BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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UV-A mediated induction of carotenoid accumulation in *Dunaliella bardawil* with retention of cell viability

Abstract The effect of adding UV-A radiation (320–400 nm) to photosynthetically active radiation (PAR, 400–700 nm) during growth of the photosynthetic marine microalga *Dunaliella bardawil* was investigated in this work in terms of cell growth and carotenoid production. Although signs of slow cell growth (slight reduction of chlorophyll and protein content) were observed after 24 h of cell exposure to UV-A (40 μmol photons m^{-2} s^{-1} and 70 μmol photons m^{-2} s^{-1}) plus 140 μmol photons m^{-2} s^{-1} PAR , 84 h exposure to these UV-A conditions slightly stimulated cell growth and increased the photosynthetic efficiency of the exposed cultures. The enhanced cell growth was coupled with an increase in total carotenoid content. Besides β-carotene as the major pigment, increases in the well-known antioxidants lutein and zeaxanthin of about 3-fold and 5-fold, respectively, were determined in cultures exposed to UV-A radiation of 70 μmol photons m^{-2} s^{-1} for 84 h. As a consequence, far from being negative to cell growth, low and medium UV-A radiation are stress factors that could be successfully applied to long-term processes for large scale carotenoid production using *D. bardawil* cultures with retention of cell viability. UV-A exposure has the advantage of being a factor either easily applied or removed as required, in contrast to other nutrient stresses, which require medium replacement for their application.

Introduction

Naturally occurring carotenoids are of significant commercial interest in the food and cosmetics industries, and as pharmaceuticals (Armstrong 1997). Because of their antioxidant properties (Miller et al. 1996) certain xantophylls have been implicated in the prevention of chronic disease (Rao and Agarwal 1999). Some of these pigments are currently produced for commercial purposes by chemical synthesis (Nelis and De Leenheer 1991) but production of some commercial pigments with microalgae still remains unexplored and unexploited (Jin et al. 2001, Margalith 1999). To find specific environmental stress conditions that lead to large accumulation of commercially interesting carotenoids is currently an attractive task in the field of microalgal biotechnology.

Conditions inducing large accumulation of carotenoids include high photon flux densities (wavelength range 400–700 nm). Such inducing conditions can change the level of e.g., β-carotene from 1% dry weight (dw) to 10% dw (Ben-Amotz and Shaish 1993). In addition, UV radiation has been proved to have both positive and negative effects on the viability of microalgal cultures (Horwitz 1994). Some of the commercially attractive carotenoids produced by the microalga *Dunaliella bardawil* absorb in the UV-A spectrum (Tsukida et al. 1982). As a consequence, the accumulation of carotenoids is one of the responses of microalgae to the oxidative stress produced by UV radiation (Jahnke 1999). In this paper, the effect of UV-A on the growth, photosynthetic efficiency, and type of carotenoids produced is characterized and discussed. Both quantitative and qualitative profiles of the carotenoids produced are analyzed, and the potential of UV-A as a stress factor in the production of carotenoids other than β-carotene is also discussed.

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Table 1 Irradiation and light quality during *Dunaliella bardawil* growth. *PAR* Photosynthetically active radiation, *PFD* photon flux density

Irradiation (µmol photons m^{-2} s^{-1}) and light quality	Total PFD[a] (µmol photons m^{-2} s^{-1})	UV-A/PAR ratios
40 UV-A + 140 PAR	180	0.28
70 UV-A + 140 PAR	210	0.50
90 UV-A + 140 PAR	230	0.64

Materials and methods

Microorganism and culture conditions

Dunaliella bardawil (UTEX 2538) was kindly provided by the ICMAN (Instituto de Ciencias Marinas de Andalucía, CSIC, Cádiz, Spain). Standard cultures were grown in mineral liquid medium at 25°C, bubbled with air containing 5% (v/v) CO_2 and continuously illuminated with cool white and daylight from fluorescent lamps (100 µmol photons m^{-2} s^{-1} at the surface of the flasks) plus UV-A from 40 to 70 µmol photons m^{-2} s^{-1}. The composition of the culture medium is described in Jiménez and Pick (1993). The UV-A incubation experiments were carried out in 38 mm ×200 mm Pyrex glass tubes within a microalgae culture chamber at 25°C. The tubes were made of UV-transmitting glass. In order to maintain a homogenous cell suspension, the cultures were continuously bubbled with air containing 5% (v/v) CO_2 at a rate of 10 l h^{-1}.

Growth rate determination

Microalgae growth rates were determined as the rate of change of the number of cells during the exponential growth phase. A specific growth rate of 2.0 indicates doubling of the algae in 24 h.

Oxygen evolution

The biological activity used to test cell viability was light-dependent photosynthetic activity. For determination of photosynthetic and respiratory activities 1 ml cell culture of the microalgae was placed in a Clark-type electrode to measure O_2-evolution. Measurements were made at 25°C under either saturating white light (1,500 µmol photons m^{-2} s^{-1}, light-dependent oxygen production) or darkness (endogenous respiration).

Analytical determinations

Chlorophyll was determined by heating and extracting with 1 ml acetone, using an absorption coefficient at 652 nm of 34.5 mg^{-1} ml cm^{-1}. Total carotenoids were determined spectrophotometrically by the following equation: $[(3,000 \times A_{470}) - 1.63(\text{Chl a})]/221$. Protein content was determined following the method described by Bradford (1976).

HPLC analysis of carotenoids

Separation and analysis of carotenoids was performed in a Merck Hitashi HPLC, column RP-18. Mobile phase: solvent A, ethyl acetate; solvent B acetonitrile/water (9:1, v/v). Flow rate 1 ml min^{-1}. Gradient: 0–16 min 0–60% A; 16–30 min 60% A; 30–35 min 100% A, as described by Young et al. (1997).
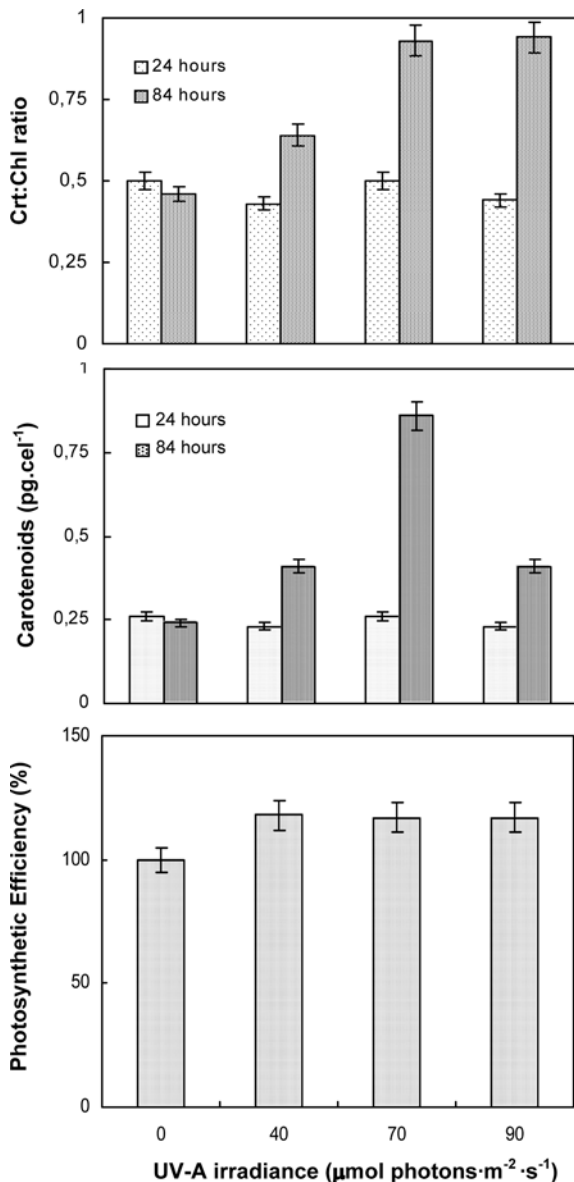
Results

Effect of UV-A on cell growth and carotenoid accumulation in *D. bardawil*

Cell cultures of *D. bardawil* were grown under UV-A radiation (Table 1) in addition to photosynthetically active radiation (PAR; 140 µmol photons m^{-2} s^{-1}). Control cultures (−UV cultures) were grown in the absence of UV-A radiation.

At a photon flux density (PFD) of 140 µmol photons m^{-2} s^{-1}, the addition of UV-A radiation induced an increase in the carotenoid/chlorophyll ratio (Table 2). The increases in the carotenoid/chlorophyll ratios are a
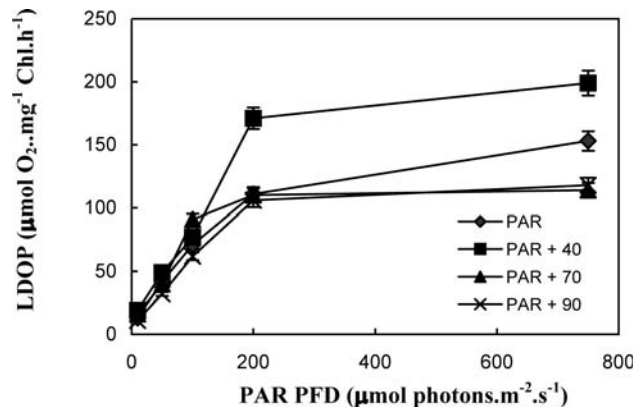
Table 2 Variation of growth parameters in *D. bardawil* cell cultures incubated under continuous illumination with PAR (140 µmol photons m^{-2} s^{-1}) plus UV-A radiations of different intensities (µmol photons m^{-2} s^{-1}). A specific growth rate of 2.0 indicates a doubling of the algae in 24 h. *Crt* Total carotenoids, *Chl* chlorophyll, *prot* total proteins

	Time (h)	Control (−UV-A)	UV-A 40	UV-A 70	UV-A 90
Crt/prot (mg/g)	24	28.89	21.76	23.23	18.99
	84	37.99	54.76	129.63	72.45
Chl/prot (mg/g)	24	56.79	50.44	45.98	42.8
	84	81.79	85	139.98	77.32
Crt/Chl (g/g)	24	0.5	0.43	0.5	0.44
	84	0.46	0.64	0.93	0.94
Crt (pg cell^{-1})	24	0.26	0.23	0.26	0.23
	84	0.24	0.41	0.86	0.41
Specific growth		1.73	1.77	2.01	1.31

Fig. 2 Light-dependent oxygen production (LDOP) saturation curves determined in *D. bardawil* cultures illuminated for 24 h with PAR plus either 0 (control cells), 40, 70, or 90 μmol photons m^{-2} s^{-1} UV-A. *PFD* Photon flux density

Fig. 1 Effect of UV-A photon flux density on the carotenoid/chlorophyll ratio, total carotenoid content and photosynthetic efficiency of *Dunaliella bardawil*. Cells grown under standard culture conditions were illuminated with photosynthetically active radiation (PAR; 140 μmol photons m^{-2} s^{-1}) plus either 0 (control cells), 40, 70, or 90 μmol photons m^{-2} s^{-1} UV-A. Total carotenoids (*Crt*), chlorophyll (*Chl*) and photosynthetic efficiency were determined at the culture times indicated. Photosynthetic efficiency of *D. bardawil* cells after 24 h growth was determined from the slopes of light-dependent oxygen production to UV-A photon flux density curves. 100% =0.119 μmol O$_2$ μmol^{-1} photons mg^{-1} Chl

protein and chlorophyll content. Such growth stimulation was observed until the end of the experiment (84 h).

The induction of significant increases in the carotenoid/chlorophyll ratios by UV-A was observed only after 3 days of growth. In the early exponential growth phase (24 h) the carotenoid/chlorophyll ratio and the carotenoid content in the cultures growing under UV-A radiation were slightly lower than that of the control (Fig. 1).

The major increase in the carotenoid/chlorophyll ratio, 2-fold that of the control culture, was observed in cultures grown for 84 h with 70 μmol photons m^{-2} s^{-1} UV-A (Fig. 1). The maximum total carotenoid content per cell was found to be 3.6-fold (pg cell^{-1}) that of the control cultures, after 84 h growth at 70 μmol photons m^{-2} s^{-1} (Fig. 1). Therefore, the maximum accumulation of carotenoids occurred in *D. bardawil* cultures growing under continuous radiation of 70 μmol photons m^{-2} s^{-1} UV-A.
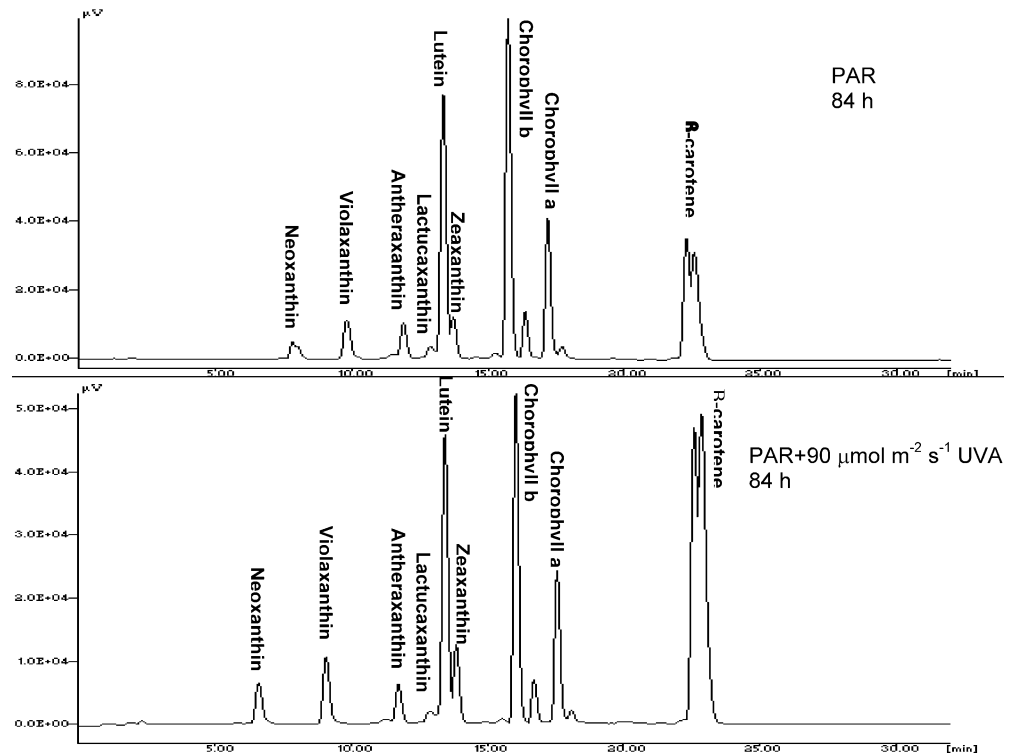
Effect of UV-A on the photosynthetic efficiency of *D. bardawil*

Far from negatively affecting cell growth, UV-A radiation added to PAR stimulated cell growth. The addition of UV-A radiation of 40 or 70 μmol photons m^{-2} s^{-1} to low photosynthetic photon flux densities (less than 200 μmol photons m^{-2} s^{-1}) enhanced the specific growth rate (Table 2), and also resulted in slight increases in light-dependent oxygen production (LDOP) by *D. bardawil* at low and medium PAR as shown in Fig. 2.

The slopes of LDOP versus PFD (Fig. 2) yielded the values of photosynthetic efficiency shown in Fig. 1. The photosynthetic efficiency in a culture of photosynthetic cells is defined as the LDOP per absorbed photon. The addition of UV-A to PFD resulted in a higher photosynthetic efficiency of *D. bardawil*, which is also in good agreement with the higher specific growth rates reported above.

As inferred from these results, the addition of low photon flux densities of UV-A to PAR, far from being

consequence of increases in the carotenoid content of the cells rather than decreases in the protein and chlorophyll contents, as inferred from the carotenoid/protein and chlorophyll/protein ratios (Table 2). Thus, addition of UV-A of 40 or 70 μmol photons m^{-2} s^{-1} to photosynthetic active radiation (140 μmol photons m^{-2} s^{-1}) slightly stimulated cell growth according to the specific growth rates of *D. bardawil* cultures incubated with UV-A (Table 2), which is in agreement with the increase in

Fig. 3 Typical profile of carotenoids from *D. bardawil*. The chromatogram was obtained following the HPLC procedure described in Materials and methods

damaging to *D. bardawil* cell cultures, results in a faster growth and in a 20% higher efficiency in the photosynthetic use of the light.

Effect of UV-A on the accumulation of specific carotenoids in *D. bardawil*

The effect on the carotenoid profile of *D. bardawil* cells of addition of UV-A to PAR was also studied in this work. Acetone extracts from *D. bardawil* cells grown under UV-A radiation were utilized for chromatographic pigment separation by HPLC as described in Materials and methods. A typical chromatographic profile of carotenoids from *D. bardawil* cells including the most significant peaks is shown in Fig. 3.

At all UV-A radiations, induction of β-carotene accumulation was observed after 3 days of growth (Fig. 4). The maximum β-carotene accumulation (243% with respect to the control culture) was observed in cultures growing under 70 μmol photons m^{-2} s^{-1} UV-A. At all UV-A radiations the maximum lutein accumulation was observed after 3 days of growth (Fig. 4). Among all cultures incubated under UV-A radiation, that of 70 μmol photons m^{-2} s^{-1} produced the maximum lutein content (180% with respect to the control culture).

Particularly interesting was the response of the xanthophyll cycle to UV-A. Incubation of *D. bardawil* with 70 μmol photons m^{-2} s^{-1} UV-A radiation yielded the maximum production of violaxanthin—234% with respect to the control culture—which was observed at 84 h growth (Fig. 4). Interestingly, after 84 h of the same UV-A irradiation the zeaxanthin content was maximal, at 407%

that of the control cells, becoming the largest increase among all the carotenoids stimulated by UV-A radiation. The zeaxanthin/violaxanthin ratio after 84 h of incubation under UV-A rose to 1.5.

Discussion

The accumulation of significant quantities of carotenoids in *D. bardawil* has been shown to occur under a number of stress factors, including low temperature, nutrient deficiency, irradiation with light of high intensity, and salinity (Ben-Amotz and Avron 1983; Borowitzka et al. 1990). In all these stress conditions, the induction of carotenoid accumulation is usually accompanied by a reduction in cell growth as a consequence of the metabolic disfunction caused by the non-optimal growth conditions applied.

UV radiation is also responsible for oxidative damage in microalgae cultures. Short duration exposures to UV-A radiation have been reported to reduce cell growth of *Dunaliella* cultures (Döhler et al. 1997) and to stimulate carotenoid accumulation (Jahnke 1999). In this paper we show that longer exposure to adequate UV/PAR ratios (Table 1) may even enhance specific cell growth of *D. bardawil* cultures while stimulating the accumulation of carotenoids as a natural response of viable cells to oxidative growth conditions (Table 2). As shown in Table 2, after a slight decrease during first 24 h of exposure, the growth of UV-A-irradiated cultures then recovered from that acclimation period to UV-A. This can be observed in terms of chlorophyll/protein content at 24 h and 84 h.
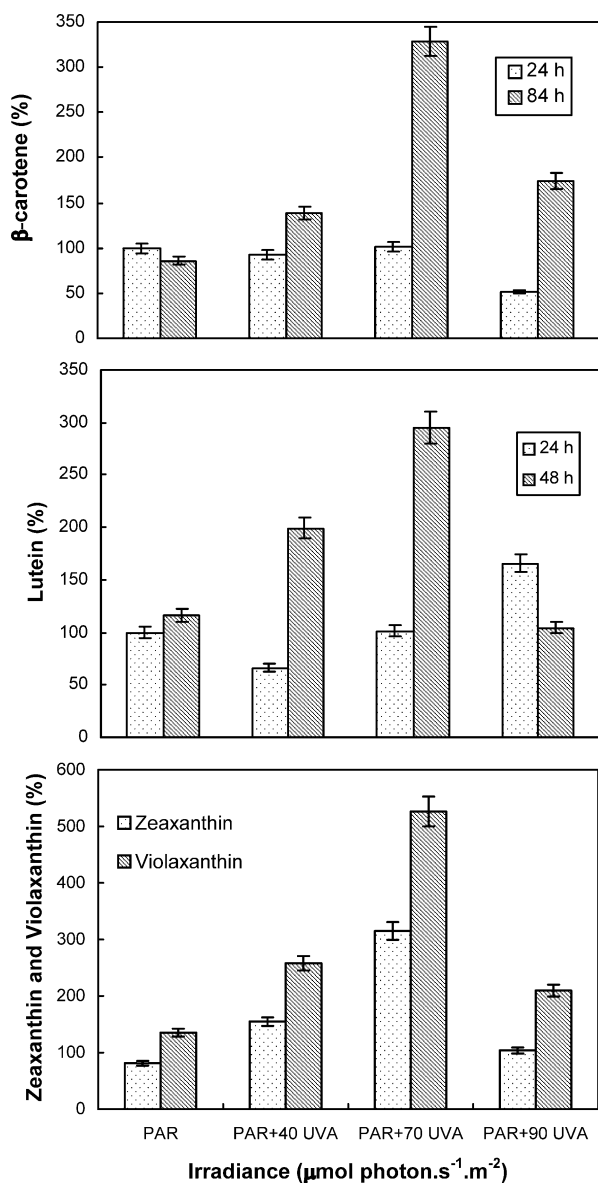
Fig. 4 Effect of UV-A photon flux density on the cell content of β-carotene, lutein, zeaxanthin, and violaxanthin in *D. bardawil*. Cells grown under standard culture conditions were illuminated with PAR (140 μmol photons m^{-2} s^{-1}) plus either 0 (control cells), 40, 70, or 90 μmol photons m^{-2} s^{-1} UV-A. Content of β-carotene and lutein at 24 h and 84 h, and violaxanthin and zeaxanthin at 84 h, were determined by HPLC as described in Materials and methods. 100% β-carotene =0.24 pg cell^{-1}, 100% lutein =0.18 pg cell^{-1}, 100% violaxanthin =6.8×10^{-3} pg cell^{-1}, 100% zeaxanthin =5.5×10^{-3} pg cell^{-1}

The positive effect of UV-A on *D. bardawil* cell growth is shown in terms of increases in the specific growth rate (Table 2). In addition, the photosynthetic efficiency calculated from LDOP data (Fig. 1) give more support to the positive effect caused by UV-A on *D. bardawil* growth, showing an increase of photosynthetic efficiency of up to 20% in those cultures irradiated with 40 or 70 μmol m^{-2} s^{-1}, revealing an even more active PSII in UV-A-exposed cultures with respect to control cells.

The accumulation of carotenoids is induced at low UV-A radiation. As shown in Table 2, the amount of total carotenoids per cell in cultures irradiated with 70 μmol m^{-2} s^{-1} was 3.6-fold the amount of the control culture. This means that UV-A-stimulated cell growth in *D. bardawil* cultures can be compatible with the induction of carotenoid accumulation by UV-A. To our knowledge, and looking at the potential application of this concept, this might be an attractive tool to drive large-scale production of carotenoids with retention of cell viability, in addition to the added value of having an easily applied stress factor that does not require replacement of culture medium.

As shown in Fig. 4, the time course of carotenoid accumulation shows first an increase in the β-carotene content rather than in xanthophylls. These results are in good agreement with previous studies in which other stress factors were applied to drive carotenoid accumulation (Phillips et al. 1995; Jiménez and Pick 1994). The protective role of carotenoids to scavenge oxygen free radicals directly, or to quench excited states of chlorophyll, is slight (Ben-Amotz et al. 1989; Jiménez and Pick 1993). This conclusion arises from the fact that the carotene-containing globules are not in photochemical contact with thylakoid pigments (Hejazi and Wijffels 2003). However, the high concentrations of carotenes accumulated in *D. bardawil* following exposure to UV-A radiations lead to significant absorption of UV-A. This can explain the fast cell reaction to UV-A exposure in terms of carotenoid accumulation shown in our results. Therefore, the production of carotenoids is possible with viable cells by exposing *D. bardawil* cultures to UV-A radiation of low–medium intensity. It can be concluded that UV-A exposure is a stress factor that drives the biocompatible production of carotenoids in *D. bardawil* cultures, with the added value that there is no need for medium replacement to stop the application of the stress: simply switching off the UV-A lamps is sufficient.

Regarding the type and quantity of specific pigments produced following UV-A exposure, the highest production of β-carotene occurred at exposure times longer than 24 h. A slight decrease in total carotenoids was observed at 24 h, which could be a consequence of slight damage produced by UV-A radiation. However, the increase in the specific growth rate and photosynthetic efficiency are evident signs of cell recover from early oxidative damage. This is consistent with the increase in the total carotenoid content after 2 and 3 days of UV-A exposure, and the subsequent higher UV-A absorption then reducing the damage caused by UV-A. After 84 h of UV-A exposure, the viable cultures of *D. bardawil* still produced β-carotene as the main carotenoid in quantity, necessary to reduce the impact of UV-A radiation.

Great attention should be paid to the significant increases observed in zeaxanthin and lutein content after 84 h UV-A exposure (70 μmol photons m^{-2} s^{-1}). Both carotenoids are well recognized antioxidants, especially zeaxanthin, which is involved in the response to oxidative stress via the xanthophyll cycle.

Finally, we conclude that UV-A radiation of low–medium intensity is a stress factor that can be successfully applied in long-term processes for large-scale carotenoid production using *D. bardawil* cultures with retention of cell viability. UV-A exposure has the advantage of being a factor either easily applied or quitted when needed, as opposed to other nutrient stresses, which require medium replacement for their application. Cell acclimation to UV-A exposure takes about 2 days, as indicated by slight increases in specific growth rates and photosynthetic efficiency. The significant absorption of UV-A radiation by carotenoids is behind this cell acclimation process. Interestingly, long-term exposure to UV-A induces significant increases in the intracellular accumulation of three commercially relevant antioxidants produced by *D. bardawil*: β-carotene, lutein, and zeaxanthin.

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