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Induction of canthaxanthin production in a *Dactylococcus* microalga isolated from the Algerian Sahara

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HIGHLIGHTS

- A *Dactylococcus* from the Sahara was characterized for carotenoids production.
- Carotenoid production was a function of light intensity and enhanced by salinity.
- Nitrate depletion enhanced lipids production but not carotenoids production.
- Production of lipids and carotenoids was greatest when stresses were combined.
- Canthaxanthin was the main secondary carotenoid.

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ABSTRACT

Secondary carotenoids are high-valued anti-oxidants which can be produced by some algae when exposed to an environmental stress (e.g. nutrient deprivation, high light intensities). To this end, we characterized the stress-induced carotenoid production of a new microalgal strain, *Dactylococcus dissociatus* MT1, which was isolated from the Sahara Desert of Algeria. Nitrate starvation, oxidative stress and varying light intensities were applied to determine the effect of illumination on carotenogenesis. Canthaxanthin was the main secondary carotenoid and light intensity had an important influence on the rate of its accumulation. The addition of NaCl also enhanced canthaxanthin production while nitrate depletion had more of an effect on lipid production. However, these two stresses in combination synergistically increased the production of both. Our results represent a step toward the development of strains suitable for secondary carotenoid production at the industrial scale.

1. Introduction

The primary and secondary carotenoids of microalgal origin are bioactive compounds with antioxidant activities which may have diverse applications in human or animal nutrition and pharmacology (Plaza et al., 2010). They are used as additives and colorants in the food industry and aquaculture, in cosmetics and as active ingredients in pharmaceutical products (Higuera-Ciapara et al., 2006). Secondary carotenoids, such as astaxanthin and canthaxanthin, can be produced in algal cell cultures and are considered to be stronger antioxidants than primary carotenoids (Jin et al., 2006).

While the primary, or photosynthetic, carotenoids are localized exclusively in the thylakoid membranes of chloroplasts, secondary carotenoids do not participate in photosynthesis and are characterized by extra-thylakoid localization. Secondary carotenoids are accumulated in specialized structures which can be localized in both the chloroplast stroma and outside the plastids (Solovchenko, 2013). The study of secondary carotenoids biosynthesis pathways, the factors of their induction and the mechanisms of their synthesis regulation are less studied in comparison with those of primary carotenoids, although progress has been made in recent years (Takaichi, 2011).

The carotenogenesis process in algae is induced by various stressors and especially their combinations. Combinations of osmotic stress, nitrogen and phosphorus deficiency and oxidative stress from high light intensities are particularly effective (Solovchenko, 2013). Under high light intensities which oversaturate the photosystem, photons are absorbed in excess and a considerable part of the incident light energy cannot be utilized in photochemical reactions (Solovchenko, 2010). As a result, the excess energy leads to the formation of highly active oxygen

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free-radicals (Bar et al., 1995). The primary carotenoids cannot scavenge the radicals sufficiently, so additional mechanisms are required to eliminate them or reduce the illumination reaching the photosynthetic apparatus under such conditions (Bar et al., 1995). Such a state is imposed by high fluxes of solar radiation and by other factors limiting the rate of CO_2 fixation (high salinity, nutrient stress, extremely high or low temperatures, etc.) (Solovchenko, 2010).

Microalgae are a very attractive and potentially cost-effective production platform for antioxidant production because of their capacity to accumulate high levels of secondary carotenoids in their biomass, simple media requirements and use of solar energy and CO_2 as primary substrates. A large number of green microalgae have already demonstrated the capacity to accumulate secondary carotenoids under species-specific stresses, such as *Dunaliella salina* (Lamers et al., 2012) and *Haematococcus pluvialis* (Boussiba et al., 1999) among others. The co-production of astaxanthin and algal biofuel has also been reported (Liu et al., 2013). However, no strains of *Dactylococcus* have been characterized for the overproduction of lipids or carotenoids and canthaxanthin production in the closely related *Scenedesmus* genus is also not well studied (Pirastru et al., 2012). The canthaxanthin market is growing, but hindered by the lack of appropriate microbial sources. Therefore, the current work examined the production of high-valued carotenoids from the perspective of developing a new species of microalgae for use in a microalgae-based biorefinery (Borowitzka, 2013; Yen et al., 2013).

Among microalgae, secondary carotenoids accumulation is typical in extremophiles, an ability which is imparted by the high content of secondary carotenoids in their cells (Solovchenko, 2013). The specific properties and adaptation mechanisms developed by extremophile strains has attracted interest in the field of phytobiotechnology.

A vast quantity of algal biodiversity remains to be discovered, so the potential to find new algal platforms for the production of high-valued compounds, such as secondary carotenoids, remains high. The great diversity of microalgal flora of the Sahara Desert of Algeria is so far untapped. To this end, strains of microalgae were isolated from this extreme environment which is characterized by intense solar radiation and high daily and seasonal temperature variations. The coldest month is January (−10 to 3 °C), the hottest months are July and August (45–56 °C). Because this is an environment of high solar intensity, it was hypothesized that strains isolated from this location would possess efficient mechanisms for producing photoprotective pigments, such as secondary carotenoids. Indeed, the current work showed the strain *Dactylococcus dissociatus* MT1 to be an efficient producer of canthaxanthin. While microalgal strains from the Sahara have been investigated for their ability to produce hydrogen (Chader et al., 2009), this study marks the first strain from the Sahara to be characterized for the production of carotenoids.

2. Methods

2.1. Microalgae strain

D. dissociatus MT1 was field collected from the Sid Ahmed Timmi oasis, altitude 252–282 m, 0° 15′E longitude, 27° 45′N latitude near Tamentit in Adrar, in the Sahara Desert of Algeria. It was initially identified as a strain of *Chlorella* in the Bioenergy and Environment Laboratory at the Center of Renewable Energy Development (Centre de Développement des Energies Renouvelables, CDER), in Algiers, Algeria, by key identification (Gayral, 1975) for single-celled green algae, because it was spherical in shape, between 2 and 10 μm in diameter, without flagella and contained one chloroplast with a typical pyrenoid structure.

Subsequently, an analysis of internal transcribed spacer (ITS-1 and ITS-2) regions of the 18S nuclear ribosomal DNA was conducted to characterize the phylogeny of the strain. Genomic DNA was extracted and purified using a DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The amplification, determination and analysis were performed according to previous work (Qiao et al., 2009) using reagents from a GoTaq Flexi DNA Polymerase kit (Promega, Madison, Wisconsin, USA) and a Sigma GenEluteTM Gel Extraction Kit (Sigma, USA). The strain *D. dissociatus* MT1 was determined to have 99.2% similarity to *D. dissociatus* UTEX #1537, accession: AY510466.1. The full characterization procedure is found in the supplementary material.

The strain was maintained as an axenic culture on solid medium comprised of modified 3N-BBM+V (Culture Collection of Algae and Protozoa, http://www.ccap.ac.uk/) plus 1.0 g L^{-1} sodium acetate trihydrate, 2.0 g L^{-1}BactoTM Tryptone, 2.0 g L^{-1}BactoTM Yeast Extract and 15 g L^{-1} agar. Subculturing to fresh solid medium was done monthly. Preparation of 3N-BBM+V is found in the supplementary information.

Suspension cultures of *D. dissociatus* MT1 were prepared by transferring one loopful of culture from the solid medium into 250 mL Cellstar® flasks (Greiner Bio-One #658195) containing 50 mL 3N-BBM+V medium which were maintained on an orbital shaker (Lab-Shaker, Kühner, Switzerland) at 90 rpm, 20 °C, and 30 μE m^{-2} s^{-1} with a light:dark period of 12:12. These sterile, liquid-phase cultures were used to inoculate the growth phase precultures.

2.2. Carotenogenesis process

2.2.1. Growth phase cultures

Green, growth phase *D. dissociatus* MT1 precultures were prepared to provide nitrate-starved inoculum to the carotenogenesis experiments. These cultures were maintained in an incubator (Lovibond Model ET 650-8) in 500 mL Duran glass bottles with a culture volume of 400 mL. The cultures were aerated by bubbling filtered air through a silicone tube (I.D. of 6 mm) at a flow rate of 200 mL min^{-1}. The temperature was constant at 25 ± 0.1 °C and the light intensity was 80 μE m^{-2} s^{-1} (measured at the exterior surface of the bottle) provided by 18 W cool white lamps. When the measured concentration of NO_3^- was less than 4 mg NO_3^- L^{-1} the green cells were used as inoculum for the carotenogenesis experiments. This happened when the nitrate-starved pre-cultures were transferred to the stress medium in the Multicultivator MC 1000-OD (see below). The carotenogenesis process was induced as described below.

2.2.2. Carotenogenesis phase

After the growth phase, the stress phase began when the microalgae were transferred to a Multicultivator MC 1000-OD (Photon Systems Instruments, Drasov, Czech Republic) for cultivation. It consisted of eight cultivation vessels, each one of which maintained 80 mL of suspension culture under controlled temperature, light and aeration conditions. Each vessel was independently illuminated by an array of warm white LEDs which were adjustable for intensity. Air was delivered to each vessel at a rate of 125 mL min^{-1} by a glass tube with an I.D. of 2 mm.

For inoculation into the Multicultivator, 20 mL of growth phase cells (<4 mg NO_3 L^{-1}) were combined with 60 mL of 3N-BBM+V stress medium, which was identical to 3N-BBM+V except it contained 10 g L^{-1} NaCl and no nitrate. The final concentration in the medium was therefore 7.5 g L^{-1} NaCl after mixing with the cell culture. This resulted in nutritional and osmotic stresses, as reported in a previous study (Gouveia et al., 1996). The cultures were grown in duplicate at 26 °C and with light intensities at the illumination source (determined by the Multicultivator software) of 100,