ISOLATION AND MOLECULAR IDENTIFICATION OF TWO STRAINS OF MICROALGAE FROM SIDI AMEUR SALT LAKE IN ALGERIA

YAICHE ACHOUR Hafsa[1*], DOUMANDJI Amel[2], BOURAS Noureddine[3,4], SABAOU Nasserdine[4], ASSUNÇÃO Patricia[5]

[(1)]Laboratoire de Recherche de Biotechnologie des Productions Végétales, Département de Biotechnologie, Faculté des Sciences de la Nature et de la Vie Université de Blida 1, 09000, Blida, Algérie

[(2)]Laboratoire de Recherche de Biotechnologie des Productions Végétales Département Agroalimentaire, Faculté des Sciences de la Nature et de la Vie Université de Blida 1, 09000, Blida, Algérie

[(3)]Département de Biologie, Faculté des Sciences de la Nature et de la Vie et Sciences de la Terre, Université de Ghardaïa, 47000, Ghardaïa, Algérie

[(4)]Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure de Kouba, Alger, Algérie

[(5)]Instituto Tecnológico de Canarias (ITC), Departamento de Biotecnología, División de Investigación y DesarrolloTecnológico, Pozo Izquierdo, 35119, Sta. Lucía, Canary Islands, Spain

E-mail: hafsa_yah@hotmail.fr

(Received 11 April 2018 - Accepted 05 June 2018)

Abstract.- *Microalgae constitute an excellent feedstock of valuable compounds which have varied applications in the nutraceutical and pharmaceutical industries. In this study, the isolation of two microalgal strains, DunaDZ$_2$ and DunaDZ$_3$, from Sidi Ameur salt lake was done by streaking water on solid f/2 medium. The strains were subjected to morphological identification as well as molecular identification by sequencing genes ITS and rbcL. The strain DunaDZ$_2$ presented a palmella stage (colonial aggregates of cells) throughout all of its life cycle (length 5.5 ± 1.8 µm and width 4.8 ± 1.2 µm). By contrast, DunaDZ3 presented ellipsoid shape of about 14.6 ± 1.7 µm length and 6.9 ± 1.3 µm width. Phylogenetic analysis based on ITS and rbcL genes revealed that both strains are members of the genus* Dunaliella*, and were grouped in the same clade with different strains of* Dunaliella viridis *(96.9 to 98.7% of similarity for ITS gene, and 96.4 to 98.1% for rbcL gene). The species* Dunaliella viridis *is considered as promising feedstock for applications in the production of biofuels and as a nutritive food source for aquatic organisms.*

Key words: Dunaliella, *isolation, salt lake, ITS, rbcL.*

ISOLEMENT ET IDENTIFICATION MOLÉCULAIRE DE DEUX SOUCHES DE MICROALGUES PROVENANT DU LAC SALÉ DE SIDI AMEUR EN ALGÉRIE

Résumé.- *Les microalgues représentent une excellente source de molécules qui sont valorisables dans l'industrie nutraceutique et pharmaceutique. Dans cette étude, l'isolement de deux souches de microalgues, Duna DZ$_2$ et Duna DZ$_3$, à partir du lac salé de Sidi Ameur a été réalisé par stries sur le milieu solide f/2. Les souches ont fait l'objet d'une identification morphologique et moléculaire par l'amplification de deux gènes, ITS et rbcL. La souche DunaDZ$_2$ se présente au stade de «Palmella» (agrégat de cellules formant des colonies) tout au long de son cycle de vie. Les dimensions des cellules sont de 5,5 ± 1,8 µm pour la longueur et 4,8 ± 1,2 µm pour la largeur. En revanche, la souche DunaDZ$_3$ possède une forme ellipsoïde, dont la longueur est de l'ordre de 14,6 ± 1,7 µm et la largeur de 6,9 ± 1,3 µm. L'analyse phylogénétique des gènes amplifiés ITS et rbcL a permis de rattacher les deux souches au genre* Dunaliella. *Ces souches sont groupées dans le même clade que les souches* Dunaliella viridis *(96,9 à 98,7% de similarité pour le gène ITS, et 96,4 à 98,1% pour le gène rbcL). L'espèce* D. viridis *est considérée comme une espèce prometteuse pour la production de biofuel et comme un aliment nutritif pour les organismes aquatiques.*

Mots clés: Dunaliella, *isolement, lac salé, ITS, rbcL.*

Introduction

Microalgae are photosynthetic unicellular organisms, which have the ability to grow rapidly. They use light energy and fix atmospheric CO_2. The wide diversity of compounds synthesized from different metabolic pathways of fresh and marine water algae provide promising sources which can support human health, as fatty acids, steroids, carotenoids, polysaccharides and lecithin [1,2]. These compounds could be used in food, pharmaceutical, and cosmetic industries, thanks to their numerous biological activities (antioxidant, anticancer, antihypertension, immunomodulatory and prevention of cardiovascular diseases) [3].

Microalgae are ubiquitous organisms. They are found worldwide and in many different environments. They can be found in fresh water (ponds, canals and lakes), as well as in marine and hyper-saline environments [4]. Ephemeral salt lakes, called Sebkhas or Chotts, are closed depressions which are periodically flooded. The depth can range from 20 cm to 4 m. During summer, salt lakes dry up and are covered with a salt crust. Sebkhas are common in Algeria, and they are located mainly in the North and the East of the country. The main salt lakes in Algeria are chott Merouane, sebkha of Arzew, sebkha of Oran, sebkha of Sidi Bouziane, chott Zahrez Gharbi and Sidi Ameur lake [5]. These salt lakes should be investigated to estimate their microalgal biodiversity.

In this study, two strains of microalgae were isolated from Sidi Ameur salt lake. The strains were described on the basis of their morphological characteristics and molecular identification, by amplification of ITS (Internal Transcribed Spacer) and rbcL (ribulose-bisphosphate carboxylase) genes.

1.- Material and methods

1.1.- Isolation, purification and morphological identification of the microalgal strains

The strains of microalgae were isolated from saline water, collected from Sidi Ameur salt lake (latitude 35°27’ North, longitude 3°68’ East), Djelfa province, Algeria (fig. 1).

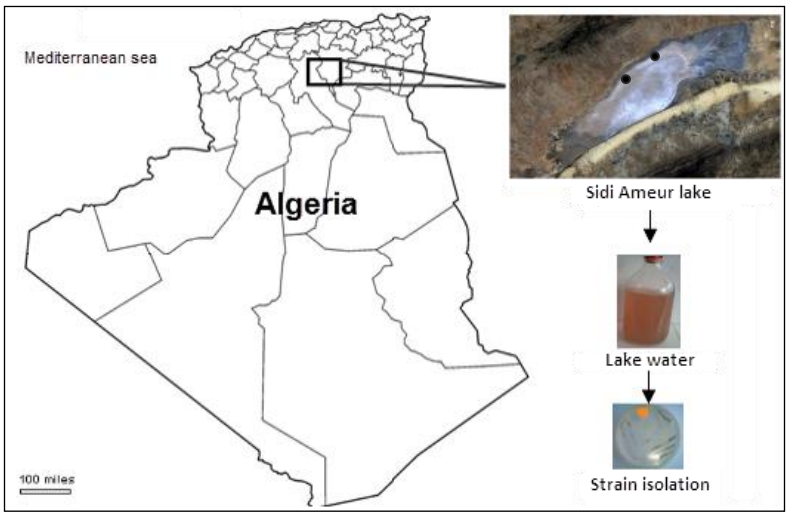

Figure 1.- Site description and geographic location of Sidi Ameur lake.
The marked parts (•) are the sample collection sites

Strains were isolated by spreading water samples on f/2 medium plates. Then plates were incubated at 22 ± 2°C and illuminated with white fluorescent light (120 µmol photons m^{-2} s^{-1}), with a 24 h photoperiod. The f/2 medium [6] contains the following nutrients: 1M NaCl; 8.82 10^{-4} M $NaNO_3$; 3.62 10^{-5} M KH_2PO_4; 1.17 10^{-5} M $FeCl_3.6H_2O$; 1.17 10^{-5} M $Na_2EDTA.2H_2O$; 3.93 10^{-8} M $CuSO_4.5H_2O$; 2.60 10^{-8} M $Na_2MoO_4.2H_2O$; 7.65 10^{-8} M $ZnSO_4.7H_2O$; 4.20 10^{-8} M $CoCl_2.6H_2O$; 9.19 10^{-7} M $MnCl_2.4H_2O$; 2% agar. The pH was adjusted to 7.5. Colonies were picked up and streaked in a new plate.

The morphology of the isolated strains was studied using a light microscope (Leica). The software LAS EZ (Leica DM500) was used to estimate cell size (length and width). This measurement was done in f/2 liquid medium containing 1 M NaCl, by averaging different repetitions.

1.2.- Molecular identification of the microalgal strains

1.2.1.- DNA extraction

DNA extraction was performed according to chelex-100 method [7]. Colonies from solid medium (monoalgal culture) were suspended in 300 µl of 10% chelex resin. The suspension was vortexed for 10 to 15 s, then incubated at 95°C for 30 min. The supernatant which contain DNA was recovered by centrifugation for 5 min at 10,000 *g*.

1.2.2.- PCR amplification and sequencing

ITS and rbcL regions were amplified using primers ITS-AB28 (5'-GGGATCCGTTTCCGTAGGTGAACCTGC-3'), ITS-TW81 (5'-GGGATCCATATGCTTAA GTTCAGCGGGT-3'), rbcL-17 (5'-ATGGTTCCACCAACAGAAAAC-3') and rbcL-18 (5'-TGTGCTTTGTAAATAGCTTCAG-3'), respectively [8, 9].

Amplification reactions were performed on a Bio-Rad thermocycler. PCR amplification was carried out in a total volume of 25 µl, containing 19.5 µl H_2O, 1.87 µl PCR buffer, 2 µl dNTPs (2.5 mM of each), 0.125 µl *Taq* polymerase (Takara Extraq Hot Start Version, in 4.5 mM of $MgCl_2$), 1 µl DNA and 0.25 µl of each primer (25 pM).

The PCR conditions were as follow for ITS: 1 cycle at 95°C for 5 min, 35 cycles at 52°C for 1 min and 72°C for 1 min. The PCR conditions for rbcL were: 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s and 1 cycle of final extension at 72°C for 5 min. PCR products were examined on 1% agarose gel and were purified using Illustra ExoProStar 1-Step from GE Healthcare Life Sciences. Sequencing was performed by Macrogen services, using Sanger's Dideoxy method.

1.2.3.- Phylogenetic analysis

The obtained sequences were aligned with others sequences available from GenBank BLAST in the NCBI database, using ClustalW algorithm in MEGA version 6 [10]. The phylogenetic tree was generated using Tamura-Nei distance parameters and the neighbor-joining (NJ) method. NJ bootstrapping was performed with 1000 resampling events. The tree was established using *Chlamydomonas reinhardtii* as an outgroup.

2.- Results and discussion

2.1.- Morphological characteristics

In this study, two strains, named DunaDZ$_2$ and DunaDZ$_3$, were isolated. The morphological characteristics of these strains were studied during exponential phase, from f/2 medium with 1 M NaCl.

For the strain DunaDZ$_2$, the cells are round, non motile and grouped together (aggregate) within a mucilaginous matrix. This stage is called palmella forms (fig. 2a). Average length and width were about 5.5 ± 1.8 and 4.8 ± 1.2 µm, respectively. The unicellular algae can develop palmella forms in their life cycle, when exposed to various extreme environment conditions, such as salinity [11] and oxidative stress [12]. According to Borowitzka and Siva [13], this stage could be caused also by a reduced salinity, less than 10% NaCl. When the salinity is around 20% cells reform their flagella and return to the motile stage. Several authors have noticed this changing in shape and behaviors for the microalgae *Euglena*, *Chlamydomonas*, *Dunaliella* and *Pediastrum* [14, 15]. Nutrient deficiency and low temperature may also trigger the formation of these palmelloid forms [13].

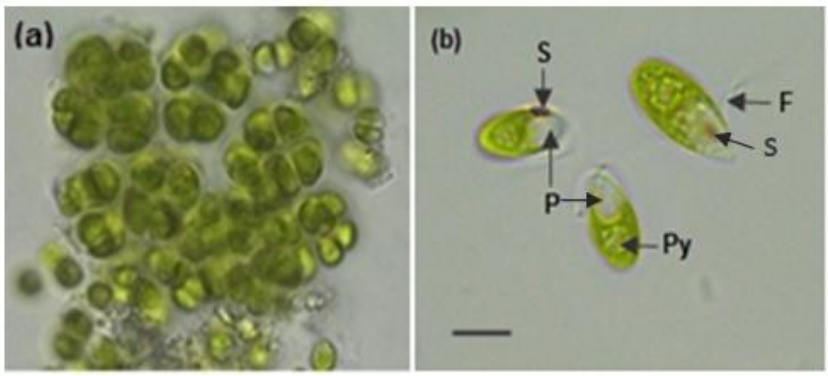

Figure 2.- Microscopic view of the isolated *Dunaliella* strains, grown on f/2 medium at 1 M NaCl.
(a) DunaDZ$_2$ at palmella stage (aggregate of cells on mucilaginous matrix).
(b) DunaDZ3: ellipsoid cells with chloroplast containing pyrenoid (Py), stigma (S), flagella (F) and Papilla (P). *Bar*, 10 µm.

However, the strain DunaDZ3 presented an ellipsoid shape, with one large red stigma elongated at the apical end of cell (fig. 2b). Cells are motile with two equal flagella. Cell size was 14.6 ± 1.7 µm and 6.9 ± 1.3 µm for length and width, respectively.

Based on the morphological characteristics, the isolated strains were assigned to the genus *Dunaliella*. When the strains DunaDZ$_2$ and DunaDZ$_3$ were subjected to stress factors, by nitrate starvation and high light, they remain green, which means that they do not have the ability to produce carotenoids. Only the species *D. salina* and *D. parva* were reported in the literature as carotenogenic species.

2.2.- Amplification and sequencing results

Based on sequences obtained by amplification of ITS and rbcL genes, the strains DunaDZ$_2$ and DunaDZ$_3$ were assigned to the genus *Dunaliella.* Amplification of ITS gene gave products of 732 bp for DunaDZ$_2$ and 761 bp for DunaDZ$_3$, respectively. While the

products obtained for rbcL gene were 866 bp for DunaDZ2 and 966 bp for DunaDZ3.

Alignment of ITS sequences obtained for both strains with those from the NCBI database, revealed that strains $DunaDZ_2$ and $DunaDZ_3$ grouped with different strains of *Dunaliella viridis*. A phylogenetic tree was established and is shown in figure 3. The strain $DunaDZ_2$ showed 96.9% identity with *D. viridis* NIOT-95. Strain DunaDZ3 showed 98.7% of similarity with *D. viridis* MSV-1. When the sequences of the isolated strains were aligned together, they showed identity of 95.3%.

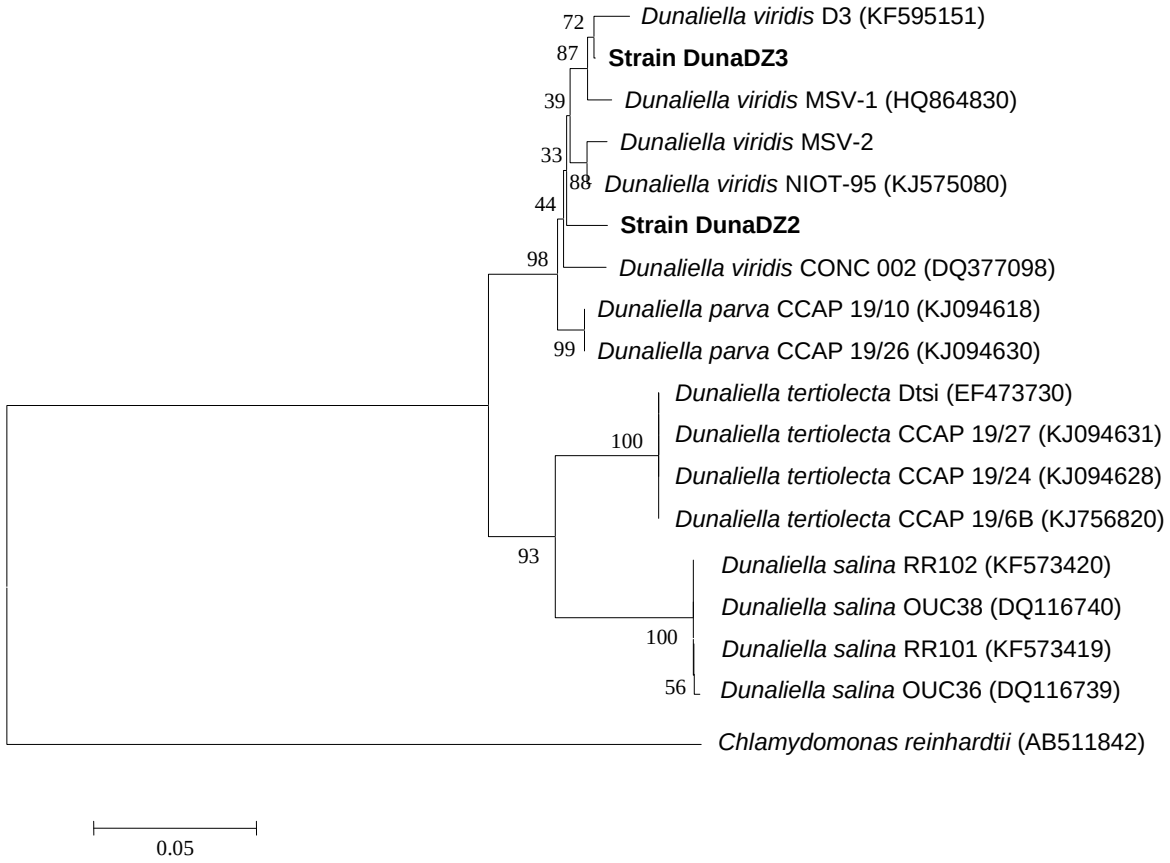

Figure 3.- Neighbor-joining tree based on ITS gene sequences showing the position of strains $DunaDZ_2$ and $DunaDZ_3$, and its related species of the genus *Dunaliella*. The numbers at the nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets; *Bar*, 0.05.

Results obtained from the amplification of the rbcL gene showed that strains $DunaDZ_2$ and $DunaDZ_3$ belong to the genus *Dunaliella*, and presented 96.4% and 98.1% of similarity, with *D. viridis* strain CONC002 and *D. viridis* strain D3, respectively (fig. 4).

Dunaliella viridis is a halotolerant green unicellular microalgae, that belong to the phylum *Chlorophyta.* It grows optimally at 60-90 g/l NaCl [13].

This species generates a great interest, by its role in the environment as well as its capacity to produce bioactive compounds under stress conditions. *Dunaliella viridis* can accumulate significant amounts of valuable lipids (15-45%), which can be exploited for the production of biofuel. It also contains proteins (up to 32%) and carbohydrates (up to 8%) [16]. This species has properties that make it a potential candidate for mass culture on a

commercial scale. It has a high growth rate and is much more productive then the other *Dunaliella* species. *D. viridis* could be exploited as nutritive food source for aquatic organisms [17]. It will be very interesting to study, in the future, the characteristics of the strains DunaDZ$_2$ and DunaDZ$_3$, and to promote the microalgae *Dunaliella*, especially those present in the Algerian salt lakes. It will be important to focus attention on other *Dunaliella* species, such as *D. salina*, known in the literature as a natural producer of β-carotenes.

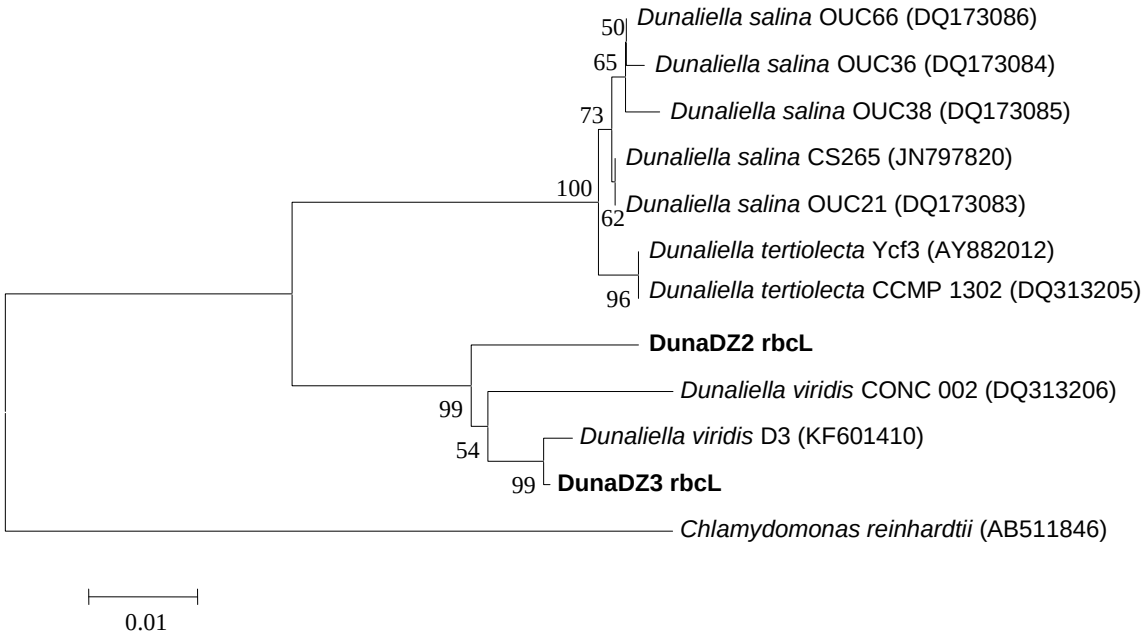

Figure 4.- Neighbor-joining tree based on rbcL gene sequences showing the position of strains DunaDZ$_2$ and DunaDZ$_3$, and its related species of the genus *Dunaliella*. The numbers at the nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets. *Bar*, 0.01.

References

[1].- García J. L., Vicente M., Galán B., 2017.- Microalgae, old sustainable food and fashion nutraceuticals. Microbial Biotechnology, 10: 1017–1024.

[2].- Sathasivam R., Radhakrishnan R., Hashem A., Abd Allah E. F., 2017.- Microalgae metabolites: a rich source for food and medicine. Saudi Journal of Biological Sciences, (in press).

[3].- Hamed I., 2016.- The evolution and versatility of microalgal biotechnology: a review. Comprehensive Reviews in Food Science and Food Safety, 15: 1104–1123.

[4].- Williams P. J. L. B., Laurens L. M., 2010.- Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics. Energy & Environmental Science, 3: 554–590.

[5].- Demnati F., Samraoui B., Allache F., Sandoz A., Ernoul L., 2017.- A literature review of Algerian salt lakes: values, threats and implications. Environmental Earth Sciences, 76: 127.

[6].- Guillard R. R., Ryther J. H., 1962.- Studies on marine planktonic diatoms. I. *Cyclotella nanta* Hustedt and *Detonula confervacea* (Cleve) Gran. Canadian

Journal of Microbiology, 8: 229–239.

[7].- Singer-Sam J., Tanguay R. L., Riggs A. D., 1989.- Use of chelex to improve the PCR signal from a small number of cells. Amplifications, 3: 11 p.

[8].- Goff L. J., Moon D. A., Coleman A. W., 1994.- Molecular delineation of species and species relationships in the red algal *Agarophytes gracilariopsis* and *Gracilaria* (*Gracilariales*). Journal of Phycology, 30: 521–537.

[9].- Nozaki H., Itoh M., Sano R., Uchida H., Watanabe M. M., Kuroiwa T., 1995.- Phylogenetic relationships within the colonial *Volvocales* (*Chlorophyta*) inferred from rbcL gene sequence data. Journal of Phycology, 31: 970–979.

[10].- Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013.- MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30: 2725–2729.

[11].- Takouridis S. J., Tribe D. E., Gras S. L., Martin G. J., 2015.- The selective breeding of the freshwater microalga *Chlamydomonas reinhardtii* for growth in salinity. Bioresource Technology, 184: 18–22.

[12].- Wang S. B., Chen F., Sommerfeld M., Hu Q., 2004.- Proteomic analysis of molecular response to oxidative stress by the green alga *Haematococcus pluvialis* (*Chlorophyceae*). Planta, 220: 17–29.

[13].- Borowitzka M., Siva C., 2007.- The taxonomy of the genus *Dunaliella* (*Chlorophyta, Dunaliellales*) with emphasis on the marine and halophilic species. Journal of Applied Phycology, 19: 567–590.

[14].- Sztrum A. A., Sabatini S. E., Rodríguez M. C., 2012.- Isocitrate lyase activity and antioxydant responses in copper-stressed cultures of *Chlamydomonas reinhardtii* (*Volvocales, Chlorophyceae*). Phycologia, 51: 135–143.

[15].- Lurling M., Beekman W., 2006.- Palmelloids formation in *Chlamydomonas reinhardtii*: defence against rotifer predators. Annales de Limnologie - International Journal of Limnology, 42: 65–72.

[16].- Ben-Amotz A., Avron M., 1990.- The biotechnology of cultivating the halotolerant alga *Dunaliella.* Trends in Biotechnology, 8: 121–126.

[17].- Guedes A. C., Malcata F. X., 2012.- Nutritional value and uses of microalgae in aquaculture. In Aquaculture, inTech, 60–78.