UV-A promotes long-term carotenoid production of Dunaliella in photobioreactors with retention of cell viability

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The effect of adding UV-A radiations (320-400 nm) to photosynthetically active radiation (PAR, 400-700 nm) during the growth of *Dunaliella bardawil* in an air-fluidized bed photobioreactor was studied to evaluate cell growth and long-term production of carotenoids. The obtained results were compared to those obtained from *D. bardawil* cultures incubated under lab standard conditions for carotenoid production, this is to say, nitrogen starvation and absence of UV-A radiation. The addition of 26.5 μmol photons m^{-2} s^{-1} UV-A radiation to 1150 μmol photons m^{-2} s^{-1} PAR stimulated the growth of *D. bardawil* cultures grown in a full nutrient culture medium. The total carotenoid content, mostly β-carotene, was higher than that of control cultures (UV-A non added cultures) along the exponential phase. The concentration of β-carotene in UV-A added cultures after 450 h was found to be about two-fold that of control cultures. From the results of this work it can be concluded that the UV-A modulated addition to PAR could be successfully applied to long-term carotenoid production processes, whereas *D. bardawil* cells accumulates carotenoids with retention of its viability. It is also shown that UV-A promotes increases of both carotenoid production per culture volume unit and the specific carotenoid production rate ($pg.cell^{-1}$), β-carotene being the major accumulated carotenoid.

Keywords carotenoids; *D. bardawil*; photobioreactor; UV-A; β-carotene; lutein

1. Introduction

The increasing interest on carotenoid production by microalgae is due to the important commercial applications of these natural compounds and to the market demand of carotenoids, specially for pharmaceutical and nutritional applications [1, 4]. Carotenoids have traditionally been commercialised as food additives including colorants, antioxidants and vitamins [2, 7, 26]. Their protective ability against oxygen free radicals seems to be responsible for some therapeutic applications of carotenoids as degenerative diseases preventives, anti-cancer agents and immune-system stimulators, claimed by several studies [21, 25, 28].

Chemically carotenoids are a wide family of isoprenoids with 40 carbon atoms than comprises carotenes and their oxygenated derivates, the xantophylls. Physiologically they act as secondary photosynthetic pigments, as provitamin factors and as protectors against photooxidative damage of the photosynthetic apparatus. Its ability of protection against photooxidative damage has been associated to the capacity of carotenoids to dissipate the excess of light acting as a filter and to their antioxidant properties. Carotenoids are effective singlet oxygen quenchers able to eliminate activated oxygen radical forms [5, 18, 19], therefore involved in protection mechanisms against excess of irradiance.

Among the conditions inducing large accumulation of carotenoids, high photon flux densities (wavelength range 400-700 nm) are included. These inductive conditions might result in increases of the level of e.g. β-carotene from 1 % dry weight to 10 % dw [3, 6, 14]. Beside this, UV radiations have been proved to have both possitive and negative effects on the viability of microalgae cultures [16]. Some of the commercially attractive carotenoids produced by the microalgae *Dunaliella bardawil* have absorption in the UV-A spectrum [30]. As a consequence, the accumulation of carotenoids can be one of the microalga responses to the oxidative stress produced by UV radiations [12, 13, 17, 29].

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In this paper, the effect of UV-A radiation and nitrogen depletion on *Dunaliella* growth, xanthophylls pool (violaxanthin and zeaxanthin) and type of accumulated carotenoids is characterized and discussed. Both quantitative and qualitative profiles of the produced carotenoids are analysed, and the possible synergic effect of UV-A and nitrogen depletion as stress factors for production of carotenoids others than β-carotene is also discussed.

2. Materials and Methods

2.1 Microorganism and culture conditions

Dunaliella bardawil (UTEX 2538) was kindly provided by the Science Marine Institute of Andalucia (ICMAN), CSIC. Standard cultures were grown in mineral liquid medium at 25ºC, bubbled with air containing 5% (v/v) CO_2 and continuously illuminated with white light from fluorescent lamps (100 μE m^{-2} s^{-1}, at the surface of the flasks) plus 26.5 μE m^{-2} s^{-1} UV-A (Philips 30W/33) . The composition of the culture medium is described by Cao et al [10].

2.2 Analytical determinations

Chlorophyll was determined by heating and extracting cell pellets with acetone, and measuring absorbance at 647 and 663 nm of 18.3 and 74.46 mg^{-1} cm^{-1} ml. The chlorophyll content of the cells was calculated by the following equations [13]:
Chl a = 5 [(12.25 Abs_{663nm}) - (2.73 Abs_{647nm})]
Chl b = 5 [(21.5 Abs_{647nm}) - (5.1 Abs_{663nm})]
Chl total = Chl a + Chl b
The total carotenoid content were determined spectrophotometrically by the following equation: [(3000 · Abs470) – 1.63 (Chl a)]/221. Protein content was determined following the method described by Bradford [8].

2.3 HPLC analysis of carotenoids

Separation and analysis of carotenoids was peformed in a Merck Hitashi HPLC, RP-18 column. 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; 30-35min, 100% A, as described by Young et al [31].

2.4 Air-fluidized bed photobioreactor

A 3.2 liters stirred tank Applikon photobioreactor was used in this study (Figure 1). The bed was fluidized by a mix of air and CO_2 (5% v/v) at a flow rate of 2.5 $l.min^{-1}$ and 150 rpm, respectively.
Main cultivation parameters (pH, dissolved oxygen concentration and temperature) were continuously measured by an Applikon control unit. The reactor was equipped with a water jacket connected to a water bath, which allowed to set and control a constant temperature of 25ºC. The pH was set at 7.5 for all the experiments.

The photobioreactor was illuminated by fluorescent lamps (PAR: Philips 15W/33, and UV-A: Philips 30W/33) and the incident light intensity at the reactor surface was 1150 μmol photon m^{-2} s^{-1} PAR and from 0 to 26.5 μmol photon × m^{-2} × s^{-1} UV-A radiation. The light intensity was determined with a quantum photoradiometer from Delta OHM HD 9021.

3. Results and Discussion

In this work, the effect of adding UV-A radiations to PAR radiations on the growth and long-term carotenoid accumulation of *D. bardawil* cultures in a fluidized-bed photobioreactor was studied. The obtained results were compared to those obtained in *D. bardawil* cultures incubated under nitrogen starvation as stress factor (standard conditions to induce carotenoid accumulation). In addition, the synergy effect produced by both UV-A radiation and nitrogen starvation on carotenoid accumulation was also evaluated.

3.1 Growth and total carotenoid accumulation in cultures of *D. bardawil* exposed to UV-A radiations in an air-fluidized bed photobioreactor operated in discontinuous mode

Cultures of *D. bardawil* grown in standard conditions and illuminated with only PAR (see Materials and Methods) were harvested, washed and resuspended into two aliquots of standard fresh culture medium. One of the cultures was added nitrate, the other one was not added inorganic nitrogen. Both cultures were incubated in an air-fluidized bed photobioreactor (Figure 1) illuminated with continuous UV-A radiations of 26.5 μmol photons m^{-2} s^{-1} added to photosynthetically active radiation (PAR, 1150 μmol photons m^{-2} s^{-1}). The choice of the UV-A irradiance, 26.5 μmol photons m^{-2} s^{-1}, was based on previous experiences at our lab [29]. The chosen UV-A irradiance produced photooxidative stress but did not have negative effect on the culture viability.

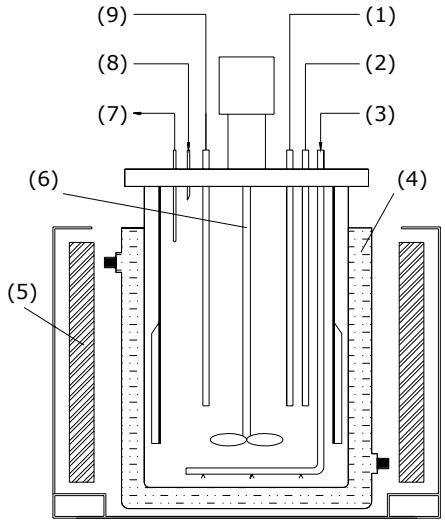

Fig. 1 Scheme of the air-fluidized bed photobioreactor used in the experiments. The photobioreactor components are: (1) O_2 probe; (2) pH sensor; (3) Air and CO_2 supply; (4) Water jacket; (5) Light jacket; (6) Stirrer; (7) Medium outlet; (8) Medium inlet; (9) Temperature sensor.

Similarly, two control cultures (non UV-added cultures) were both grown in the absence of UV-A radiation and either in the presence or in the absence of inorganic nitrogen (nitrate), respectively. The initial cell density in all of the cultures was 10^6 cells.ml^{-1}.

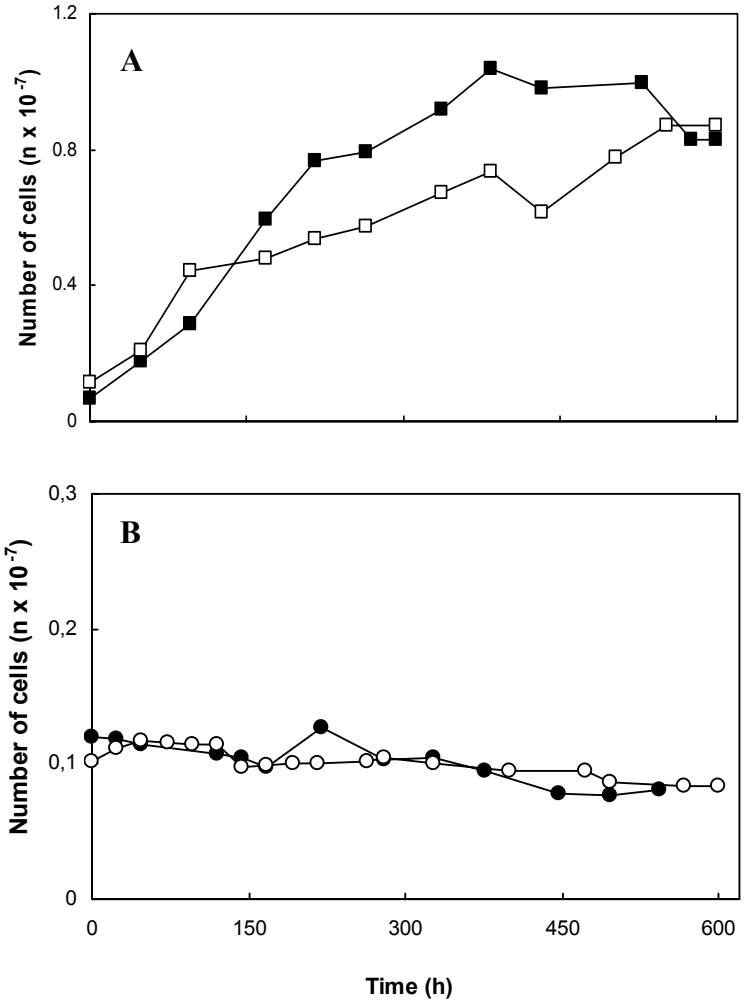

Fig. 2 Growth of UV-A exposed *D. bardawil* cell cultures compared to non-exposed control cultures. Cells grown in standard culture conditions (see Materials and Methods) were harvested, washed, resuspended in fresh culture medium and incubated under the following conditions: (part A) full nutrient medium and illuminated with 1150 μmol photons m^{-2} s^{-1} PAR plus 26.5 μmol photons m^{-2} s^{-1} UV-A (□), full nutrient medium and illuminated with 1150 μmol photons m^{-2} s^{-1} PAR only (■), (part B) inorganic nitrogen-starved medium and illuminated with 1150 μmol photons m^{-2} s^{-1} PAR plus 26.5 μmol photons m^{-2} s^{-1} UV-A (○), inorganic nitrogen-starved medium and illuminated with 1150 μmol photons m^{-2} s^{-1} PAR only (●). Number of cells was determined at the indicated times.

As shown in Figure 2A, the addition of 26.5 μmol photons m^{-2} s^{-1} UV-A radiation to PAR slightly stimulated cell growth of those *D. bardawil* cultures in the presence of nitrate during the early exponential growth phase, up to 100h growth. This is in good agreement with previous results obtained from short-term experiments [17,29] which showed the enhancement of *Dunaliella* growth in cultures irradiated with UV-A radiations. After 100h growth the evolution of the number of cells in those cultures illuminated with UV-A remained lower than those of the control cultures (UV non-added cultures). However, both cultures showed similar growth slopes along the late exponential phase (up to 25 days of growth) and reached similar final cell densities after 600h incubation.

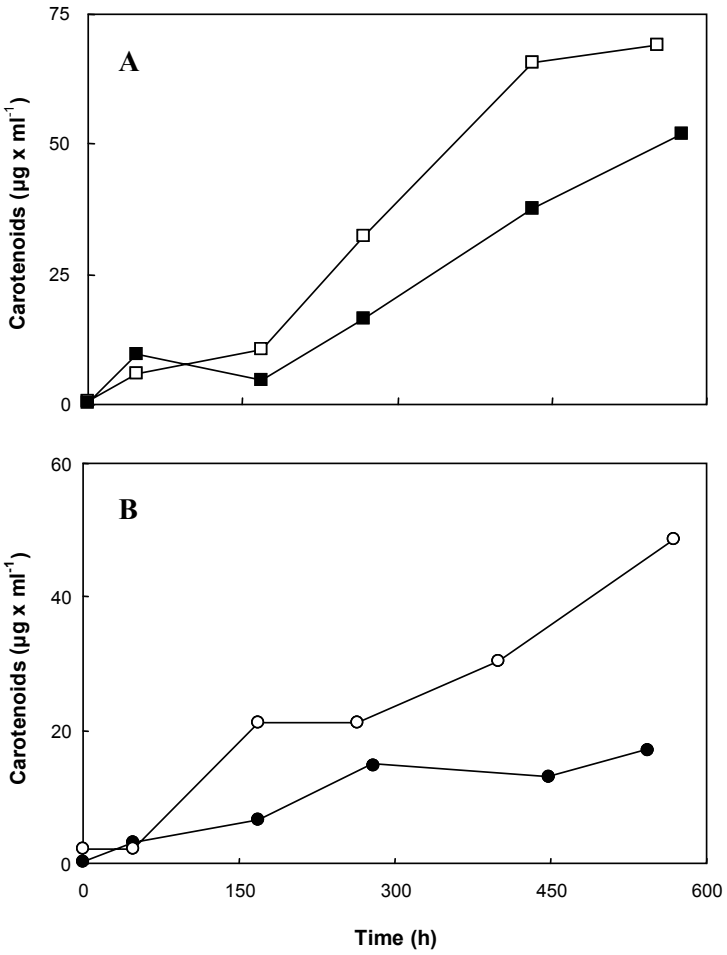

Fig. 3 Total carotenoid content of UV-A exposed *D. bardawil* cell cultures compared to non exposed control cultures. Cells grown in standard culture conditions (see Materials and Methods) were harvested, washed, resuspended in fresh culture medium and incubated under the following conditions: (part A) full nutrient medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR plus 26.5 µmol photons m^{-2} s^{-1} UV-A (□), full nutrient medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR only (■), (part B) inorganic nitrogen-starved medium and illuminated with 1150 □mol photons m^{-2} s^{-1} PAR plus 26.5 µmol photons m^{-2} s^{-1} UV-A (○), inorganic nitrogen-starved medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR only (●). Total carotenoid content was determined at the indicated times.

Those cultures incubated in nitrogen-starved culture medium showed no growth, as expected (Figure 2B). Therefore, addition of low intensity UV-A radiations to PAR has no negative effect on *D. bardawil* growth.

Figure 3 shows the time course carotenoid content for each one of the *D. bardawil* cultures. Along the exponential growth, the total carotenoid content of those cultures growing in a full nutrient culture medium and illuminated with UV-A radiation (Figure 3A) is higher than that of control cultures (non UV-A added cultures), resulting in a higher carotenoid accumulation with less biomass. The addition of UV-A radiation to PAR enhances carotenoid accumulation of *D. bardawil* all its growth along.

The carotenoid content of *D. bardawil* cultures grown in the absence of inorganic nitrogen and illuminated with only PAR (Figure 3B) showed, up to mid exponential phase, a similar profile than that

of UV-A irradiated cultures. However, the combination of two oxidative stress factors as UV-A and lack of inorganic nitrogen promotes the long.term carotenoid production of *D. bardawil* but it did not result in a higher amount of accumulated carotenoids when compared to *D. bardawil* growing in a full nutrient culture medium and exposed to UV-A radiation (Figure 3B). Consequently, the addition of UV-A to PAR radiations enhances carotenoid accumulation in *D. bardawil* growing in an airlift photobioreactor and prolongs the carotenoid production with viable cells up to the end of the exponential phase. The metabolic deficiencies produced in the absence of nitrate can not be overcome by the stimulating effect of UV-A on cell growth [24]. Table 1 shows the carotenoid production rates under the different conditions studied.

Table 1 Average carotenoid production rate of *Dunaliella bardawil* cultures in an air-fluidized bed photobioreactor illuminated with UV-A radiations.

Culture conditions	Production rate per volume unit $mg.l^{-1}.d^{-1}$	Production rate per cell $pg.cel^{-1}.d^{-1}$
PAR	2.01	0.24
PAR + UV-A	3.45	0.31
PAR + (-N)	1.86	3.00
PAR + UV-A + (-N)	1.99	4.10

3.2 Analysis of specific carotenoids of UV-A exposed cultures of *D. bardawil*

The analysis of specific carotenoids (Figure 4) reveals that the highest accumulation of β-carotene is produced in those *D. bardawil* cultures growing with nitrate and illuminated with PAR plus UV-A. The concentration of β-carotene in these cultures after 450 h is about two-fold that of control cultures. Violaxanthin and zeaxanthin accumulation also seems to be stimulated by UV-A radiations, the biosynthesis of violaxanthin being strictly dependent on the presence of inorganic nitrogen (Figure 4). In all of the cultures, the highest carotenoid production rate both per volume unit and in terms of specific production rate ($pg.cell^{-1}$), occurred at the end of the exponential growth phase. This is according to the fact that carotenoids are accessory pigments which are produced by secondary metabolic pathways [24], usually expressed when the culture medium is exhausted.

Zeaxanthin and violaxanthin are the main pigments involved in the xanthophyll cycle. The xanthophyll cycle is ubiquitous in higher plants and is implicated in protection against photodamage [11,15]. Zeaxanthin is a power antioxidant and violaxanthin is rather involved in light capture than in the antioxidative response [9, 20, 27]. The lack of nitrogen sustains an oxidative situation which the cell faces by increasing carotenoid production, including zeaxanthin and β-carotene. Carotenoids have significant absorption in the UV-A spectrum [22,30]. This can explain the fast reaction to UV-A exposure in terms of the rapid accumulation of zeaxanthin in those UV-A exposed cultures (Figure 4).

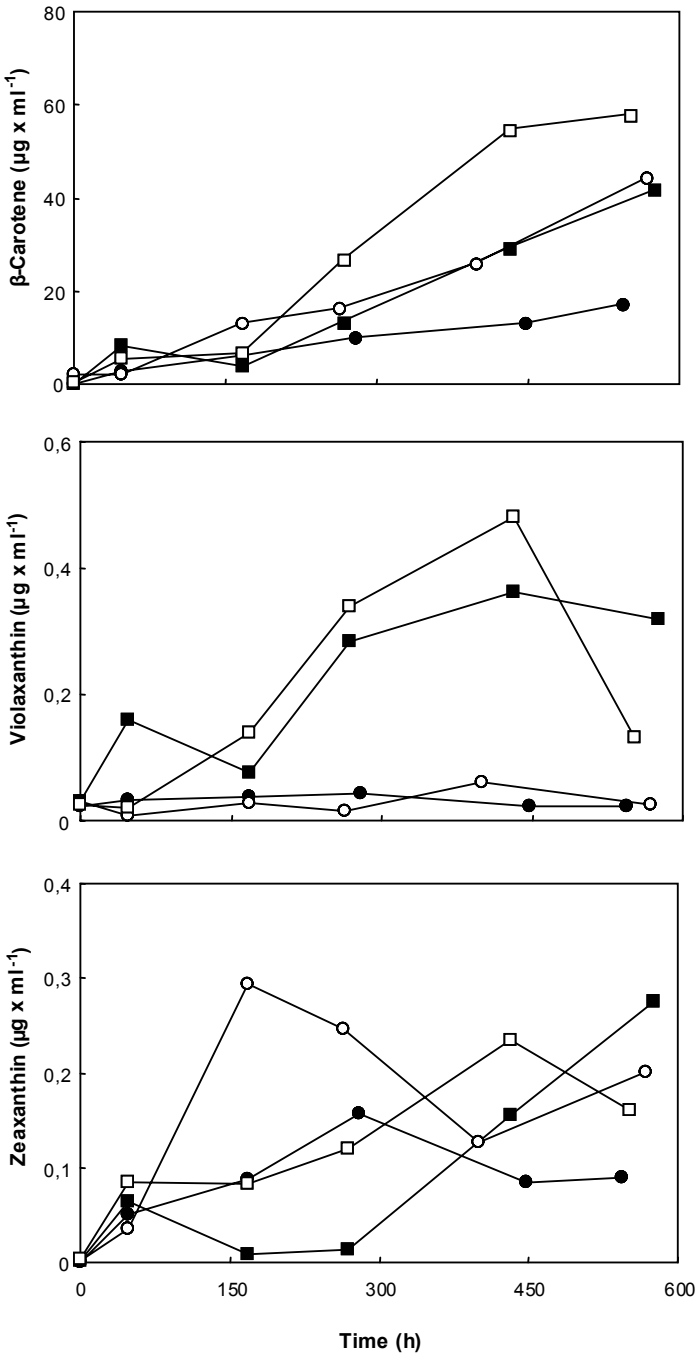

Fig. 4 Carotenoid profile of UV-A exposed *D. bardawil* cell cultures compared to non exposed control cultures. Cells grown in standard culture conditions (see Materials and Methods) were harvested, washed, resuspended in fresh culture medium and incubated under the following conditions: full nutrient medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR plus 26.5 µmol photons m^{-2} s^{-1} UV-A (□), full nutrient medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR only (■), inorganic nitrogen-starved medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR plus 26.5 µmol photons m^{-2} s^{-1} UV-A (○), inorganic nitrogen-starved medium and illuminated with 1150 µmol photons m^{-2} s^{-1} PAR only (●). Cell content of β-carotene, zeaxanthin and violaxanthin was determined at the indicated times.

From the results of this work it can be concluded that addition of UV-A radiation to PAR could be successfully applied to long-term carotenoid production processes, where *D. bardawil* cells accumulate carotenoids with retention of its viability. It has also been shown that UV-A promotes increases of both carotenoid production per culture volume unit and the specific carotenoid production rate ($\mu g.cell^{-1}$), β-carotene being the major accumulated carotenoid.

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