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β-Carotene production enhancement by UV-A radiation in *Dunaliella bardawil* cultivated in laboratory reactors

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β-carotene is an antioxidant molecule of commercial value that can be naturally produced by certain microalgae that mostly belong to the genus *Dunaliella*. So far, nitrogen starvation has been the most efficient condition for enhancing β-carotene accumulation in *Dunaliella*. However, while nitrogen starvation promotes β-carotene accumulation, the cells become non-viable; consequently under such conditions, continuous β-carotene production is limited to less than 1 week. In this study, the use of UV-A radiation as a tool to enhance long-term β-carotene production in *Dunaliella bardawil* cultures was investigated. The effect of UV-A radiation (320–400 nm) added to photosynthetically active radiation (PAR, 400–700 nm) on growth and carotenoid accumulation of *D. bardawil* in a laboratory air-fluidized bed photobioreactor was studied. The results were compared with those from *D. bardawil* control cultures incubated with PAR only. The addition of 8.7 W · m^{-2} UV-A radiation to 250 W · m^{-2} PAR stimulated long-term growth of *D. bardawil*. Throughout the exponential growth period the UV-A irradiated cultures showed enhanced carotenoid accumulation, mostly as β-carotene. After 24 days, the concentration of β-carotene in UV-A irradiated cultures was approximately two times that of control cultures. Analysis revealed that UV-A clearly induced major accumulation of *all-trans* β-carotene. In N-starved culture media, β-carotene biosynthesis in UV-A irradiated cultures was stimulated. We conclude that the addition of UV-A to PAR enhances carotenoid production processes, specifically *all-trans* β-carotene, in *D. bardawil* cells without negative effects on cell growth.

[**Key words:** Carotenoids; *Dunaliella bardawil*; Photobioreactor; UV-A; β-carotene]

The increasing interest in carotenoid production by microalgae is related to the commercial applications of these natural compounds and market demands for carotenoids, especially for pharmaceutical and nutritional applications (1, 2). Traditionally, carotenoids have been used commercially as food additives, including as colorants, antioxidants, and vitamins (3–5). Their ability to protect against oxygen free radicals has resulted in their use in some therapeutic applications as degenerative disease preventives, anti-cancer agents, and immune system stimulators (6–8).

Carotenoids are effective singlet oxygen quenchers able to eliminate activated oxygen radical forms (9–11), and therefore are involved in protection against excess irradiance. Among the conditions that induce accumulation of carotenoids are high photon flux densities (wavelength range, 400–700 nm). Such inductive conditions have been reported to result in increases of β-carotene levels from 1% dry weight (dw) to 10% dw (2, 12, 13).

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 the microalgae *Dunaliella bardawil* (9, 11, 12). UV radiations have been shown to have both positive and negative effects on the viability of microalgae cultures (14), and some of the commercially attractive carotenoids produced by *D. bardawil* have absorption in the UV-A spectrum (15). Moreover, increased carotenoid accumulation in microalgae has been reported to occur when microalgae are exposed to low-intensity UV radiation added to photosynthetically active radiation (PAR) (16, 17). As a consequence, the accumulation of carotenoids might be one of the microalgal responses to the oxidative stress produced by UV radiations (18–20).

Carotenoid accumulation also increases in microalgae cultures under nitrogen starvation (21–23), and relationships between N deficiency and oxidative stress have been reported. In N-deprived mulberry (*Morus alba*) plants, lipid peroxidation and activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase increased (24), while in rice (*Oryza sativa*) plant leaves, changes in the level of antioxidant enzyme activities were found (25). This suggests that a combination of UV-A and N starvation could be a tool for enhancing carotenoid production in microalgae.

In this paper, the effects of UV-A radiation and N-depletion on growth, β-carotene accumulation, and on selected xanthophylls (violaxanthin and zeaxanthin) in *D. bardawil* are characterized and discussed. Both quantitative and qualitative profiles of the produced carotenoids are analyzed, and the possible combined effects of UV-A

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radiation and N depletion as stress factors for increasing long-term production of carotenoids are discussed. In this paper, long-term production of carotenoids refers to carotenoid accumulation in *D. bardawil* cultures illuminated with UV-A radiation during their entire growth phase. Improving long-term carotenoid production in cultures illuminated with UV-A radiation is aimed at determining production conditions that could be useful in trial commercial production systems.

MATERIALS AND METHODS

Microorganism and culture conditions *D. bardawil* (UTEX 2538) was kindly provided by the Marine Science Institute of Andalusia (ICMAN), Spanish National Research Council (CSIC). Standard cultures in a culture room were grown in mineral liquid medium at 25 °C, bubbled with air containing 5% (v/v) CO_2, and continuously illuminated with white light from fluorescent lamps (22 $W \cdot m^{-2}$, at the surface of the flasks). The composition of the culture medium was described by Cao et al. (26).

Analytical determinations Chlorophyll was determined by heating and extracting cell pellets with acetone and measuring absorbance at 647 and 663 nm ($\varepsilon = 18.3$ and 74.46 ml $mg^{-1} \cdot cm^{-1}$, respectively). Using equations from (19), the chlorophyll content of the cells was calculated by

$$\text{Chl}-\text{a} = 5[(12.25\text{Abs}_{663\ \text{nm}}) - (2.73\text{Abs}_{647\ \text{nm}})]$$

$$\text{Chl}-\text{b} = 5[(21.5\text{Abs}_{647\ \text{nm}}) - (5.1\text{Abs}_{663\ \text{nm}})]$$

$$\text{Chl total} = \text{Chl}-\text{a} + \text{Chl}-\text{b}.$$

The total carotenoid content was determined spectrophotometrically by [(3000 Abs_{470}) − 1.63 (Chl-a)] / 221.

HPLC analysis of carotenoids Separation and analysis of carotenoids were performed in a Merck Hitachi HPLC, RP-18 column. In the mobile phase, solvent A was ethyl acetate and solvent B was acetonitrile and water (9:1, v/v). The flow rate was 1 ml $\cdot$ min^{-1}. The gradient schedule was 0–16 min, 0–60% solvent A; 16–30 min, 60% solvent A; 30–35 min, 100% solvent A, as described by Young et al. (27).

Air-fluidized bed photobioreactor A 3.2 l stirred tank Applikon photobioreactor was used. The bed of the photobioreactor was fluidized by air containing CO_2 (5% v/v) at a flow rate of 2.5 l $\cdot$ min^{-1} and 150 r $\cdot$ min^{-1}, respectively.

The cultivation parameters (pH, dissolved oxygen concentration, and temperature) were continuously measured by the Applikon control unit. The reactor was equipped with a water jacket connected to a water bath and maintained at a constant 25 °C. The pH was set at 7.5 for all of the experiments.

The photobioreactor was illuminated by fluorescent lamps (PAR: 15 W/33 and UV-A: 30 W/33; both from Philips) and the incident light intensity at the reactor surface was 250 $W \cdot m^{-2}$ PAR and 8.7 $W \cdot m^{-2}$ UV-A. The light intensity was determined using a quantum photoradiometer (HD 9021; Delta Ohm).

Cell counting The number of cells was determined by counting *Dunaliella* cells in a Neubauer chamber using a CX41 microscope (Olympus, Tokyo).

Statistics Unless otherwise indicated, the presented data are the mean values from three independent experiments.

RESULTS AND DISCUSSION

Carotenoid production under UV-A radiation Cultures of *D. bardawil* grown in standard conditions and illuminated with PAR only were harvested, washed, and then suspended in fresh standard culture medium with nitrate as the N source. The cultures were then incubated in 3.2 l air-fluidized bed photobioreactors illuminated with 8.7 $W \cdot m^{-2}$ of continuous UV-A radiation added to 250 $W \cdot m^{-2}$ PAR. The use of 8.7 $W \cdot m^{-2}$ UV-A irradiance was based on a previous report (17) in which the photooxidative stress produced by the selected irradiance did not have a negative effect on photosynthetic activity or cell viability.

As shown in Fig. 1, the addition of 8.7 $W \cdot m^{-2}$ UV-A radiation to PAR increased the exponential growth of *D. bardawil* cultures at day 4 of cultivation in nitrate. This agrees with the results obtained in other short-term experiments with UV-A and PAR radiations (11, 20). At 8 days of growth and later, the cell numbers in cultures illuminated with UV-A and PAR remained lower than those in the control cultures (PAR only) with both cultures showing similar cell densities during the late exponential growth phase (24 days).

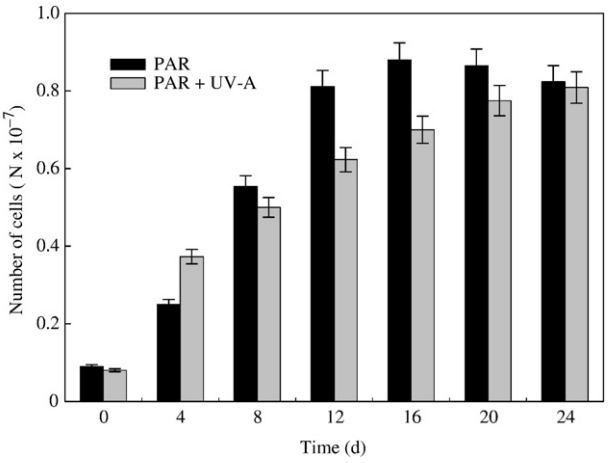

FIG. 1. Time-course of number of cells per milliliter of *Dunaliella bardawil* cultures exposed to UV-A and PAR radiation. Cultures were incubated under the following conditions: full nutrient medium and 250 $W \cdot m^{-2}$ PAR plus 8.7 $W \cdot m^{-2}$ UV-A (gray bars), full nutrient medium and 250 $W \cdot m^{-2}$ PAR only (solid bars). Columns represent averages and bars indicate SD derived from three independent experiments.

Fig. 2 shows temporal changes in carotenoid cell content of UV-A and PAR exposed *D. bardawil* cultures growing on nitrate. Throughout the exponential growth period tested, carotenoid cell content was higher in UV-A and PAR exposed cultures than in the control (PAR only) cultures. This produced a higher carotenoid accumulation in the UV-A exposed cultures (Table 1; Fig. 3). Therefore, the addition of UV-A radiation to PAR enhanced long-term carotenoid accumulation of *D. bardawil* throughout its exponential growth period, rather than through the enhancement of carotenoid biosynthesis via increased biomass production. This suggests that illumination with UV-A added to PAR can be used to induce sustained, long-term antioxidative responses in microalgae cultures, without producing a negative effect on cell growth. Based on short-term experiments (<4 days) performed by our group (17), use of UV-A radiation should consist of applying UV-A to PAR in ratios of ≤1:25 (data not shown). Using such moderate radiation mixtures, enhanced carotenoid accumulation can be continuous and sustained in UV-A illuminated cultures. Furthermore, the results indicate that both carotenoid production rate and maximum carotenoid cell content may be up to 30% higher when using UV-A and PAR than when using PAR alone (Table 1).

Combined effect of UV-A radiation and N-starvation on carotenoid production The absence of inorganic N has been used as a tool to induce increased carotenoid accumulation in microalgae (2), and oxidative stress is a consequence of incubating *Dunaliella* cultures under N-starvation (21). In this work, the combination of N-starvation and UV-A radiation on carotenoid accumulation in *Dunaliella* cultures was investigated. As expected, when *Dunaliella* was incubated in the absence of inorganic N, the number of cells remained almost constant (data not shown) and illumination under UV-A and PAR had no effect on that result (data not shown). Thus, the metabolic deficiencies resulting from the absence of nitrate could not be overcome by the growth stimulating effect of UV-A radiation (28). As shown in Fig. 4, the combination of two stressors, UV-A and N-starvation, promoted long-term carotenoid biosynthesis of *D. bardawil*. Maximum carotenoid accumulation in *Dunaliella* cells incubated in the N-starvation culture medium and illuminated with PAR only was 24.23 pg $\cdot$ $cell^{-1}$ after 24 days, while maximum carotenoid accumulation in *Dunaliella* cells incubated in the N-starvation culture medium and illuminated with UV-A added to PAR was 57.34 pg $\cdot$ $cell^{-1}$ after 24 days (Table 1). Therefore, the combined effects of UV-A and N-starvation promoted a 2.5-fold increase in carotenoid

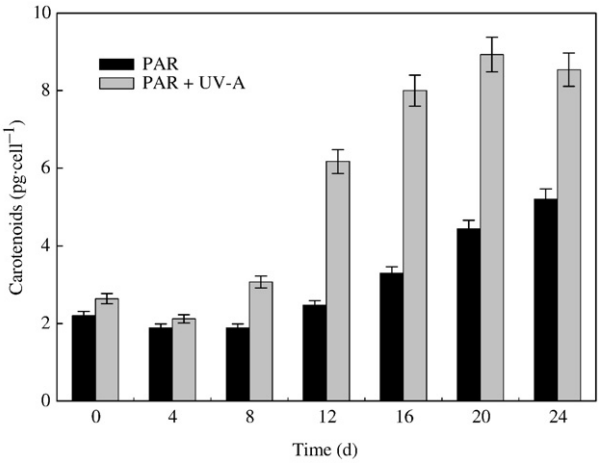

FIG. 2. Time-course carotenoid cell content in *Dunaliella bardawil* cultures exposed to UV-A and PAR radiation. Cultures were incubated under the following conditions: full nutrient medium and 250 W · m^{-2} PAR plus 8.7 W · m^{-2} UV-A (gray bars), full nutrient medium and 250 W · m^{-2} PAR only (solid bars). Carotenoids were extracted and their concentration determined as described in Materials and methods. Columns represent averages and bars indicate SD derived from three independent experiments.

accumulation in *Dunaliella* cells compared with that in *Dunaliella* cells incubated with PAR only.

The addition of UV-A radiation to PAR in N-starved carotenoid producing *Dunaliella* cultures resulted in an increased accumulation of carotenoids (Table 1). In terms of carotenoid concentration produced per reactor volume unit (mg · l^{-1}), cultures of *Dunaliella* incubated for 24 days in the N-starvation culture medium and illuminated with both UV-A and PAR (i.e., PAR + UV-A + (−N) in Table 1) accumulated up to 55 mg · l^{-1}, which is approximately three times the carotenoid concentration accumulated in cultures of *Dunaliella* incubated in N-starved culture medium and illuminated with PAR only (19 mg · l^{-1}) (i.e., PAR + (−N), in Table 1).

The stimulating effect of UV-A radiation on carotenoid accumulation in *Dunaliella* cells may also be shown as a carotenoid biosynthesis rate (Table 1). In *Dunaliella* cells incubated in N-starved culture medium and illuminated with UV-A added to PAR, carotenoids were produced at a rate of 2.05 pg · $cell^{-1}$ · d^{-1}, 2.4-fold higher than that in

TABLE 1. Total carotenoid accumulation parameters of *Dunaliella bardawil*.

Culture conditions	Production rate (mg · l^{-1} · d^{-1})	Maximum concentration (mg · ml^{-1})	Biosynthesis rate (pg · $cell^{-1}$ d^{-1})	Maximum cell content (pg · $cell^{-1}$)
PAR	2.01 ± 0.10	45 ± 2	0.18 ± 0.01	5.05 ± 0.25
PAR + UV-A	2.72 ± 0.14	69 ± 3	0.31 ± 0.02	8.92 ± 0.45
PAR + (−N)	0.85 ± 0.04	19 ± 1	0.85 ± 0.04	24.23 ± 1.21
PAR + UV-A + (−N)	2.46 ± 0.12	55 ± 3	2.05 ± 0.01	57.34 ± 2.87

D. bardawil cells were incubated 24 days under the conditions indicated. Carotenoid accumulation rates (i.e., production and biosynthesis rates) were calculated from the carotenoid production phase of the cultures, following the acclimation phase (i.e., 4–5 days after culture initiation). Light intensity: PAR, 250 W · m^{-2}; UV-A, 8.7 W · m^{-2}. PAR, photosynthetic active radiation; UV-A, ultraviolet A radiation; (−N), nitrogen starvation. Values are averages ± SD of three independent experiments.

Dunaliella cells incubated in N-starved medium and illuminated with PAR only (0.85 pg · $cell^{-1}$ · d^{-1}). The results show that in carotenoid-producing (N-starved culture medium) *Dunaliella* cells, the addition of UV-A radiation resulted in a greater amount and a faster rate of carotenoid accumulation compared to those cultures illuminated with PAR only. Therefore, in growth limiting conditions, UV-A radiation can have a significant impact on carotenoid accumulation, and its impact can be stronger than that produced by N-starvation only.

As chlorophyll biosynthesis does not occur in N-starved cultures (21), the oxidative damage produced by UV-A radiation is reported to be counteracted by enhanced production of other antioxidants, including carotenoids (17). In support of that report, Fig. 4 shows that the cell antioxidant response – in terms of carotenoid content – in cultures illuminated with PAR only did not increase as markedly as that which occurred in N-starved cultures illuminated with UV-A added to PAR.

Analysis of specific carotenoids Fig. 5 shows an HPLC chromatogram obtained from a *Dunaliella* extract; it indicates the presence of chlorophylls and some major carotenoids. Analysis of the carotenoids (Table 2) revealed that β-carotene was the major carotenoid accumulated under the assayed conditions. The highest carotenoid accumulation after 24 days was found in cultures grown on nitrate and illuminated with UV-A added to PAR (69 mg · l^{-1}) and in cultures grown in N-starved medium and illuminated with UV-A

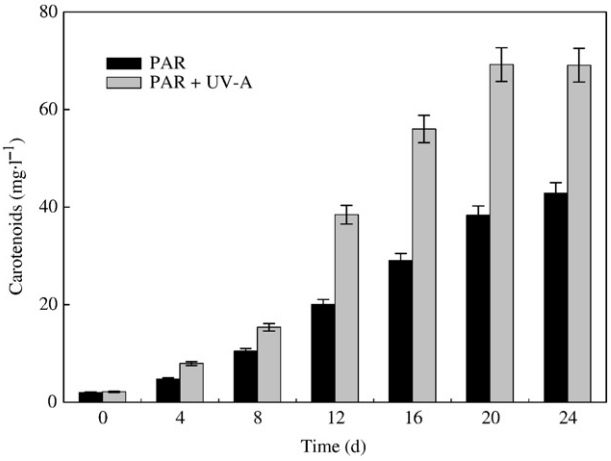

FIG. 3. Time-course of carotenoid concentration in *Dunaliella bardawil* cultures exposed to UV-A and PAR radiation. Cultures were incubated under the following conditions: full nutrient medium and 250 W · m^{-2} PAR plus 8.7 W · m^{-2} UV-A (gray bars), full nutrient medium and 250 W · m^{-2} PAR only (solid bars). Carotenoids were extracted and their concentration determined as described in Materials and methods. Columns represent averages and bars indicate SD derived from three independent experiments.

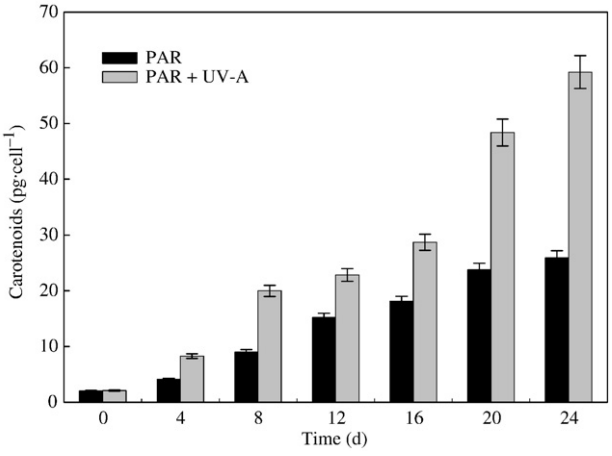

FIG. 4. Time-course of carotenoid cell content of *Dunaliella bardawil* cultures exposed to PAR and UV-A radiation in N-starved culture medium. Cells grown in standard conditions were cultivated under the following conditions: N-starved medium and 250 W · m^{-2} PAR plus 8.7 W · m^{-2} UV-A (gray bars); and N-starved medium and 250 W · m^{-2} PAR only (solid bars). Carotenoids were determined as described in Materials and methods. Initial cell density: 9×10^5 cell · ml^{-1}. Columns represent averages and bars indicate SD derived from three independent experiments.

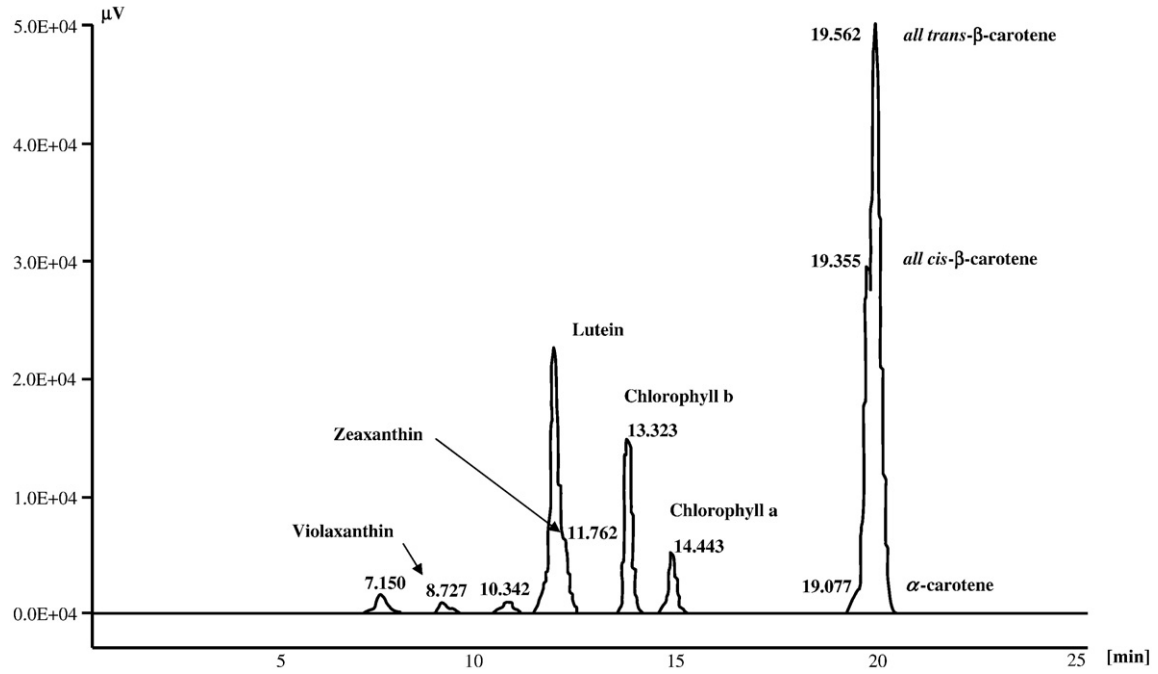

FIG. 5. HPLC chromatogram from a *Dunaliella bardawil* extract. The culture was grown in a full nutrient culture medium and illuminated with PAR 250 W m^{-2}.

added to PAR (55 mg $\cdot l^{-1}$). However, maximum β-carotene content (57 pg $\cdot$ cell^{-1}) was produced in cultures grown in N-starved medium and illuminated with UV-A added to PAR and accounted for more than twice the β-carotene content of cells incubated in N-starved medium and illuminated with PAR only. In cultures incubated under UV-A, about 75% of the β-carotene was in the *all-trans* form (remaining 25% (approx.) was *all-cis* β-carotene). Under PAR only, the *all-trans* percentage decreased to 60%. In addition, chlorophyll cell contents in N-containing cultures incubated under PAR and PAR plus UV-A were determined and no significant differences were found (Table 2).

Accumulations of other carotenoids, including violaxanthin and zeaxanthin, were significantly lower than that of β-carotene. Violaxanthin accumulation in *Dunaliella* cultures was dependent on the presence of inorganic nitrogen (data not shown) (22), and the maximum violaxanthin concentration (0.5 pg $\cdot$ cell^{-1}) was observed after 20 days of growth on nitrate and with illumination of UV-A added to PAR. On the contrary, the maximum zeaxanthin accumulation in *Dunaliella* cells (0.4 pg $\cdot$ cell^{-1}) was observed after 6 days of incubation in N-starved culture medium with UV-A added to PAR illumination, i.e., the most oxidative stress conditions applied. Zeaxanthin and violaxanthin are the main pigments involved in the xanthophyll cycle, which is ubiquitous in higher plants and has been implicated in protection against photodamage (28–30). Zeaxanthin is reported to play an antioxidant role in the cell response against oxidative damage caused by excess light and is known to facilitate dissipation of excess light energy by thermal relaxation (29). In addition, the antioxidant effect of the xanthophyll cycle, with zeaxanthin as singlet oxygen quencher, has been demonstrated (29). Thus, a large increase (from 0.02 pg $\cdot$ cell^{-1} to 0.25 pg $\cdot$ cell^{-1}, Table 2) of zeaxanthin in *Dunaliella* cells illuminated with UV-A radiation is consistent with the cell response to an imposed oxidative stress. In contrast, the role of violaxanthin is reported to be related to light capture (31–33). This is consistent with the results that showed no significant increase in violaxanthin content ($\pm$0.02 pg $\cdot$ cell^{-1}) in cells cultivated in N-starved culture medium (Table 2).

The results obtained suggest the presence of a key regulatory step linking accumulation of β-carotene and UV-mediated induction of carotenoid biosynthesis gene expression in microalgae. However, the effects of UV-A radiation on the β-carotene biosynthesis pathway and the corresponding molecular mechanisms in microalgae are not clear. The successful isolation of genes for carotenoid biosynthesis in microalgae and plants should help to identify key regulatory steps of carotenoid biosynthesis (34). Recently, the genes responsible for *hp1* and *hp2* (mutations conferring high carotenoid levels) have been shown to encode the proteins UV-DAMAGED DNA-BINDING PROTEIN 1 (DDB1) and DEETIOLATED 1 (DET1), components that are involved in the light-signal transduction pathway (35). This is consistent with the observation of enhanced production of carotenoids in microalgae cultures incubated under UV radiation and also with the results obtained here.

TABLE 2. Maximum cell content of the main carotenoids and chlorophyll of *Dunaliella bardawil*.

Culture conditions	Maximum β-carotene content (pg $\cdot$ cell^{-1})	Maximum chlorophyll content (pg $\cdot$ cell^{-1})	Maximum violaxanthin content (pg $\cdot$ cell^{-1})	Maximum zeaxanthin content (pg $\cdot$ cell^{-1})
PAR	4.5 $\pm$ 0.2 (60% *all-trans*)	5.41 $\pm$ 0.27	0.04 $\pm$ 0.02	0.02 $\pm$ 0.01
PAR + (−N)	21.7 $\pm$ 1.1 (60% *all-trans*)	–	0.04 $\pm$ 0.02	0.13 $\pm$ 0.01
PAR + UV-A	7.9 $\pm$ 0.4 (75% *all-trans*)	6.85 $\pm$ 0.34	0.05 $\pm$ 0.02	0.03 $\pm$ 0.01
PAR + UV-A + (−N)	51.5 $\pm$ 2.6 (75% *all-trans*)	–	0.04 $\pm$ 0.02	0.25 $\pm$ 0.01

D. bardawil cells were incubated for 24 days under the conditions indicated. Content of the main carotenoids was determined by HPLC (see Materials and methods). Maximum content per cell of each of the main carotenoids occurred at the following times: PAR, 24 days; PAR + UV-A, 20 days; PAR + (−N) and PAR + UV-A + (−N), 24 days for β-carotene and violaxanthin, and 6 days for zeaxanthin. Light intensity: PAR, 250 W $\cdot$ m^{-2}; UV-A, 8.7 W $\cdot$ m^{-2}. PAR, photosynthetic active radiation; UV-A, ultraviolet A radiation; (−N), nitrogen starvation. Values are averages $\pm$ SD of three independent experiments.

There is controversy regarding the effects of specific UV wavelengths on photosynthetic system responses and the mechanisms of the associated damage. Research has demonstrated that exposure to UV-A may result in direct photosynthetic damage, and research has found that the accumulation of β-carotene may be a protective mechanism for photosynthesis under high-intensity blue and white light conditions, thus suggesting that β-carotene protects the light-harvesting complexes through absorption of blue/UV-A wavelengths (16, 36, 37).

In some microalgae, carotenoid accumulation can also be induced by N deficiencies in the culture medium. Furthermore, N deficiency can produce oxidative damage in illuminated cultures as reported for higher plants (38). Thus, carotenoid accumulation might be a biological response to induce oxidative damage. It was shown that nitrogen deficiency affected the photosynthetic apparatus in leaves of rice plants, leading to lowered fluorescence parameters (photosynthetic yield, quantum yield for photosystem II, PSII, electron transport rate, and quenching) as well as lowered photosynthetic capacity at saturation irradiance, which are indicators of photoinhibition. In addition, the carotenoid-to-chlorophyll ratio increased and the activities of antioxidant enzymes were lowered, which led to aggravation of membrane lipid peroxidation (i.e., oxidative damage) (38).

From our results, we conclude that the addition of UV-A radiation to PAR illumination could significantly enhance carotenoid production in long-term production processes by using two stages: (i) carotenoid-enriched biomass production (N-containing nutrient medium and UV-A radiation), and (ii) massive carotenoid biosynthesis (N-starved medium, UV-A radiation). Such enhancement would occur in both production per culture volume unit ($g \cdot L^{-1}$, stage 1) and in the carotenoid production rate ($pg \cdot cell^{-1}$, stage 2), with β-carotene (mostly the *all-trans* form) being the major carotenoid accumulated.

ACKNOWLEDGMENTS

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