Effect of the medium culture on cells growth and accumulation of carotenoids in microalgae hypersaline Dunaliella sp. isolated from salt ponds of the region of Essaouira in Morocco

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Abstract
The green microalgae Dunaliella acquires very significant economic values for its richness in carotenoids (β-carotene), glycerol and other accessory pigments, applied in various sectors such as food, pharmaceutical and nutraceutical activities.

In this work, we have evaluated the effect of culture medium on production of biomass and carotenoids from this microalgae. Two strains isolated from two different sebkhas (salt ponds) located in the region of Essaouira (Morocco) are tested. Microalgae are grown during 21 days at natural and artificial culture media maintained at 25 ± 1 °C in controlled room and under continuous white light.

The obtained results show that both strains presented a high growth and high accumulation of carotenoids in the medium F/2. Besides, the contents of carotenoids registered for DUN 3 strain are higher than those obtained for the strain DUN 2.

Keywords: Biomass, Carotenoids, Dunaliella sp, Microalgae, Sebkha.

1. Introduction
In recent years, the research activity in the field of microalgae has increased and their potential industrial interest was significantly increasing. Their culture has received much attention worldwide and became one of the most important aspects in the trade of modern biotechnology [1], [2],[3]. Microalgae can also be used for environmental protection using their ability to fix carbon dioxide and heavy metals and to produce energy (biofuel production) without releasing greenhouse gases [4].

Actually, the microalgae are cultured as a source of valuable molecules such as pigments, lipids and proteins that can be incorporated in important areas [5]. The growing interest in the culture of microalgae has emerged because of their many advantages over higher plants. Microalgae have higher productivity than plants (rapid growth, doubling biomass few hours to days) [6] and are capable of converting solar energy into biomass with a yield 2 to 5 times higher than higher plants [7].

The unicellular green algae of the genus Dunaliella have been studied since the early 19th century, and many species have been characterized and classified since.

This eukaryotic organism has the largest known halo-tolerance that promotes culture under laboratory conditions and natural (race-way). An increased salinity, decreased predation problems are observed, competition with other species and reduced pathogenicity [8], [9].

Dunaliella stands out as an organization that accumulates the stress response in carotenoids. For this reason, it is successfully applied to the production of carotene on an industrial scale.
Bartly and Scolnik [10] have demonstrated that the carotenoid pigment plants act as an accessory for photosynthesis, a sort of protection against the photo-oxidation by absorption of light in the blue region of the spectrum and the energy absorbed can be transformed into chlorophyll. Ben-Amotz [11] reported that among the algae, one photosynthetic eukaryotic organism capable of growing in a medium containing a wide range of salt concentrations of 0.5% to saturation (about 35%) is Dunaliella. The salts in Morocco are only exploited for the production of salt, and few studies have been conducted on the biotechnological potential of microalgae Dunaliella. The main objective of this study is to evaluate the effect of culture medium on the growth and carotenoids production of two strains of Dunaliella under controlled conditions.

2. Materials and Methods

The experiments were conducted in the laboratory of marine biotechnology of the Specialized Center of Valorization and Technology of Marine Products (CSVTPM), National Hauteui Research Institute (INRH), Agadir, Morocco.

2.2. Biological material and culture conditions

The study was conducted on two strains isolated from continental salt production tables in the region of Essaouira in South East of Morocco. The DUN 2 strain isolated from the saline Azla (Latitude 31.160723, 31°9'38, 6''N. Longitude -9.705030, 42'18, 11''W 9°), and strain DUN3 isolated from the saline Idao Iaza (Latitude 31.145431, 8°31'43.55''N Longitude -9.737920, 44'16, 51''W 9°). Samples are collected from the bottom and the surface of the salt production tables, in cylinders with a capacity of 1000 ml labeled; taken in summer (summer 2014), coinciding with the period in which solar radiation is high, the temperature is about 33 °C and salinity is around 210 gl-1 of NaCl. Cell isolation was done by successive dilution and plaque isolation to obtain mono-specific strains. Morphological identification was performed by means of an optical microscope. Experimental cultures were grown in triplicate in 250 ml of culture medium in Erlenmeyer flasks of a capacity of 500 ml under continuous white light generated by the fluorescent lamps of 36W for 21 days at a temperature of 25 ±1°C, under constant agitation to ensure consistency and avoid sedimentation.

2.3. Culture medium

Two types of culture medium were prepared with a different water source. The culture medium F/2 is prepared from sea water [12]; and Semenenko medium (SMK) is prepared with distilled water amended with nutrients and NaCl [13]. The pH was adjusted to 7.5 and 8 for the SMK medium and the medium F/2, respectively, before autoclaving (121 ° C for 20 minutes). The media are cooled to room temperature and added aseptically inoculum taken in exponential phase.

2.4. Measurement of Cell Growth

The growth kinetics is identified, in accordance with the temporal evolution of cell densities under light microscopy, using the direct counting with hemocytometer (Thomas cells) after fixing the cells with Lugol. The cells counted are in cells.ml-1. Cell number is calculated using the following formula:

\[ \text{Number of Cells.ml}^{-1} = \text{total number of cells} \times 10^4 \times \text{dilution factor} \]

The specific growth rate and doubling time and cell productivity was determined by calculating the constant \( \mu \), \( t_d \) and \( P \), using the method of [14]:

\[ \mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \]

with \( \mu \): growth rate, \( X_2 \) and \( X_1 \): initial and final cell density of the logarithmic growth phase in culture and \( t_2 - t_1 \): the time interval between observations

and \( t_d = \frac{\ln 2}{\mu} \)
and \[ P = \frac{X_2 - X_1}{t_2 - t_1} \]

Monitoring the growth kinetics determines the different phases of growth.

2.5. Determination of production of total carotenoid pigments

An aliquot of Dunaliella sp. cells suspension was centrifuged at 5000 rpm for 5 min. The pellet obtained after removal of water is mixed with acetone 80\% (80: 20 v/v) and vortexed for 1 to 2 min until the appearance of a white precipitate. Rupturing the cell membrane in contact with the organic solvent (acetone) which allows the extraction of carotenoids and chlorophyll. The acetone extract is separated from cell debris by centrifugation again at 5000 rpm for 5 min. The concentration of chlorophyll a, b and total carotenoids was determined spectrophotometrically and calculated according to equations recommended by [15].

2.6. Statistical analysis

Statistical analysis and graphic illustrations were performed using the Microsoft Excel program. Data was tested by one-way ANOVA analysis using STATISTICA software. All significant levels were set at \( p < 0.05 \).

3. Results and Discussions

3.1. Isolation and microscopic identification of the species of Dunaliella

Strains of Dunaliella sp. isolated and identified were observed under optical microscope. Morphological examination showed unicellular green algae, biflagellate with two equal and long flagella. Chloroplast occupies half of the cell volume. Our observation is comparable to [16]. DUN 2 cells have an oval shape while the strain DUN 3 is fusiform. The apical region is narrower while the posterior region is wide.

During step of seeding the liquid medium after the addition of nutrient salts, growth is therefore observed by color change which changes from colorless to a green color from the 5th day of culture. On the agar medium, the colonies appeared 10 days after inoculation. Crops are planted every 2-3 months on agar medium.

3.2. Effect of culture medium on microalgal growth

3.3.

The evolution of the growth kinetics of the two strains of Dunaliella sp. separately grown in both media (F/2 and SMK) shows a different profile. The growth of strains DUN 2 is slow or almost zero on both media. Besides, the growth of the strain DUN 3 on both media go through three phases; a latency period due to the adaptation of the strains to new cultures conditions, the exponential phase reflects a fast duplication of young cells, and the stationary phase expresses the aging of cells in relation to the depletion of nutrients from the culture medium.
Figure 1: the growth kinetics of *Dunaliella* strains (DUN 2 and DUN3) in two types of culture media F/2 and SMK during 21 days of culture at T = 25 ± 1 °C.

Nevertheless, DUN 3 strains grown on medium F/2 behave differently from those grown on the medium Semenenko. The physiological response of the cells is instantaneous on the medium F/2, and expresses a fast duplication. The cell densities obtained are significantly higher. The highest cell concentration recorded on the medium F/2 is in the order of 8,629.10^6 Cells.ml^{-1} against 3,752.10^6 Cells.ml^{-1} in the same strain grown on the medium Semenenko. This difference in behavior could be attributed to the medium composition (natural and artificial) culture and/or salinity and pH.

The results of cultures showed a high production of biomass exceeding 8.106 Cells.ml^{-1} cell suspension in the medium F/2. These results are better than those reported by [17] and [18], the cell density of microalgal cells on the same medium hardly exceeds 1.2.10^6 Cells.ml^{-1}. And this can be explained by the fact that the species of the same genus but different geographically isolated from natural environment may have specific physiological responses to each strain. This observation is similar to that reported by [19] explains that the species Dunaliella haven’t all the ability to respond the same way to environmental conditions. Also, the origin of the strain may be responsible for this difference in cellular production; some authors such [20], reported that the continental origin strain showed better performance than the marine strains that have attracted many researchers.

Table 1: Growth parameters for two strains DUN2 and DUN 3 cultivated under laboratory conditions on two culture media F/2 and SMK

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Strains</th>
<th>Specific growth rate μ (Division/day)</th>
<th>Duplication time t_d (hours)</th>
<th>Production of algal cells P (10^6 Cells.ml^{-1}day^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/2</td>
<td>DUN 2</td>
<td>0,026</td>
<td>26,659</td>
<td>0,011</td>
</tr>
<tr>
<td></td>
<td>DUN 3</td>
<td>0,090</td>
<td>7,701</td>
<td>0,349</td>
</tr>
<tr>
<td>SMK</td>
<td>DUN 2</td>
<td>0,061</td>
<td>11,363</td>
<td>0,010</td>
</tr>
<tr>
<td></td>
<td>DUN 3</td>
<td>0,113</td>
<td>6,134</td>
<td>0,162</td>
</tr>
</tbody>
</table>

The growth parameters (μ, t_d and P) are calculated from cell densities of strains cultivated during 21 days on both media F/2 and SMK. They inform us about the rate of growth and biomass production by microalgae tested. These parameters are illustrated in the table above. (Table 1)

The specific growth rate of the strains differs in each of the media and between the cultured strains. The highest was observed for strains grown on SMK medium; with a maximum growth rate of 0.113 div.jr^{-1} for the strain DUN 3 and 0.061 div.jr^{-1} for the strain DUN 2. These results remain twice more significant than those observed on the F/2 medium. On the other hand, this variation in growth rate is dependent on the initial cell density.

The duplication time is proportional to the rate of cell growth. It is observed that the duplication time is better on the SMK medium, the cost of which remains high compared with the medium F/2 for a mass culture. Moreover, this medium would be suitable for the launching of low volume cultures while accelerating cell growth.
In terms of cell biomass production, the maximum productivity of the algal cells is recorded on the F/2 medium with a better concentration of 0.329 \times 10^6 \text{Cells.ml}^{-1} \text{jr}^{-1} labeled in strain DUN 3 against 0.011 \times 10^6 \text{Cells.ml}^{-1} \text{jr}^{-1} in the strain DUN 2. It is observed that the strain DUN 3 is 30 times more efficient than the strain DUN 2 on the F/2 medium; this is probably due to a distinct physiological adaptation of each strain to the different nutritive components of the culture medium.

3.3. Effect of culture medium on the content of chlorophylls a and b

The most important pigments analysis is carried out throughout the culture period by evaluating the contents of Chlorophyll (a) and Chlorophyll (b); we observe almost the same profiles for the two pigments measured.

The results obtained from the accumulation profile of Chl (a) show that the strain DUN 3 exhibits better accumulation ability on the two media tested; with a concentration of 17,355 μg.ml\(^{-1}\) at the 18th day of culture on the SMK medium and 14,790 μg.ml\(^{-1}\) at the end of the 21st day of culture on the F/2 medium. On the other hand, the highest concentrations recorded for the DUN 2 strain on the F/2 medium are of the order of 10,591 μg.ml\(^{-1}\) against a productivity of 8,035 μg.ml\(^{-1}\) on the SMK medium.

![Figure 2: Content of chlorophylls a and b in strains of Dunaliella (DUN2 and DUN3) in two types of culture media F/2 and SMK during 21 days of culture at T = 25 ± 1°C.](image)

The same results for Chl (b) productivity are recorded. Indeed, the accumulation of Chl (b) in strain DUN 3 follows a similar behavior as that reported for Chl (a). With maximal concentrations reached on the 18th day of the order of 13,811 μg.ml\(^{-1}\) and 12,323 μg.ml\(^{-1}\) on the medium SMK and F/2, respectively.

The amount of chlorophyll is an indicator of the process of photosynthesis. The levels of Chl (a) (17.355 μg.ml\(^{-1}\)) and Chl (b) (13.811 μg.ml\(^{-1}\)) reached their maximum for strain DUN 3 on SMK medium with a pH of 7.5 and 1M of concentration of NaCl, compared to a concentration of 14.790 μg.ml\(^{-1}\) in chl (a) and 12.323 μg.ml\(^{-1}\) in chl (b) for the same strain on the prepared sea-water F/2 medium. Muthukannan [21] has reported that the maximum concentration of Chl (a) (6.302 μg.ml\(^{-1}\))
and Chl (b) (5,002 μg.ml\(^{-1}\)) on the 21st day of culture is reached on a synthetic culture medium pH 7.5 and salinity of 1M. This confirms our results but with more significant concentrations of these pigments.

3.4. Effect of culture medium on carotenogenesis

We see a gradual evolution of the production of carotenoids in the days of culture. The total carotenoids productivity for both algal strains it grown on the medium F/2, with strong accumulation in pigment observed in the strain DUN 3. However, the lowest concentration of total carotenoids was observed on the medium SMK for both strains. The maximum concentration of carotenoids obtained on the medium F/2 is 5,298μg.ml\(^{-1}\) and 4,418μg.ml\(^{-1}\) for DUN 3 and DUN 2 respectively. These strains are proving a potential source of carotenes production.

Figure 3: Carotenoids production in strains of Dunaliella (DUN2 and DUN3) in two types of culture media F/2 and SMK during 21 days of culture at T = 25 ± 1 °C.

The culture medium is one of the economic factors that affect the cost of producing biomass and bioactive molecules for commercial purposes. In this study, the culture medium F/2 used, which is a natural environment at a lower cost compared to the Smk medium, demonstrates its great performance in the maintenance, cultivation as well as the production of carotenoids of the two strains. However, this high production of carotenoids can be influenced by the chemical composition of the medium and moreover its salt concentration. Fazeli [22] studied the production of carotenoids on a synthetic medium with different NaCl concentrations ranging from 0.3 to 2M. The highest carotenoid production varies between 3,06 μg.ml\(^{-1}\) and 3,75 μg.ml\(^{-1}\), and is observed on a low salinity medium (0.3 and 0.7 M NaCl) against a production of 2,96 μg.ml\(^{-1}\) at 1M salinity. Muthukannan et al. [21] also observed that the highest rate of carotenoids is obtained in a culture medium with salinity of 0.5 M NaCl. Those works were in agreement with our results since the highest levels of carotenoids are reached in the F/2 culture medium at 0.5 M salinity compared to the SMK medium whose salinity is 1 M. However, the contents recorded in carotenoids in this study remain more significant.

4. Conclusion

It’s clear that the reference strain DUN 3 brings the best growth potential and carotenoids accumulation on the two culture media tested, confirming its validity for productivity. Therefore, it can be considered as a microorganism with a high potential for the biotechnology production carotenoids. All the results obtained indicate that the culture medium is also an economic factor which conditions the mass production of the microalgae. The F/2 medium proves to be a simple, inexpensive formulation culture medium which can be used as a better alternative for mass cultures for carotenogenic strains.
Microalgae culture conditions should be checked in order to identify pro-growth factors (nitrates, salinity, and light intensity). The further studies will be optimizing those contents, and to identify an experimental model to algal biomass combined with a high concentration of carotenoids.

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References