Growth and Carotenoid Production in *Dunaliella* spp. Isolated from Hypersaline Habitats in the Region of Essaouira (Morocco): Effect of NaNO₃ Concentration

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Abstract: In order to determine the impact of nitrogen deficiency in medium, growth rate and carotenoids contents were followed during 15 days in two strain *Dunaliella* spp. (DUN2 and DUN3), isolated respectively from Azla and Idaouia saltworks in the Essaouira region (Morocco). These microalgae were incubated at 25 ± 1 °C with a salinity of 35‰ and continuous light in four growth media with different concentrations of sodium nitrate (NaNO₃): 18.75 g/L, 2.5 g/L, 37.5 g/L and 75 g/L. Maximum of cell density was observed under high sodium nitrate concentration during logarithmic phase of growth. The highest specific growth rate was $0.450 \times 10^6 \pm 0.006$ cells/mL and $2.680 \times 10^6 \pm 0.216$ cells/mL, respectively for DUN2 and DUN3. Carotenoids production mean were not stimulated under nitrogen deficiency, and the highest content was showed in DUN2 at high nitrogen concentration (3.210 ± 0.261 µg·mL⁻¹) compared with DUN3 strain.

Key words: Carotenoids, *Dunaliella* spp., growth, nitrogen deficiency, Essaouira, Morocco.

1. Introduction

The microalgae biotechnology has been under research and valorization in last decade. In both marine and freshwater ecosystems, microalgae species were highly considered and have acquired a significant economic and industrial interest. These microorganisms have become the source of many biocompounds, each with large applications in various sectors, such as food, pharmaceutical and nutraceutical [1-3]. Indeed, microalgae can be used to produce a wide range of compounds, such as proteins,

lipids, carbohydrates, carotenoids or vitamins for health, food and feed additives, cosmetics and for energy production [4].

In microalgae group, *Dunaliella* species have taken importance and acquired a significant economic value for their most profitable business in the biotechnological industry and their richness of highly bioactive metabolites that might be useful in various sectors, such as food, pharmaceutical and nutraceutical [5, 6]. These microorganisms are the predominant algae in saltworks and naturally occurring hypersaline environments and are known by their ability to adapt to various changes in medium. *Dunaliella* species were widely distributed in the world and live in an extremely wide range of

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habitats [7]. These microorganisms were cultivated in many countries to produce carotenoids [8, 9], glycerol [8], proteins and vitamins [10], feeding for fish and human consumption [11, 12], biofuel production [13, 14] and pharmaceutical formulations [15]. *Dunaliella* species were also used in bioremediation for removing heavy metals and play an important role in detoxification and accumulation of heavy metals [16-18].

Because of interest mentioned above, capacity of growth and biocompounds production of *Dunaliella* were largely studied and exploited in various sectors. Optimization of culture conditions has received much attention worldwide in last decade. In this context, researchers have focused on isolation and physiological characterization of species or strains to evaluate their characteristics which can be exploited for mass cultivation, like rapid growth rate, broad tolerance of environmental conditions, high product content of biocompounds, carotenoid production and/or engineering of culture systems.

Many studies have been reported on optimal culture conditions of *Dunaliella* strains and growth and carotenogenesis have generated much interest by many researchers. These biological functions were best in cultures where balanced concentrations of nitrogen are present. This macronutrient is considered as important factor [19] and supplied as the source of nitrogen in growth and carotenoidogenesis [20, 21].

Therefore, the present study was undertaken to study the growth and carotenoid production in two Moroccan strains of *Dunaliella* under different concentrations of nitrates. Our objective is to determine the effect of nitrate deficiency on these parameters, with perspective to use results of this study for future commercial production of *Dunaliella* and its biocompounds in Morocco.

2. Materials and Methods

2.1 Strains Isolation and Culture Conditions

Our study was conducted on samples of strain

Dunaliella collected in 2014 from Azla (DUN2) (Latitude 31.160723 31°9'38.6" N, Longitude -9.705030 9°42'18.11" W) and Idao Iaaza (Latitude 31.145431 8°31'43.55" N, Longitude -9.737920 9°44'16.51" W) (DUN3) saltworks in the Province of Essaouira (Morocco) (Fig. 1).

Samples were collected from the bottom and the surface of the salt production tables, in summer 2014. Cell isolation was done by successive dilution and isolation to obtain monospecific strains. The identification of strains *Dunaliella* was based on a morphostructural study under binocular microscope. Strains were purified and identified according to Oren [22].

Experiments about growth and carotenoid content analysis were conducted during 15 days under continuous white light generated by the fluorescent lamps of 36W at a temperature of 25 ± 1 °C and salinity of 35‰, under constant agitation to ensure consistency and avoid sedimentation. In each bottle (capacity: 1 L), 50 mL of the stock suspension was added to 150 mL experimental medium [23], and four treatments (75, 37.5, 25 and 18.75 g/L of NaNO₃) with tree replicates each were considered.

2.2 Growth Determination

Growth rate of *Dunaliella* strains was followed every two days and during 15 days by cell counting using a light microscope and a Thomas haemocytometer. Number of cells was calculated as follows:

$$\text{Number of cells/mL} = \frac{\text{total counted cells} \times 10^6}{\times \text{dilution factor}}$$

2.3 Pigment Extraction and Analysis

Algal suspension, taken from each culture medium after mixing thoroughly, was centrifuged at 5,000 rpm (revolutions per minute) for 5 min. The pellet was then extracted with acetone 80% (80/20: v/v) after the mixture vigorously shaken to recover the maximum amount of extract and centrifugation again at the

Fig. 1 Geographical situation of Azla and Idao Iaaza saltworks (Province of Essaouira, Morocco).

same speed. The colorless biomasses were discarded and pigment concentrations were calculated according to Lichtenthaler [24]:

$$\text{Total carotenoids } (\mu\text{g/mL}) = [1,000 \times A_{470} - (1.82 \times 12.25 \times A_{663.2} - 2.79 \times A_{646.8}) - (85.02 \times 21.5 \times A_{646.8} - 5.10 \times A_{663.2})] / 198$$

where, A_{470} , $A_{663.2}$ and $A_{646.8}$ stand for sample absorbance at 470, 663.2 and 646.8 nm, respectively.

2.4 Statistical Analysis

Data were expressed as mean $\pm$ SD (standard error). The statistical significance of the differences between samples was determined by the *t*-test using the Statistical software. A *p* value of less 0.05 was considered as statistically significant.

3. Results

To determine if the number of cells and carotenoid content could be influenced by inorganic nitrogen

deficiency in *Dunaliella* strains, sodium nitrate was added at the following concentrations: 75 g/L (medium F/2 "control"), 37.5 g/L (medium F/4), 25 g/L (medium F/6) and 18.75 g/L (medium F/8).

The growth and carotenoid accumulation were analyzed by cell counting and exploring the carotenoid concentration respectively in two strains of *Dunaliella* spp. (DUN2 and DUN3 isolated respectively from Azla and Idao Iaaza saltworks).

As shown in Figs. 2a and 2b, cell number per milliliter tracking growth of DUN2 and DUN3 increased exponentially and during the culture period in F/2, F/4, F/6 and F/8 mediums. The amplitude of growth is more significantly marked in DUN3 comparatively to DUN2.

The highest densities of cells were observed on medium F/2 with an important production at the 9th and 13th day of culture respectively in DUN2 and DUN3.

Decreasing nitrate concentration in medium has

reduced slightly the growth of the DUN2 and DUN3 strains of *Dunaliella*.

The results of monitoring of cell density in strain DUN3 show pronounced temporal changes in cell production in nitrates concentrations tested. The

highest cell density is reached on the control medium F/2 (nitrates: 75g/L) at the 13th day of culture reaching a maximum of 2.680×10^6 cells/mL.

The growth presented a rapid cell multiplication trend in the first three days in F/2, F/4 and F/6 medium

Fig. 2 Effect of different concentrations of NaNO₃ on growth of *Dunaliella* strains: (a) DUN 2; (b) DUN3.

Each number is mean $\pm$ SE (standard error) (75 g/L (medium F/2 "control"), 37.5 g/L (medium F/4), 25 g/L (medium F/6) and 18.75 g/L (medium F/8)).

but an adaptation phase appeared until the 5th day in F/8 medium. The speed of growth was then accelerated to the 13th day reflecting exponential growth trends and maximum cell multiplication. After the 13th day, there was the beginning of the decline

phase in all medium culture excepted in F/8.

Concerning carotenogenesis, the present study shows that total carotenoids content increased, during the incubation period, in F/2, F/4, F/6 and F/8 mediums (Figs. 3a and 3b).

Fig. 3 Effect of different concentrations of NaNO₃ on carotenoids content of *Dunaliella* strains: (a) DUN 2; (b) DUN3.

Each number is mean ± SE (standard error) (75 g/L (medium F/2 “control”), 37.5 g/L (medium F/4), 25 g/L (medium F/6) and 18.75 g/L (medium F/8)).

For strain DUN2, the depletion of nitrate concentration in culture medium seems to increase the carotenoids content in this strain alga, but in DUN3, this parameter shows an almost constant production on all media tested. The highest concentration of this pigment, is produced at high nitrate concentration (medium F/2: NaNO₃ 75g/L) and the reduction of nitrate concentration in culture medium seems to decrease the carotenoids content in DUN3.

4. Discussion

The result obtained in our preliminary work, despite its limitation in time and space, indicates a significant influence of nitrogen deficiency in culture medium. Indeed, growth rate was improved under high nitrate concentrations in the two strain *Dunaliella* (DUN2 from Azla saltwork and DUN3 from Idao Iaaza saltwork), but the growth in DUN3 was more important. The highest growth rate was $0.450 \times 10^6 \pm 0.006$ cells/mL and $2.680 \times 10^6 \pm 0.216$ cells/mL, respectively for DUN2 and DUN3. Otherwise, highest carotene production was obtained by DUN3 at high nitrogen concentration compared with DUN2 which was stimulated under nitrogen deficiency.

Above results showed that growth and cartonogenesis of *Dunaliella* strains are a function of physicochemical factors in culture medium as light, salinity, temperature [25, 26], pH, CO₂ [27], the availability of nutrients and carbonates [28, 29].

These results are in agreement with those obtained, in several species of *Dunaliella*, by many researchers [30-34].

Native strain DUN2 showed low cell density with a tendency of cells to large size and virage color to orange due to deficiency nitrates in culture medium. This change constitutes an algal strategy for survival under stress conditions.

Although, high cells densities are observed in nitrate deficiency conditions in the DUN3 strain. This result, which seems to be contradictory, indicates that

not all strains of *Dunaliella* possess the same ability to respond similarly to the same culture conditions [35]. Indeed, in our experimental conditions, the cells densities have reached a production of 2×10^6 cells/mL. These results are better than those reported by Lopez-Elias et al. [34] who also studied the effect of nitrate deficiency on cellular biomass production; and have obtained cell densities ranging from 1.2×10^6 cells/mL to 1.28×10^6 cells/mL.

For carotenoid production capacity in the native strain DUN2 isolated from the saline Azla, carotenogenesis was probably generated by environmental stress conditions in this saltwork. This result confirmed the effect of nitrate deficiency on cell growth and carotenogenesis the two strains tested. This later factor induced carotenogenesis in strain DUN 2 in favor of inhibition of cell growth. While the native strain DUN3 showed a decreased ability of carotenogenesis against cell production stimulation.

These observations agree with findings of Semenenko and Abdullaev [36], Ben-Amotz et al. [37] and Phadwal and Singh [38] who described increase in the cellular level of β -carotene under conditions of nitrogen starvation in green micro algae *Dunaliella* spp. Indeed, it is well known that when some *Dunaliella* species or strains are submitted to environmental stress conditions as high salt concentrations, high light intensities or nutrient deprivation, these organisms can overproduce and accumulate very large amounts of β -carotene [39-44].

In this context, Cifuentes et al. [45] and Guevara et al. [46] have reported that the environmental conditions may be partly responsible for the development carotenogenesis in a particular strain of *Dunaliella* spp.

This kind of response to environmental stress was also described for other physiological function in *Dunaliella* spp. and other algae species, like lipid synthesis [13, 47], Chlorophyll accumulation and photosynthesis.

Difference response between DUN2 and DUN3

traduce the hypothesis that the DUN3 strain is another type of the species of the genus *Dunaliella*. This hypothesis is approved by small size observed in DUN3 cells comparatively to DUN2 cells. Further investigations about this hypothesis are ongoing in our laboratory. This kind of observation and results were reported in *Dunaliella salina* from northern Chile that carotenoids biosynthesis and accumulation were not stimulated but inhibited when cells were grown under low nitrate concentrations [48].

The concentrations of carotenoids obtained in DUN2 reached 3.21 µg/mL but only 1.020 µg/mL in DUN3. This carotenogenesis rate appeared to be better than those reported by Serpa Ibáñez and Calderón-Rodríguez [49] who studied the effect of different sources of nitrate on the production of carotenoids and obtained rates between 0.164 µg/mL and 1.417 µg/mL in *Dunaliella* spp. in Mexico.

5. Conclusions

Above results showed that the growth and carotenoid content in native strains *Dunaliella* of Azla (DUN2) and Idao Iaaza (DUN3) saltworks in the region of Essaouira (Morocco) were affected by nitrate concentration. These organisms could produce large amounts of carotenoids in nitrate deficiency conditions, but with great potential observed in the strain DUN2.

This result can be, therefore, considered and exploited in order to consider these strains and particularly DUN2 as a carotenoidogenic microalgae that can be exploited as natural renewable resource with biotechnological potential to become an alternative of natural resource of carotenoids.

However, other more detailed studies on taxonomic determination of *Dunaliella* spp. strains and on other factors affecting the productivity of carotenoids will be conducted to prove their biotechnological potential biocompounds productivity.

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