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Original article

Effect of stressful conditions on the carotenogenic activity of a Colombian strain of *Dunaliella salina*

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ABSTRACT

The objective was evaluate the carotenogenic activity of *Dunaliella salina* isolated from the artificial salt flats of municipality of Manaure (Department of La Guajira, Colombia). Two experimental testings were designed, in triplicate, to induce the reversibility of the cell tonality depending on the culture conditions. In the first test (A), to induce the reversibility from green to red tonality in *D. salina* cells, these were cultured in J/1 medium at a concentration of 4.0 M NaCl, 390 μmol m^{-2} s^{-1}, 0.50 mM KNO_3. In the second test (B), to induce the reversibility from red to green cell tonality, the cultures were maintained in J/1 medium 1 M NaCl, 190 μmol m^{-2} s^{-1}, 5.0 mM KNO_3 and pH 8.2. The population growth was evaluated by cell count and the pigment content was performed by spectrophotometric techniques. It was found that in both tests the culture conditions influenced the population growth and the pigments production of *D. salina*. There was a significant difference between the mean values of total carotenoids in the test A with 9.67 ± 0.19 μg/ml and second test with 1.54 ± 0.08 μg/ml at a significance level of $p < 0.05$. It was demonstrated that the culture conditions of test A induce the production of lipophilic antioxidants, among these carotenoids. The knowledge of the stressful conditions for the production of carotenoids from *D. salina* isolated from artificial saline of Manaure opens a field in implementation of this biotic resource for biotechnological purposes, production of new antibiotics, nutraceuticals and/or biofuels production.

1. Introduction

The microalgae prospecting over the last four decades has diversified due to its applications in the pharmaceutical, cosmetics, nutraceutical, biomedical and concentrate animal production industries (Eriksen, 2008, Vigani et al., 2015), treatment of domestic wastewater, production of biofertilizers (Faheed and Fattah, 2008), aquaculture (Hemaiswarya et al., 2011, Villa et al., 2014) and obtaining biofuels (Chisti, 2007, Deng and Coleman, 1999). The advantage of use of microalgae lies mainly in its photosynthetic capacity and metabolic plasticity. Advances in genetic engineering and biology techniques and procedures have allowed the selection and improvement of strains adapted to stressful culture conditions (Mostafa, 2012; Vílchez et al., 2011). This has been favorable for reduction of costs in relation to the competitiveness of new markets (Gimpel et al., 2013) for the industrial sector related to the generation of green products (for example, bioenergy, bioproducts and natural products). The majority of the biomass used for industrial production of proteins, astaxanthin, ß-carotene, glycerol, liquid fuels and fine chemicals (Raja et al., 2007; Benemann, 2013; Bhattacharjee, 2016) based on biotechnology of microalgae comes mainly of cultivated species of the genera *Botryococcus*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Spirulina* (Hallmann, 2007).

However, research efforts have focused on producing of microalgae biomass rich in carotenoids since this type of compound in terms of actual or potential applications are attractive for commercialization. Indeed, this has generated a strong demand on the global market where the price of β-carotene obtained from microalgae reaches a commercial value of 700 €/kg while its synthetic counterpart does not reach half of that figure (Guedes et al., 2011). The preference for the health market of β-carotene natural -a secondary metabolite that consists of a mixture of stereoisomers 9 *cis* and *trans*, with 40 and 50% respectively-, is

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due to its properties as a stimulator of the immune system and its uses in the treatment of more than 60 deadly diseases, including different types of cancer, heart disease and neurodegenerative diseases, premature aging and arthritis (Emtyazjoo et al., 2012) and pathologies with hyperlipidemia and hypercholesterolemia effects (Priyadarshani and Rath, 2012; Kumar, 2014).

The genus *Dunaliella* was described in 1905 by Teodoresco and is traditionally included within the family Polyblepharidaceae in the order Volvocales. Ettl (1983) proposed the new order Dunaliellales and the Dunaliellaceae family for this genus. Massyuk (1973) divided the genus into two subgenus, *Pascheria* and *Dunaliella*. Among the *Dunaliella* species of commercial interest, *D. salina*, *D. bardawil*, *D. parva*, *D. tertiolecta* and *D. viridis* have been reported. The taxonomy of the genus includes morphological and physiological attributes. Among its main morphological attributes are naked cells, isocontos flagella and a pyrenoid; chloroplast in cup form rich in chlorophyll *a* and *b*, xanthophylls (violaxanthin, zeaxanthin and lutein), carotenes (β, α and γ) with or without aplanospores; reproduction by longitudinal or sexual division (Oren, 2005). *D. salina* has developed adaptive strategies that favors its survival in aquatic environments with high salt concentrations, nutrient deficiency and high solar irradiation (Borowitzka, 1995). In these metabolic stress conditions, this microalga accumulates high concentrations of β-carotene as an antioxidant photoprotector and glycerol as an ormoregulatory agent, both at intracellular level (El-Baky et al., 2004). Since these species have a chemical composition of approximately 50% protein, 20% carbohydrates and 8% fatty acids, *D. Salina* was the first microalga cultivated for β-carotene industrial production, a valuable substance as a raw material for pharmaceutical products therapeutic and cosmetic (Pulz and Gross, 2004; Borowitzka, 1995).

Currently, *D. salina* biomass production has become an attractive opportunity for companies selling green products whose varieties and demands have increased in recent years on the large shopping centres, cosmetics stores and pharmacies in large cities (Benemann, 2013; Raja et al., 2008; Spolaore et al., 2006). For this reason, in order to obtain higher yields in algal biomass rich in carotenoids is developing studies that include design and implementation of closed (for example, photobioreactors) or open "raceways" systems, genetic improvement of strains, metabolomics research to obtain new molecules, cultivation physiology associated with the overproduction of metabolites, many scientific and technological efforts have been carried out (Lamers et al., 2008; Zhu and Jiang, 2008; Kleinegris et al., 2011; Wichuk et al., 2014; Andersen and Kawachi, 2005; Gallego et al., 2013).

In this way, bioprospecting has been proposed as a strategy to evaluate and recognize the ecological and economic relevance of biodiversity. For this reason, it has been imperative from different scientific approaches to promote research related to the prospecting of microalgae, a natural resource that until now has been little explored and undervalued (only 15 species are recognized for biotechnological purposes among 25,000 reported specimens). The aim of this study was to evaluate the carotenogenic activity of isolated and taxonomically characterize a strain of *D. salina* from artificial salt flats of the municipality of Manaure (Department of La Guajira, Colombia). To establish the relationship between the carotenoids production and chlorophyll in cultures at laboratory scale, different experimental tests were carried out. This study contributes to the prospection of photosynthetic microorganisms of biotechnological interest.

2. Materials and methods

2.1. Sample collection

D. salina strain was isolated from water samples collected in glass jars (previously cleaned with HNO_3, rinsed with distilled water and sterilized in an autoclave) of artificial salt flats located at coordinates 11°46′01.6″N 72°28′00.8″W of the municipality of Manaure (Department of La Guajira, Colombia) (see Fig. 1). The samples were enriched with J/1 medium (Borowitzka, 1988), preserved in thermos coolers until their laboratory transfer. The samplings coincided with the dry season of year (December 2016), high solar radiation with a photon flux density of approximately 900 μmol m^{-2} s^{-1}, temperature between 50 °C and 60 °C on surface of water, salinity of 4.0 M NaCl and pH between 7.55 and 7.77.

2.2. Isolation and identification of Dunaliella salina

The non-axenic monoclonal isolate of *D. salina* was carried out by combined use of microbiological techniques: capillary pipetting and seeding of stretch marks (Andersen and Kawachi, 2005). Before the implementation of each technique, 5 ml of each sample of water collected were taken, then they were added to Erlenmeyer flasks with 40 ml of J/1 medium. Each sample was analyzed with an inverted microscope (Leica DMI 600, Wetzlar, Germany). Given the tonality of the saline water at the time of sampling, the detection of cellular forms of the genus *Dunaliella* was facilitated (see Fig. 1).

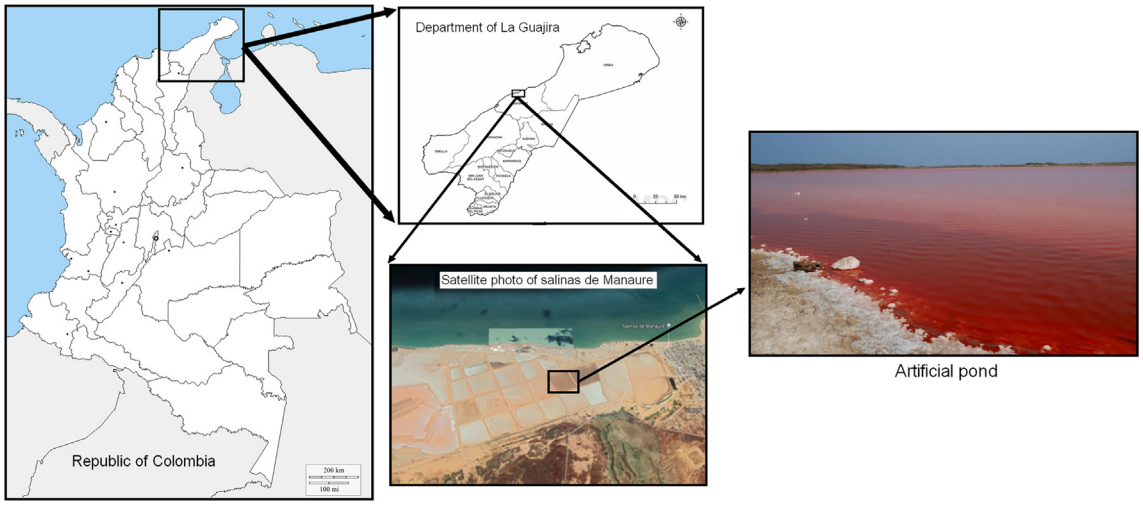

Fig. 1. Sampling site, Salinas de Manaure (Municipality of Manaure, Department of La Guajira, Colombia).

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2.2.1. Isolation by capillary pipetting

The isolate of *Dunaliella* sp. using the capillary pipetting technique, it was separated with a Pasteur micropipette (previously sterilized and immersed in 70% ethyl alcohol), individual cells from the Erlenmeyer flasks and transferred in vials with 5 ml of J/1 medium with independent concentrations of 1.5, 2.5 and 3.5 M NaCl. After 12 days, the algae growth was transferred to a 50–500 ml Erlenmeyer flask with 300 ml of J/1 medium with the same salinity concentrations of vials.

2.2.2. Scratch technique on agar plates

For this purpose, cultures samples (obtained in the previous procedure) with bacteriological needle previously sterilized were taken. Then they were seeded forming stripes on surface of agar medium enriched with J/1 medium in Petri dishes. The plates were maintained in a photoperiod incubation chamber of 14:10 h with a photon flux density of 120 μmol m^{-2} s^{-1} (Feilo Sylvania, Bogotá, Colombia), temperature of 21–23 °C and salinities of 1.5 and 3.0 M NaCl for 12 days. Once the algal colonies were observed, they were placed with a sterile inoculation needle on slides for microscopic verification of pure strains of *Dunaliella* sp. Finally, the identified strains were inoculated in 500 ml Erlenmeyer flasks (Duran Group, Wertheim/Main, Germany) with 300 ml of J/1 medium under the same conditions as the capillary pipetting method.

For the taxonomic viability of *Dunaliella* sp. microscopic observations were compared with the morphological, biochemical and suggested taxonomic descriptions Borowitzka and Borowitzka, 1988; Ginzburg, 1987; Loeblich, 1982).

2.3. Evaluation of carotenogenic activity

2.3.1. Preparation of the culture medium

In the tests, modified Johnson medium or J/1 medium was used. Its chemical formulation has specific nutrient concentrations for growth microalgae of genus *Dunaliella sp*. (Borowitzka, 1988). This medium consists a mixture solutions of macronutrients, micronutrients and iron chelated with EDTA. The solution of macronutrients contains in one liter of distilled water 1,5 $MgCl_2{\cdot}6H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.2 g $CaCl_2{\cdot}2H_2O$, 1.0 g or 10 mM of KNO_3, 0.043 g $NaHCO_3$ and 0.025 g KH_2PO_4; solution of micronutrients contains in a liter of deionized water 62.0 mg H_3BO_3, 38.0 mg $(NH_4)_6Mo_7O{\cdot}4H_2O$, 6.0 mg $CuSO_4{\cdot}5H_2O$, 5.1 mg $CoCl_2{\cdot}6H_2O$, 4.1 mg $ZnCl_2$, 4.1 mg $MnCl_2{\cdot}4H_2O$ (Brand Merck KGaA, Darmstadt, Germany); iron solution contains 189.0 mg Na_2 EDTA and 244.0 $FeCl_3{\cdot}H_2O$ in a liter of deionized water. Once prepared and sterilized in the autoclave, the nutrient solution was adjusted to pH 8.20 with HCl or NaOH to 0.1%, respectively.

2.3.2. Culture conditions for experimental testing

The monoclonal cultures of *Dunaliella salina* obtained in the isolation procedures were scaled to 500 ml Erlenmeyer flasks destined for reversibility tests of tonality and evaluation of the carotenogenic activity. The experimental testing were designed: (A) Green to red phase reversibility test and (B) Red to green phase reversibility test. For Test A, the following culture conditions were maintained: J/1 medium with 4.0 M NaCl, photon flux density of 390 μmol m^{-2} s^{-1}, 0.50 mM KNO_3 (Lopez-Elijah et al., 2013). In the case Test B, the cultures were maintained with medium J/1 to 1.0 M NaCl, photon flux density of 190 μmol m^{-2} s^{-1}, 5.0 mM KNO_3 and pH 8.2. All cultures were maintained at a temperature of 24 °C ± 1 °C. The duration of each test was twenty-one days.

2.3.3. Analytical determinations of growth and pigment production

The population growth of *D. salina* in cultures was determined by daily cell count with Neubauer chamber (Marienfeld Superior, Colonia, Germany) from the beginning of adaptation stage to the initial stationary stage. According to the equations proposed by (Guillard, 1973) cell density, specific growth rate and duplication time were estimated.

The quantification of the production of chlorophyll α and β and total of *D. salina* according to culture conditions with biomass samples was carried out. Acetone 90% analytical grade was used for the pigments extraction. The assessment of extracts were carried out at absorbance readings of 480, 647 and 664 nm wavelengths in a UV–visible spectrophotometer (Genesys™ 20-Thermo Scientific, Basingstoke, Hampshire UK) against an analytical grade acetone white to 90%. The concentration of chlorophyll *a* and *b* was calculated according to the equation of (Jeffrey and Humphrey, 1975) and that of carotenoids with the equation suggested by Strickland and Parsons (1972).

2.4. Statistical analysis

Comparisons of cultivation conditions with the production of carotenoids using the model I ANOVA were carried out, once checked the assumptions of normality and homoscedasticity to P-value < 0.05 level of significance. The statistical software used for processing of data was SPSS Statistics 17.0.

3. Results and discussion

3.1. Morphological characteristics of the isolated strain

Microscopic cells observations of the genus *Dunaliella* isolated from artificial salt flats of the municipality of Manaure (Department of La Guajira, Colombia) indicated the morphological characteristics of *Dunaliella salina* species. The microscopic analyses to (40X and 100X) showed unicellular, photosynthetic and mobile biflagellate cells, without a rigid cell wall. However, are surrounded by a mucilaginous layer of glycoprotein called glucocalix (Ben-Amotz and Avron, 1983, Sathasivam and Juntawong, 2013). The cells are reddish and orange, ovoid, spherical, pear-shaped, sometimes spindle-shaped or ellipsoidal, with semi-acute anterior edges, radially symmetrical round, and posterior borders. Moreover, cells appear with a single chloroplast in the form of a cup that mostly has a central pyrenoid surrounded by starch granules. Likewise, *D. salina* has an anterior stigma, diffuse and difficult to distinguish, especially in red cells; with a length ranging between 6.1 ± 0.5 and 27.0 ± 1.2 μm and a width between 4.0 ± 0.3 and 20.3 ± 2.1 μm ($p < 0.05$).

3.2. Carotenogenic activity tests

The UV–visible spectrophotometric analyses showed that stress conditions defined for test A (Green-red reversibility) are related to the variation of chlorophyll and carotenoid production over the days (see Fig. 1). In test A, stress culture conditions significantly induce the gradual increase of carotenoids until reaching a concentration of 9.67 ± 0.19 μg/ml on day 20 ($p < 0.05$) (see Fig. 2). Table S1 from Supplementary Material shows the inverse relationship between the production of carotenoids and cell growth in test A. Given this trend, it is quite likely that the culture conditions implemented for this study (4.0 M NaCl, photon flux density of 390 μmol m^{-2} s^{-1}, 0.50 mM KNO_3) are determinants in the carotenogenic activity of the isolated strain (Dipak and Lele, 2005). This behavior in the production of carotenoids in strains of *D. salina* has been evidenced in culture conditions relatively similar to this study (Cifuentes et al., 1996).

The induction of carotenogenesis of *D. salina* in its natural habitat has been shown to be a physiological response to stressful environmental conditions, such as extreme temperatures (−25 °C to 65 °C),

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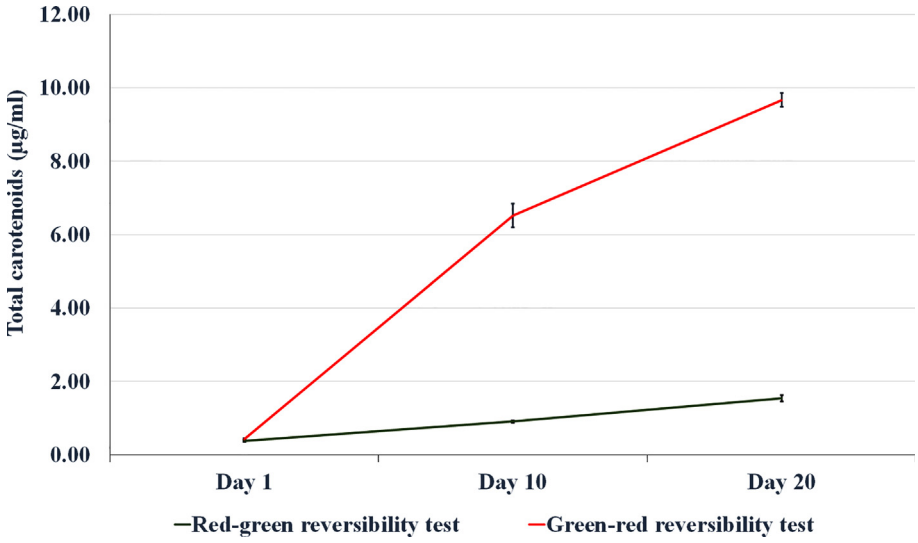

Fig. 2. Total carotenoids concentration in *D. salina* biomass grown under stress and non-stress conditions.

high intensities of sunlight (from 300 to 1200 µmol m^{-2} s^{-1}), nutrient deficiency (0.010–50 mM KNO_3) and high salt concentrations (30–40%). In this sense, it is clear the proliferation of a minimum number of halotolerant microorganisms, among them, this microalga that pigments the waters with an intense reddish colour, which are evident characteristics of the salinas at the sampling time (see Fig. 1). Indeed, the environmental conditions knowledge has generated a scientific interest involving the elucidation of biochemical and physiological processes, evolutionary mechanisms of adaptation and metabolic regulation of the carotenoids synthesis (Lopez-Elijah et al., 2013) and even for the design of experiments for biotechnological purposes (Raja et al., 2007; Kleinegris et al., 2011).

3.2.1. Effect of nitrogen

The stress conditions of the culture in *D. salina* in test A directly influenced the green-red reversibility in cells. Many studies have shown the effects of high light intensities and nutrient deficiency on the growth and carotenogenic activity of *D. salina* exposed to similar culture conditions (Borowitzka and Borowitzka, 1988, Gómez and González, 2005, Fazeli et al., 2006; Fu et al., 2014). The promoting and accumulation of carotenoids responsible for the reddish colour in cells in test A, with special attention on day 20, could be explained due to the accelerated nutrients consumption and excessive production of biomass during the first seven days of culture. Moreover, the low concentration of 0.50 mM KNO_3 for this test is likely to be adequate for the intracellular accumulation of carotenoids (Dhanam and Dhandayuthapani, 2013; Wu et al., 2016). The implementation of these stress conditions suggests an alternative to improve the carotenoids production based on the simulation of the natural stress conditions of this strain (Mishra and Jha, 2009).

3.2.2. Effect of luminous intensity

On the other hand, the lipophilic antioxidants accumulation in the genus *Dunaliella* is also attributed to metabolic activities of antioxidant enzymes such as superoxide dismutase, catalase and peroxidase. Moreover, other factors such as the production of malondialdehyde metabolites and radicals, in response to a high exposure of high light intensities, UV-B electromagnetic radiation (Kleinegris et al., 2011), (typical of the aquatic habitat of the isolated strain) have also influence on the lipophilic antioxidants accumulation. The high light intensity can damage the cell development and restrict the algae growth. Therefore, the photosynthetic mechanism of *D. salina* was activated to produce greater quantities of carotenoids, one of the most important protective pigments located among the thylakoids and stored in droplets of neutral lipids, capable of filtering the abundant harmful light (Khoyi et al., 2009). Consequently, the high light intensity was adequate for carotenoids biosynthesis, but it was disadvantageous for algae growth (Lamers et al., 2010).

To evaluate the red-green reversibility of *D. salina* in test B, the total chlorophyll production per culture volume as a function of time is greater than the achieved by test A (see Fig. 3). The comparison between growth rates and pigment production for both tests showed significant differences ($p < 0.05$). The evolution of the chlorophyll content in test B (red-green reversibility) showed a correlation with the cell densities from the initial stage of the culture until reaching a maximum cell density (MCD) of $3.12 \pm 1.09 \times 10^6$ cel ml^{-1} on day 10. The initial red cells of *D. salina* inoculated from the first day to the second, turned orange. In the day 4, the cells in the culture, presented a dark greenish colour and experienced an accelerated growth until obtaining the highest concentration of total chlorophyll on day 10 (see Fig. 3). However, the evolution reached in the carotenoids concentration in this test, showed an increase over time: from 0.38 ± 0.02 µg/ml on the first day to 1.54 ± 0.08 µg/ml on day 20. This indicates that the total chlorophyll production and the population growth of *D. salina* production, in this test, are related to a photosynthetic rate and to cellular biomass production (Lopez-Elijah et al., 2013). The observations under the microscope revealed that during the exponential phase, from day 8, the cells showed greater mitotic activity, large size and intense green color, which suggests that the culture conditions studied (1.0 M NaCl, photon flux density of 190 µmol m^{-2} s^{-1}, 5.0 mM KNO_3 and pH 8.2) are probably favorable for the physiological activities of the strain (Lopez-Elijah et al., 2013; Cifuentes et al., 1996).

3.2.3. Effect of salinity

It is known that the salinity factor affects the metabolic processes and cellular replication. This correlation was evident between the maximum growth rate of *D. salina* and the salinity in tests A and B (see Figs. 2 and 3). This condition has been demonstrated in studies with *D. salina* strains (Borowitzka and Borowitzka, 1988; Gómez and González, 2005; Cifuentes et al., 1996). This stress in salinity restricts the capacity of *D. salina* to acquire the necessary sources for its growth and productivity.

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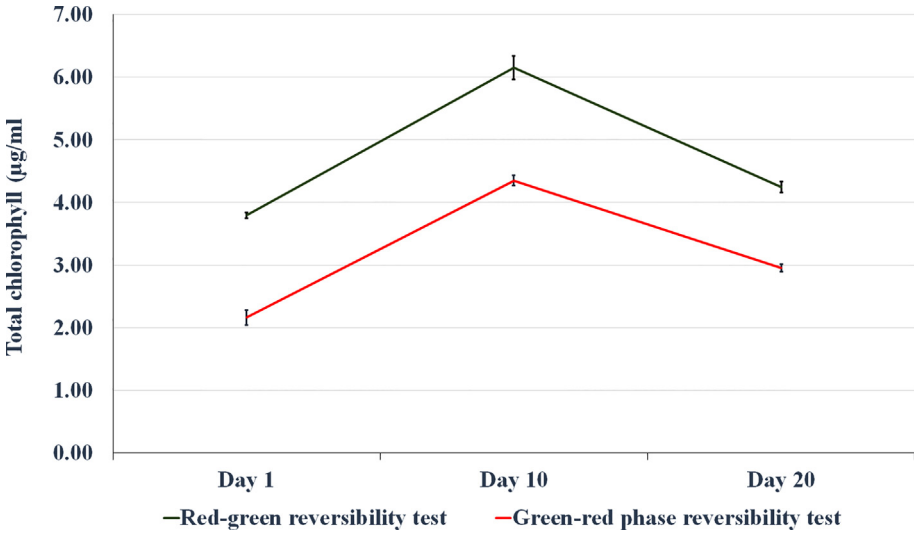

Fig. 3. Total chlorophyll concentration in *D. salina* biomass grown under stress and non-stress conditions.

This response to the increase in total carotenoids content is in first place, depending on the decrease of the nitrogen source in the culture medium that accompanied with an increase of pH, promotes the phosphate salts precipitation causing a deficiency state. In response to this, cells decrease the activity of the photosystem II and photosynthesis (Chen and Jiang, 2009). In second place, when an algal strain is influenced by a strong radiation can cause damage in the centers of reaction and in primary response to this factor promotes the carotenoids accumulation with a possible photo protector role (Ben-Amotz and Avron, 1983; Giordano and Bowes, 1997; Wykoff et al., 1998).

In general, the carbon fixation by photosynthesis is delayed in cells exposed to a variety of stresses. In spite of what was said above, in cells of *D. salina* there is a stimulation in the rate of acquisition of inorganic carbon and it has been determined the presence of a carbonic anhydrase adapted to high salinities (Azachi et al., 2002). A characteristic response of *D. salina* to stress by salinity is the adjustment in the intracellular concentration of glycerol by regulating the flow of carbon between the production of starch in the chloroplast and the synthesis of glycerol in the cytoplasm. Thus, the flow of carbon is channeled from starch toward the glycerol with a concomitant increase in the biosynthesis of plastidic isoprenoids, that is, a carotenoids increase (Cowan and Rose, 1992, 1991). Additionally, the microscopic observations showed a contrast in the average of the cell size when the microalgae was exposed to the osmotic changes. After a change, hyperosmotic cells increased their volume gradually depending on the NaCl concentration of the culture medium and of the exposure time (average cell volume of approximately $1380.36 \pm 0.03\ \mu m^3$, 1.6 times greater than those of logarithmic phase) (see Fig. 4). These observations matched with the ones presented by Fu et al. (2014), in where the *D. salina* cells exposed to osmotic stress conditions, reached to carotenoids concentrations significantly homogeneous.

4. Conclusions

The stress conditions, such as high luminous intensity ($390\ \mu mol\ m^{-2}\ s^{-1}$), high NaCl concentrations (4 M) and NO_3^- deficiency (0.50 mM KNO_3^-) exert a different effect between the population growth and the biosynthesis and intracellular accumulation of carotenoids from the *D. salina* strain isolated from Manaure's saline.

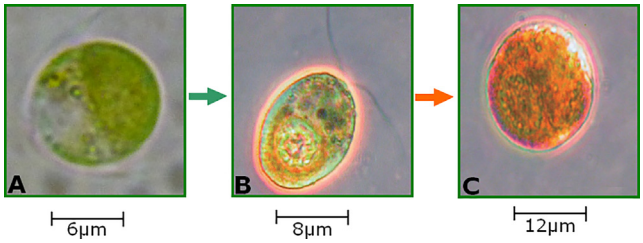

Fig. 4. Reversibility of tonality of *D. salina*. (A) Isolated from *D. salina*; (B) *D. salina* by changing its tonality (day 10); (C) *D. salina* in late stationary phase (day 20).

Stress conditions evaluated in cultures such as nitrogen source limitation, NaCl concentration increase and light intensity induced the carotenogenesis of the *D. salina* strain isolated from the Manaure's saline.

The isolated *D. salina* strain exhibits phenotypic traits that allow it to survive in extreme conditions such as extreme temperatures for other microorganisms less than 25 to 45 °C, high luminous intensities and salinities between 30 and 40% NaCl.

The biochemical and metabolic characteristics exhibited by *D. salina* due to the studied cultures conditions suggest an alternative of improvement in the carotenoids production in biotechnological and industrial terms.

The *D. salina* strain isolated from Manaure artificial saline could represent a biological resource of importance for the pharmaceutical, cosmetic and nutraceutical industries due to its great capacity for lipophilic antioxidants, enzymes, biopolymers or compatible solutes. Finally, its physiological properties and ease of cultivation in closed systems like photobioreactors, make them an attractive and economically viable technological alternative for commercial exploitation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2019.07.010.

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