other temperatures ($p > 0.05$) (Fig. 3D). The total carotenoids to chlorophyll a ratio for D. salina at day 15 increased with temperature from 2.1 at 22 °C to 38 °C (data not shown). The carotenoid to chlorophyll a ratio in D. salina at 18 °C was 3.8 higher than that at 22 °C. In D. viridis, this ratio increased from 0.7 at 30 °C to 1.7 at 38 °C, and for temperatures $\leq 26 ^\circ C$ it ranged from 0.8 to 1.3.

The P–E curves at optimal growth temperatures of 26 °C for D. viridis and at 22 °C for D. salina showed saturating irradiances ($E_s$) ranging from 110 to 140 $\mu$mol photons m$^{-2}$ s$^{-1}$ (D. viridis) and from 457 to 854 $\mu$mol photons m$^{-2}$ s$^{-1}$ (D. salina Fig. 4A). Photoinhibition in D. viridis was evident above 300 $\mu$mol photons m$^{-2}$ s$^{-1}$. Maximum photosynthetic rates ($P_{\text{max}}$) were not significantly different between D. viridis (150.11 ± 31.13 nmol O$_2$ $\mu$g chl a h$^{-1}$) and D. salina (148.04 ± 48.49 nmol O$_2$ $\mu$g chl a h$^{-1}$) ($p > 0.05$), though significantly higher respiration rates were found for D. viridis ($p < 0.05$) at low irradiance (<25 $\mu$mol photons m$^{-2}$s$^{-1}$). In D. salina there was an increase in the light saturated photosynthetic rates in response to increasing temperatures from 18 to 30 °C, but

<table>
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<th>Species</th>
<th>$\alpha$-carotene</th>
<th>$\beta$-carotene</th>
<th>Reference</th>
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<td>D. salina</td>
<td>0.041 ± 0.004</td>
<td>0.288 ± 0.043</td>
<td>Gómez-Pinchetti et al. (1992)</td>
</tr>
<tr>
<td>D. salina</td>
<td>0.083 ± 0.003</td>
<td>0.598 ± 0.020</td>
<td>This study</td>
</tr>
<tr>
<td>D. viridis</td>
<td>0.018 ± 0.002</td>
<td>0.136 ± 0.012</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 1: Content of $\alpha$-carotene and $\beta$-carotene (mg 100 g$^{-1}$ dry wt) in D. salina and D. viridis from Yucatan in comparison to D. salina from Spain grown under same experimental conditions used in this study.

Fig. 3. Chlorophyll a concentrations per cell (A–B) and total carotenoids concentrations per cell (C–D) measured at 5, 10 and 15 cultivation days in D. salina (A + C) and D. viridis (B + D) grown at different temperatures.
1. Introduction

The Dunaliella species isolated from the solar saltworks on the Yucatan Peninsula differed in their capacity for growth and physiological acclimation to varying culture conditions (i.e. NaCl concentration, temperature and light). For D. salina from Yucatan the optimum NaCl concentration for growth was 10% NaCl. In general, growth rates were lower when compared to Dunaliella bardawil (Ben-Amotz and Avron, 1989), and D. salina (Giordano and Bowes, 1997). Similar growth rates have been reported for eight strains isolated from Chile (Cifuentes et al., 1992).

In contrast, Marín et al. (1998) reported very low growth rates at this salinity for D. salina isolated from salt-ponds in Araya, Venezuela. The different growth patterns exhibited by geographically distinct strains confirm the hypothesis that these algae do not adapt to a specific saline condition, but can tolerate a wide range of salinities. Johnson et al. (1968) found that Dunaliella is able to grow in saturated brine (as it occurs in natural conditions), that its optimum growth always occurs at lower salinity, and that higher or lower halophytism is intrinsic to each strain. On this regard, reduced growth rates at 30–35% NaCl may be directly related to the high NaCl concentration that caused larger cells (Fig. 1B), and thus faster nutrient depletion in the culture medium.

Dunaliella viridis has also been described as a hyperhaline species growing in the 6–10% NaCl range (Massyuk, 1973). Dunaliella viridis from an athalassic lake in Spain has been reported to grow over 4 M (23.3%) NaCl with an optimal growth at 1 M (5.8%) NaCl (Jiménez and Niell, 1991). These figures are lower than the NaCl growth ranges found for the D. viridis strain isolated in Yucatan (15% and 20% NaCl). Davis (1990), however, reports similar results for D. viridis as being one of the main components of a planktonic community in a solar saltworks at intermediate salinities (10–20% salts).

The low growth rates observed for both D. salina (0.28 d−1) and D. viridis (0.17 d−1) in the salinity experiment may be related to the low irradiances used (80 μmol photons m−2 s−1). This would apparently support the report of Gordillo et al. (2001) of D. viridis maximum growth rates at 250 μmol photons m−2 s−1. However, lower growth rates have also been reported for four D. salina strains grown at 25 °C with maximum specific growth rates ranging from 0.093 to 0.23 d−1 (Markovits et al., 1993).

Dunaliella salina cells exposed to increasing NaCl concentration from 10% to 35% showed a linear increase in cell volume, changing from ovoid to spherical in shape and from green to reddish in colour. These changes in D. salina cell volume are well documented and are due to osmoregulation, in which the cell regulates the increase of solute in the medium through glycerol synthesis (Avron and Ben-Amotz, 1992). These changes were not observed in D. viridis since no difference was noted in cell volume with increasing NaCl concentrations. A mean cellular volume of 114 μm3 at 20% NaCl was recorded for D. viridis, which coincides with other studies (Jiménez and Niell, 1991). Also, Avron and Ben-Amotz (1992) report D. viridis cell volume to be below 200 μm3, which is within the volume range for the strain isolated in Yucatan.

4. Discussion

The Dunaliella species isolated from the solar saltworks on the Yucatan Peninsula differed in their capacity for growth and physiological acclimation to varying culture conditions (i.e. NaCl concentration, temperature and light). For D. salina from Yucatan the optimum NaCl concentration for growth was 10% NaCl. In general, growth rates were lower when compared to Dunaliella bardawil (Ben-Amotz and Avron, 1989), and D. salina (Giordano and Bowes, 1997). Similar growth rates have been reported for eight strains isolated from Chile (Cifuentes et al., 1992).
22 °C. This is only slightly lower than the 25–35 °C optimum temperature for growth in D. bardawil reported by Ben-Amotz and Avron (1989). Growth rates ranging from 1.5 to 2.0 d−1 at 25 °C have been reported for D. bardawil (Ben-Amotz et al., 1991), however, these authors used higher irradiances and increased nitrogen content in the culture medium. Giordano (1997) observed higher growth under optimal growing conditions (38 °C) than those found for eight strains of D. salina grown at 30 °C under 175 μmol photons m−2s−1 similar to the present study, but at lower salinity (9% NaCl) and different nitrogen enrichment NH4Cl). Cell densities in D. salina from Yucatan (0.43–3.06 × 10⁶ cell mL−1) were higher than those found for eight strains of D. salina from Chile (0.67–1.7 × 10⁶ cell mL−1) grown at 26 °C (Cifuentes et al., 1992). In D. viridis from Yucatan the highest cellular growth was found at 26 °C, whereas, Jiménez and Niell (1991) observed optimal growth at 30 °C with 1 M (5.8%) NaCl.

4.2. Effect of temperature on pigment concentration

Development of mass culture conditions for Dunaliella requires both an optimization of growth and of the amount of carotene produced per unit of time and culture volume. In the present study, the carotenoid content per cell obtained in D. salina at 38 °C (35.14 pg cell−1) was higher than carotenoid values reported by others (Ben-Amotz and Avron, 1989; Giordano and Bowes, 1997; Marín et al., 1998). In contrast, Cifuentes et al. (1992) reported higher carotenoid content (8.1–42.2 pg cell−1) in strains from Chile. Higher α-carotene and β-carotene content were found in D. salina from Yucatan in comparison to D. salina from Spain grown under the same experimental conditions (Gómez-Pinchetti et al., 1992). In another study, these authors have shown that suboptimal temperatures in combination with high light intensities are highly effective in enhancing carotenogenesis and thus be used for the control of β-carotene biosynthesis in D. salina (Mendoza et al., 1996). This indicates that pigment content in D. salina from Yucatan should be considered significantly higher, since cultivation time was shorter in the present study. The highest carotenoid concentrations were obtained under suboptimal growing conditions (38 °C), which confirms that induction and accumulation of high value products in Dunaliella have always been associated with stress conditions.

The chlorophyll a content in D. salina (6.7 mg L−1 and 4.29 pg cell−1) was similar to that reported by Ben-Amotz and Avron (1989), but lower than those found by Giordano et al. (1994, 1997). Pigment yield in D. viridis increases as temperature increases (Jiménez and Niell, 1991). Indeed, D. viridis from Yucatan exhibited increases in both pigments (carotenoids and chlorophyll a) up to 30 °C. The highest carotenoid concentrations in D. viridis were also obtained at suboptimal growing temperatures (30 and 38 °C), though the concentrations were three to ten times lower than those obtained in D. salina (7.06 mg L−1 and 3.12 pg cell−1). This seems to support the non-carotenogenic nature of D. viridis, although some authors have proposed its use for oxygenated carotenoids production (Moulton and Burford, 1990).

4.3. The short term effect of temperature on photosynthesis

The results demonstrated a physiological advantage of D. salina over D. viridis in relation to short term temperature changes. Differences in size and morphological features between these species may affect photosynthetic limits to irradiation and temperatures (Coles and Jones, 2000). Saturation irradiance (Ea) was higher in D. salina (304 μmol photons m−2 s−1) than in D. viridis (110 μmol photons m−2 s−1), which may be directly related to the photoprotective action of the carotenoids in the former species. Because D. viridis is a non-carotenogenic species it was photoinhibited at higher irradiances (>300 μmol photons m−2 s−1), which coincides with the photoinhibition at 700 μmol photons m−2 s−1 reported by Gordillo et al. (2001). Photosynthetic activity in D. viridis was reduced when subjected to temperatures ≥34 °C. Although D. viridis can acclimate to and grow at these temperatures, its tolerance is low and its respiration rates increased when exposed to suboptimal temperatures. Tolerance to sudden temperature changes was more evident in D. salina. These physiological adjustments in response to temperature changes may be short-term acclimation responses or long-term, fixed genotypic adaptations (Iglesias-Prieto et al., 1992).

Intraspecific physiological variability has been reported in D. salina (Cifuentes et al., 1992; Markovits et al., 1993), which can lead to erroneous assumptions about the industrial potential of this microalga when information from one strain is extrapolated to the entire species. Understanding the physiological response of these species to unique environmental conditions may help in choosing the right culture conditions as well as in selecting strains to improve biological process efficiency. In this study we reported differences in growth, pigment content, photosynthesis and morphological features between D. viridis and D. salina from the Yucatan Peninsula. Photosynthetic responses to temperature variations indicated physiological adjustments in both species with higher tolerance in D. salina. Data on the physiological responses of D. salina and D. viridis from Yucatan will help in better understanding the production system needed, either extensive cultures in large unstirred outdoor ponds (i.e. abandoned saltworks), or more intensively in paddlewheel stirred raceway ponds, depending on the desired product (biomass or fine chemicals) and, at a later stage, provide insight into potential genetic modification of desired strains.

Acknowledgements

This study was financed by CONACyT (36056-B) and SAGARPA (2002-C01-1057). Thanks to M.L. Zaldivar
and C. Chávez for technical assistance, Elvira Rios for β-carotene HPLC analysis, and Sergio Ortiz of Industria Salina de Yucatan, S.A. (ISYSA) for facilitating access to the saltworks.

References


