Electronic Journal of Biotechnology 54 (2021) 77–85

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

Research Article

Effect on growth and productivity of lutein from the chlorophyta microalga, strain MCH of *Muriellopsis* sp., when grown in sea water and outdoor conditions at the Atacama Desert

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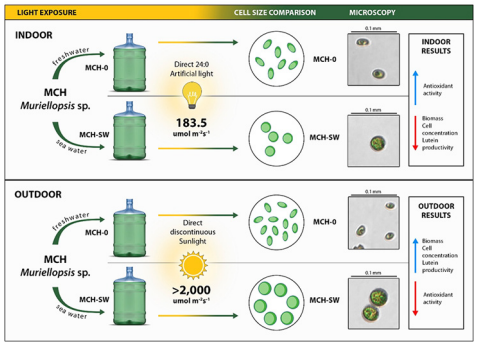
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 March 2021
Revised 30 May 2021
Accepted 8 September 2021
Available online 15 September 2021

Keywords:
Antioxidant activity
Atacama Desert
Carotenoids
Cellular stress
Lutein
Microalgae
Muriellopsis sp
Pigments
Sea water

ABSTRACT

Background: Microalgae are microorganisms that produce various products, for example, pigments, mainly carotenoids. This study aimed to used the strain of *Muriellopsis* sp. and to evaluate their behavior when grown in freshwater and seawater, along with indoor and outdoor conditions for both cultures. Growth of the strain was evaluated by determining its biomass, lutein productivity with high-performance liquid chromatography (HPLC), and antioxidant activity by using the 2,2-diphenyl-1-picrilhydrazil (DPPH method).

Results: *Muriellopsis* sp. strain in indoor cultures showed an increased antioxidant activity. In outdoor conditions, both cultures showed increased cells number, concentration of biomass, and lutein productivity. The percentage of lutein obtained from the strain MCH in indoor conditions was 25 times higher than that reported for calendula, reaching 0.75% of lutein in *Muriellopsis* sp. cultured in seawater, followed by 0.6% in *Muriellopsis* sp., cultures in freshwater at day 12 of both cultures. These values exceed that of microalgae *Scenedesmus almeriensis*, which reaches 0.53% lutein.

Conclusions: The results show that the native strain of the Atacama Desert is one of the largest producers of lutein as compared to those reported to date. The study demonstrated the feasibility of producing this carotenoid with well-known properties to prevent some diseases due to its high nutritional value. *Muriellopsis* sp. cultivation in open-air seawater is a good precedent for developing mass production of this species in an area where freshwater is scarce and costly.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

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https://doi.org/10.1016/j.ejbt.2021.09.001

How to cite: Cruz-Balladares V, Marticorena P, Riquelme C. Effect on growth and productivity of lutein from the chlorophyta microalga, strain MCH of *Muriellopsis* sp., when grown in sea water and outdoor conditions at the Atacama Desert. Electron J Biotechnol 2021;54. https://doi.org/10.1016/j.ejbt.2021.09.001

1. Introduction

Microalgae are the most abundant and the most phylogenetically diverse living organisms present on the planet, and they are capable of growing in both freshwater and seawater, hypersaline environment, wet surfaces, and even on rocks [1,2,3]. These organisms generate bioproducts such as amino acids, lipids, carbohydrates, and pigments of great commercial value [4]. The pigments include carotenoids, which have traditionally been used as food coloring agents or supplements [5,6,7,8,9]. The health benefits of carotenoids include reducing the risk of some degenerative diseases, cancer, diabetes, and other disorders [10,11]. The global carotenoid market is growing each year, with an annual growth rate of 5.7% and is expected to reach USD 2.0 billion in 2022 [12] due to an increased demand for carotenoids as a bioproduct. Among the various carotenoids, the most required and valuable ones are ß-carotene, astaxanthin, canthaxanthin, lycopene, and lutein [10].

Lutein is an important carotenoid that can be produced by some microalgal species; nonetheless, the production of this pigment can be improved by modifying the cultivation conditions. It can also be obtained from dry and wet biomass, and extraction can also be improved with different solvents [13,14,15,16]. However, the main source of lutein is still the marigold flower (a common name for the genus *Tagetes*) [17]. The growth rate of microalgae is 5–10 times higher than that of plants. Therefore, less time consumption is one of the advantages of using microalgae for the production of lutein. Furthermore, they can be cultivated in seawater or brackish-water and even on non-cultivable lands; thus, microalgae do not compete with conventional agriculture for their resources [18,19].

Microalgae are phototrophic organisms that develop an effective protection system against oxidative stress factors and free radicals, which is also known as antioxidant defense system [20,21,22,23]. The stimulation of antioxidant defense system in microalgae presents adaptive responses to the oxidative stress in them. Their antioxidant defense system consists of enzymatic and non-enzymatic mechanisms. In enzymatic mechanisms, superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase are key enzymes; non-enzymatic counterparts include mediating compounds such as ascorbic acid, reduced glutathione, tocopherols, carotenoids, and phycocyanin [24,25]. Furthermore, the microalgae biochemical composition is closely related to nutritional or environmental stress. In various species of microalgae, factors such as nutrient availability, light intensity, temperature, and salinity can induce the accumulation of carotenoids [26,27].

Muriellopsis sp. in photoautotrophic batch culture, accumulates high levels (35 mg L^{-1}) of lutein [28] and yields high values (up to 8×10^{10} cells L^{-1}) of cell biomass [29]. This microalgal species has a high content of total carotenoids, reaching approximately 1% by dry weight [28]. Lutein is present in its free form and represents around 50% of the total carotenoids. Hence, lutein is the main carotenoid in *Muriellopsis* sp. microalgae. Other carotenoids present in these microalgae are β-carotene, neoxanthin, and violaxanthin. Growth rate and lutein production are mainly based on their ability to tolerate moderate salinity, high irradiation, and a wide pH range [30]. In the open culture system, particularly in sunlight, there is a significant increase in lutein content (accumulating between 0.4% and 0.6%) occurring in response to very high levels of irradiation. It has been proposed that the pigment lutein contributes to the dissipation of excess light energy; thus, it plays an active role in photoprotection against stress caused by light [28,30]. The objective of the present study is to evaluate cell viability, oxidative stress, and productivity of pigments such as lutein from freshwater microalgae, strain MCH of *Muriellopsis* sp., when cultivated outdoors in seawater under the extreme conditions of the driest desert in the world, i.e., high solar irradiation and scarcity of water resources. This present study is the key to assess the feasibility of developing massive cultures of this species in the driest desert of the planet.

2. Materials and methods

2.1. Microalgal strain used and culture conditions

Muriellopsis sp. MCH was isolated from freshwater bodies of the coastal zone of the Antofagasta Region, available from the culture collection of the Applied Microbiology Unit of the Antofagasta University. MCH was deposited in the Spanish algae bank with accession number BEA_IDA_0063B. Cells were cultured photoautotrophically in the modified *f*/2 culture medium [31] named UMA5 [32], in separate samples of freshwater (MCH-0) and seawater (MCH-SW) within flat-bottom glass balloons of 1 L each. These 1 L cultures were maintained until an exponential phase of growth was reached within them. As the next step, the cultures were transferred to another flat-bottom glass of 18 L each. Indoor cultures were maintained at 20°C at constant light intensity of 183.5 μmol m^{-2} s^{-1} (2 fluorescent tubes, Universal, OSRAM) and constant air supply of 0.2 vvm. For the outdoor system, the temperature was maintained between 15°C and 25°C, with variations in light intensity that can exceed 2000 μmol m^{-2} s^{-1} and air supply of 0.2 vvm. The tests were carried out for 14 d.

2.2. Culture monitoring

Cultures were started at the concentration of 10^5 cells mL^{-1} from an exponential phase culture. The cell number was counted with the Neubauer chamber and observed under a microscope (B-800/B100 Series, OPTIKA, Italy). The biomass concentration was determined by filtering 100 mL of culture through a 0.2-μm fiber-glass membranes. Filters used were dried in an oven (UM600-2400 W, Memmert, Germany) at 105°C for 24 h. The variation in pH throughout the experiment was monitored with a pH sensor (Series 5342 T, Crison, Spain). The maximum potential quantum efficiency of PSII (Fv/Fm) and non-photochemical quenching (NPQ) were measured with a chlorophyll fluorometer (Junior-PAM, Walz, Germany).

2.3. Cell viability and oxidative stress

To evaluate cell viability and its oxidative stress, fluorescent propidium iodide (PI) and dihydroethidium (DHE), respectively, were used. Further analyses of cultures were done using a confocal laser microscope (TCS SP8, Leica, Germany) and the flow cytometer

(FACSJazz, BD Bioscience, USA). For this procedure, a microalgal suspension at the concentration of 10^6 cells mL^{-1} was prepared in which 6 μL of 0.014 mM PI and 1 μL of 8 mM DHE were added; it was then incubated for 1 h at ambient temperature, 25°C, and protected from light. It was then analyzed in a 585/42 nm channel flow cytometer at 488 nm excitation. As a control for the fluorochrome PI, the strain MCH was previously incubated at 95°C for at least 3 h and followed with the staining protocol. As a control for DHE staining, MCH was previously incubated with 3 mL of 30% hydrogen peroxide (H_2O_2) for 1 h at room temperature. It was centrifuged at 3500 rpm for 5 min, and the supernatant obtained was removed and resuspended in the microalgae culture medium; subsequently, the process of staining was followed by the described protocol.

2.4. Analysis of the composition of biomass

2.4.1. Lutein extraction and compound separation by high-efficiency liquid chromatography (HPLC)

Lutein content was determined by treating freeze-dried biomass with alumina in a 1:1 w/w ratio for 5 min in a mill, where alumina acts as a disintegrating agent. Each test was performed with 10 mg of the total sample, which contained 5 mg of dry biomass [33] Saponification of the sample was carried out in XXTuff reinforced microvials for mini-beadbeater-24 by adding 1 mL of tricomponent solution (composed of ethanol:hexane:water in the ratio of 77:17:6 v/v/v as described by Fernández-Sevilla et al. [34] with 4% w/v potassium hydroxide (KOH). Microvials with the samples were processed for 2 min. The tubes were then centrifuged for 2 min at 12000 rpm in a centrifuge (Mini Spin Plus, Eppendorf), and the supernatant obtained was transferred to a vial ready to be analyzed using HPLC. Lutein from microalgal extracts was analyzed using the HPLC (LC-4000, Jasco, Japan), equipped with a quaternary pump (PU-2089 s Plus, Jasco, Japan), a diode array detector (MD-4010, Jasco, Japan), an automatic injection system (AS-2055 Plus, Jasco, Japan), and ChromNAV Control Center V.2 software (Jasco, Japan). A reverse-phase column (LiChrosphere RP-18 HPLC column, 5 μm particle size, L × I.D 150 mm × 4.6 mm) was used. The gradient program used was as reported by Cerón et al. [33]. The injected volume of each sample was 20 μL. The mobile phase consisted of a mixture of two solutions: solution A containing water/methanol in the ratio of 2:8 v/v and solution B containing acetone/methanol in the ratio of 1:1 v/v. Carotenoids were eluted at the rate of 1 mL min^{-1}, and lutein was quantified by integration at 450 nm. The identification of lutein was carried out by comparing its retention times, calibration curve, and its absorption spectrum in UV–Vis, with a lutein standard (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Antioxidant activity

2.5.1. 2,2-Diphenyl-1-picrilhydrazil (DPPH) assay

A solution was prepared by dissolving 0.2 mM of DPPH (Sigma-Aldrich, St. Louis, MO, USA) in methanol, and 150 μL of this solution was then added to 100 μL of extract. Trolox (Sigma-Aldrich, St. Louis, MO, USA) was used as the referent antioxidant. The final solution was incubated for 30 min in dark, and its absorbance was measured at 517 nm [15].

Inhibition percentage (%) = (Blank Absorbance-Extract Absorbance) / (Blank Absorbance) × 100

2.6. Statistical analysis

All tests were carried out in triplicate, and all statistical analyses were carried out using Statgraphics Centurion, X. V. I. (2013). Statgraphics centurion XVI software version 16.1.03 (Virginia, USA). A one-way analysis of variance was used to compare each dataset, with a confidence level of 95%, and values of p ($p \leq 0.05$) were considered statistically significant.

3. Results and discussion

3.1. Monitoring of cultures, cell count, biomass concentration, and pH

Muriellopsis sp. MCH was evaluated in two salinities, i.e., freshwater (MCH-0) and natural seawater (MCH-SW), both in indoor and outdoor conditions. The modification of abiotic conditions such as temperature, light, and salinity is implemented to induce stress in the strain to be studied in order to increase the biocomposites of interest [28,30,32,35,36]. In Fig. 1, it is observed that the microalga changes its morphology when cultivated in seawater, increases cell size and weight, and assumes a spherical shape. A similar phenomenon was reported for microalgae of *Chlorococcum* sp., *Scenedesmus obliquus* sp., and *Skeletonema* sp., when cultivated in seawater [36,37,38]. It is evident that the size difference depends on the salinity of the culture medium and can be explained through a process of internal homeostasis in which some contents such as carotenoids, lipids or proteins increases, which further increases their cell size and cytoplasmic volume [39,40]. In the culture conditions of MCH-0, the strain had an approximate diameter of 0.010 mm and cell weight of 0.1 pg $cell^{-1}$; in the culture conditions of MCH-SW, the strain size measured was up to 0.019 mm in diameter and reached a cell weight of 0.4 pg $cell^{-1}$. If we compare these cell weights, MCH-SW has biomass four times higher than that of MCH-0.

In Fig. 2, a greater number of cells is observed in outdoor cultures than in indoor cultures as described by Blanco et al. [28], reaching the highest concentration of 6.2 × 10^6 cells mL^{-1} in MCH-0 and 3.4 × 10^6 cells mL^{-1} in MCH-SW at day 14 of the culture. On the other hand, the lowest count, not exceeding 3.5 × 10^5 cells mL^{-1}, is observed in MCH-SW indoor at day 14 of culture.

The biomass concentration was determined, and the culture with the highest concentration of 0.88 g L^{-1} was MCH-0 with outdoor conditions on day 14 of the culture (Fig. 2), this may be due to a photo-acclimatization by the microalgae to natural sunlight conditions and outdoor temperatures [35,41]. Exponential growth is observed in both indoor and outdoor crops; especially, MCH-0 is consistent with its freshwater aquaculture nature. MCH-SW outdoors registered a biomass concentration of 0.64 g L^{-1}, showing a slower growth than for indoor crops whose values are quite close but do not exceed 0.2 g L^{-1}. This can be related to their slow cell growth when exposed to outdoor conditions, which could be because their photosynthetic apparatus is not fully competent until their photo-acclimatization [41].

The cultures maintain their pH in the range of 6.00–10.00 due to the uptake of nutrients and the fixation of CO_2 through photosyn-

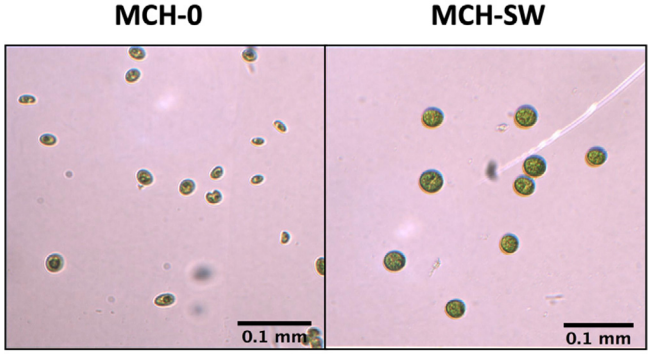

Fig. 1. Chlorophyte strain MCH of *Muriellopsis* sp. in freshwater (MCH-0) and seawater (MCH-SW).

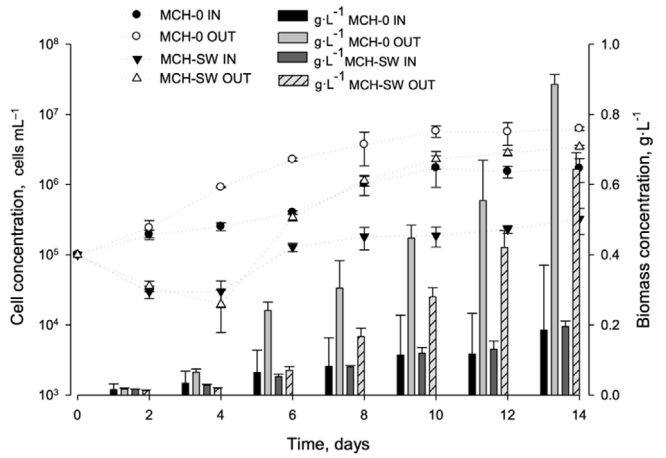

Fig. 2. Cell concentration (lines) and biomass concentration (bars) of MCH *Muriellopsis* sp. on the different days of indoor and outdoor cultivation in 20 L culture systems.

thesis. More is the alkalinity of the cultures, the higher is their photosynthetic activity. The process is largely explained by the consumption of HCO^{3-} ions, which dissociate to provide CO_2 needed for the growth of the strain MCH and, in turn, an accumulation of OH^- ions occurs, which causes a gradual increase in pH. On the other hand, pH maintained its influence on a large number of biochemical processes associated with the growth and metabolism of microalgae, including the ionization of metabolites, solubility, and the bioavailability of CO_2 and nutrients [42].

3.2. Maximum quantum efficiency and non-photochemical dissipation

Photosynthetic efficiency is a measurement that indicates the flow of noncyclic electrons through photosystem II (PSII) during photosynthesis. Under normal conditions, the values range from 0.5 to 0.8. In Fig. 3A, the photosynthetic efficiency values are observed throughout the cultures: a) Indoor cultures have values that are within the normal range, which indicates that the photosynthetic machinery is working properly, and although it could be subjected to stress, it does not affect the PSII of the microalgae; b) On the other hand, outdoor cultures showed important changes in which MCH-SW being the most affected and yield photosynthetic efficiency values between 0.2 and 0.6. The decrease in these values could be related to the adaptation period of the cultures, consequently causing a gradual loss of P^{680} reaction centers [43,44].

Under normal conditions, photosynthesis predominates over other metabolic processes. However, under stress conditions, the microalgae cannot work at their full performance level when there is excess light exceeding the photosynthetic capacity and causing damage at the cellular level (specifically in the PSII reaction centers). As a result, nonphotochemical quenching (NPQ) process increases. NPQ is an indicator of the degree of photo-protection, generally referring to both protection processes and damage where the xanthophyll cycle is activated and by which excessive light is dissipated as heat to avoid negative impacts on the electron chain or photo-inhibition at the molecular level [45,46]. In Fig. 3B, it can be observed that MCH-0 indoor does not show great changes (values close to 1) with the increase in the photosynthetically active radiation (PAR) pulses measured in μmol m^{-2} s^{-1}. On the other hand, the NPQ values of MCH-0 outdoor were increased three times (Fig. 3B), implying that the irradiance is affected by the culture conditions. Regarding MCH-SW, it has similar behavior in indoor conditions, with values close to 1 (Fig. 3D) and slightly increasing after 14 d of cultivation, whereas, if the cultivation is performed in outdoor conditions, the NPQ value increases 4 times (Fig. 3E). Therefore, it is worth mentioning that the irradiance to which indoor cultures are exposed is only 183.5 μmol m^{-2} s^{-1}, and in real conditions (outdoor), the irradiance can reach 2000 μmol m^{-2} s^{-1} which indicates that these cultures have higher photochemical stress. MCH-SW obtained the highest NPQ values, thus demonstrating its high level of stress and protection machinery that is stimulated to protect itself and survive.

3.3. Cell viability and oxidative stress

3.3.1. Cell viability

To evaluate the physiological state of the microalgae, flow cytometry was used. When cells die, their enzymatic activity is reduced, and their cellular integrity is also degraded. Dyes enter the cell when its wall is damaged. One of the most used stains is PI. Although the interference of PI with auto-fluorescence of the microalgae has been reported, previous studies have confirmed that it can be used without any issue [47] because this stain enters the microalgae when its membrane is damaged [48]. Outdoor cultures were analyzed on days 0, 7, and 14. A control was performed for both salinities, where the cells (dead) exposed to temperature 95°C were located in quadrant b (Fig. 4). Regarding cultivation, it is observed that the microalgal populations, for both MCH-0 and MCH-SW, are located in quadrant a (alive) and quadrant b (dead). Cultures were started with 95% living cells in both salinities (Fig. 4A). After 14 d of culture inoculation (Fig. 4B and 4C), a slight decrease in viability is evident for both MCH-0 and MCH-SW, reaching up to 20% mortality. It can be suggested that the effect on the viability of cells for both conditions may be due to the high and local irradiance; however, in the MCH-SW condition, despite having additional stress due to salinity, no great effects on cell viability were observed as compared with that of MCH-0 [47,48,49,50]. In addition, an analysis was performed using confocal microscopy. Fig. 4D shows the control (dead microalgae), exposed to temperature and stained with PI emitted red fluorescence when excited by the blue laser (488 nm). On the other hand, because dead cells are stained red, the cell viability of the culture is evident; therefore, here interaction of the living and dead cells can be observed in the same plane.

3.3.2. Oxidative stress

To demonstrate cellular integrity, the presence of reactive oxygen species (ROS) is identified. The presence of ROS gives an indication of the cellular oxidative stress that microalgae can generate during stress conditions, either due to concentrations of salinity, chemical or due to high-intensity light. ROS are byproducts of oxygen metabolism in chloroplasts, mitochondria, and peroxisomes. Under normal conditions, ROS and cellular antioxidants are in a balanced state; however, cells subjected to biotic or abiotic stress produce excessive ROS. In microalgae, when stress is generated by high irradiance, it will tend to protect itself by increasing the production of antioxidant pigments [22,23]. In this study, dihydroethidium (DHE) was used as a staining agent to detect intracellular hydrogen peroxide (H_2O_2), superoxide (O^{2-}), or hydroxyl (OH^-) anions in the cell. DHE enters the cell where it is selectively oxidized by superoxides and binds with DNA to emit a fluorescence signal [51,52]. A control was performed where it was observed that the microalgae exposed to H_2O_2 are located spatially in quadrant b, thus indicating the ROS-producing population (Fig. 5). When analyzing the culture, it is observed that MCH-0 and MCH-SW are located spatially in both quadrants, i.e., ROS generating (quadrant b) and ROS non-generating (quadrant a). It is also observed that at day 0 (Fig. 5A), the cultures have a low percentage of ROS and reaches up to 4% in MCH-SW, which indicates that they were in

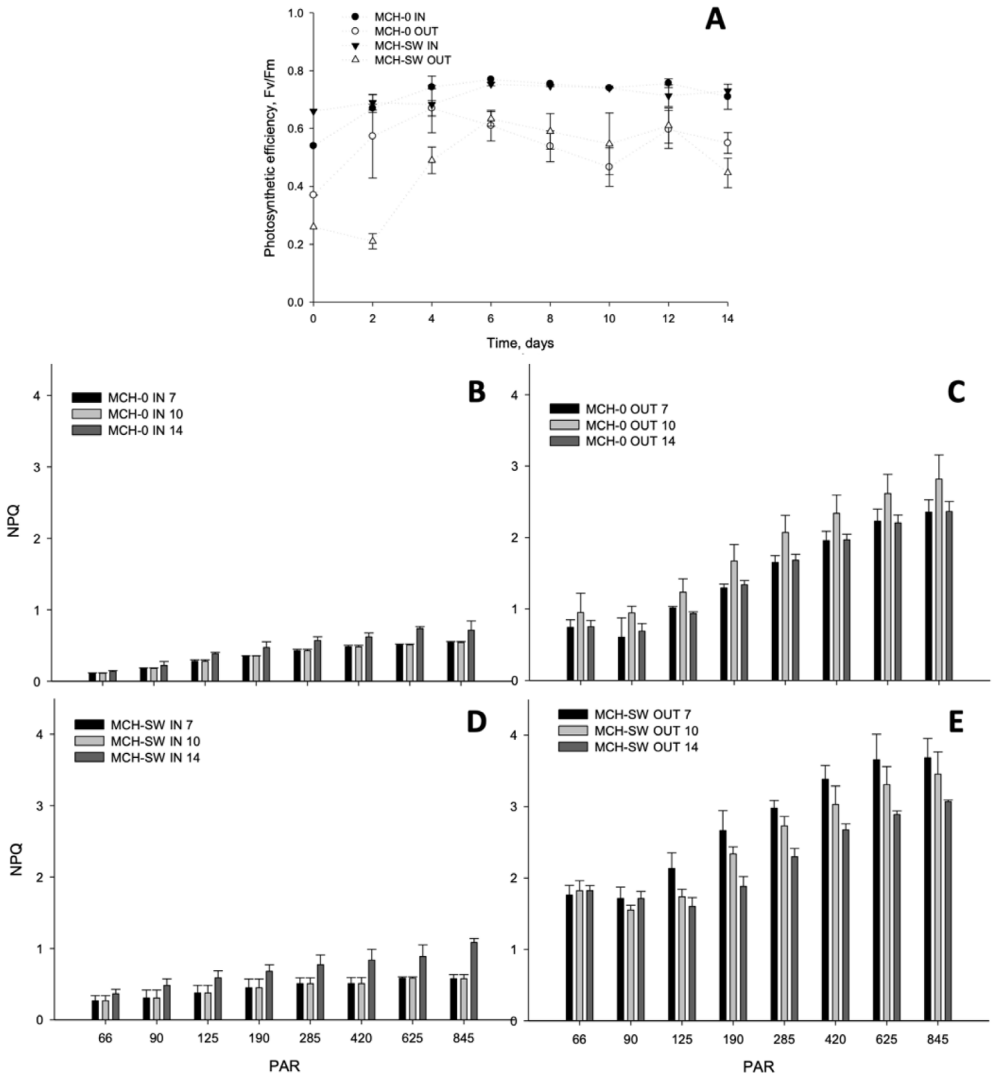

Fig. 3. Indoor and outdoor photosynthetic efficiency (Fv/Fm) values, (A) and non-photochemical dissipation (NPQ), (B) and (C) indoor; (D) and (E) outdoor of *Muriellopsis* sp., strain MCH ($p \leq 0.05$).

good condition at the beginning; after 7 d of culture, an increase is observed in ROS production (Fig. 5B), which reaches 32% in MCH-SW and remains the same on day 14 of the culture (Fig. 5C). This indicates that the microalgae were in the adaptation or acclimatization stage. The high oxygen concentration of the chloroplast ensures the rapid re-oxidation of the radicals in the microalgae, which regenerates and promotes the continuous formation of O^{2-}; therefore, this explains the high percentage values of ROS. Regarding MCH-0, the ROS production does not exceed the ROS percentage value of 12% throughout culture due to its cellular antioxidant mechanisms that were still capable of addressing ROS overproduction. ROS production resulting from various stress factors is known to affect almost all cellular processes, such as the structural stability of functional macromolecules, including DNA, proteins, and structural lipids. Because the life cycle of green algae depends on their photosynthetic activity and cellular integrity, it is crucial to protect them against oxidative stress [47,51,52]. Fig. 5D shows that ROS producing microalgae observed through confocal microscopy, emitted red fluorescence when excited with the 488 nm laser. The control is also observed, in which the cells were treated with H_2O_2 in order to induce ROS production, whereby the cells got completely stained. When analyzing the microalgae under a confocal microscope, ROS producing cells and ROS non-producing cells can be observed together. In conjunction with cytometric analysis, it was possible to determine that MCH-SW is the culture producing the highest percentage of ROS, which means that MCH-SW is more stressed than MCH-0.

3.4. Analysis of microalgal biomass

3.4.1. Lutein extraction and HPLC analysis

Microalgae are more productive than plants in terms of biomass because their photosynthetic conversion is 6 to 12 times more efficient than that of plants. Nevertheless, microalgae quickly adapt to various growth conditions and harvest systems; therefore, it is more feasible to manipulate their biosynthetic pathways for the production of bio-products. In this case, the product of interest is lutein, which is a carotenoid that is currently obtained mainly from plants, specifically from the marigold flower (*Tagetes erecta*), and marketed. However, this plant has disadvantages such as more harvest time and low lutein productivity as reaching only 0.03% of its total weight [53,54,55,56,57]. The percentage of lutein obtained from the strain MCH in indoor conditions was 25 times higher than that reported for calendula, reaching 0.75% of lutein in MCH-SW (Fig. 6A), followed by 0.6% in MCH-0, at day 12 of both cultures. These values exceed that of microalgae *Scenedesmus alme-*

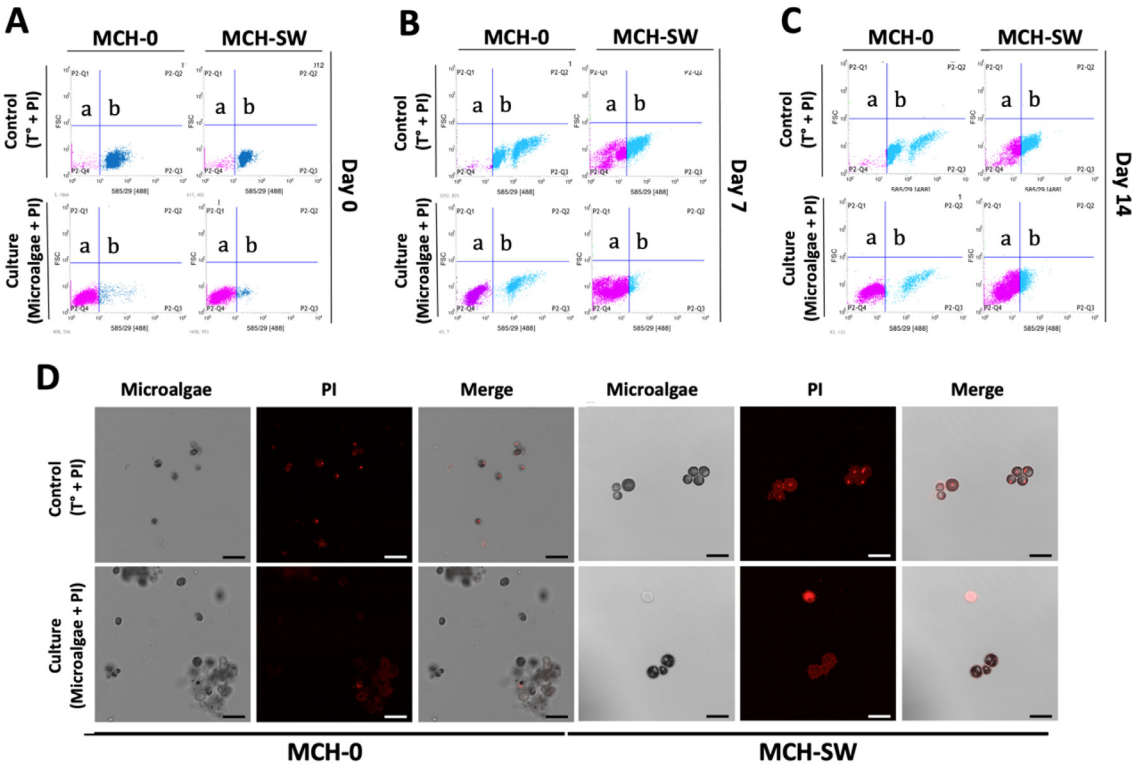

Fig. 4. Flow cytometry of MCH *Muriellopsis* sp. outdoor on different days of culture to determine cell viability. (A) day 0, (B) day 7 (C) day 14, where living (a) and dead cells (b) are evident. In addition, (D) confocal microscopy was used to determine cell viability and morphology of MCH *Muriellopsis* sp., MCH-0 and MCH-SW, outdoor. (Scale bar: 25 μm). T°: temperature, PI: propidium iodide, Merge: image overlay.

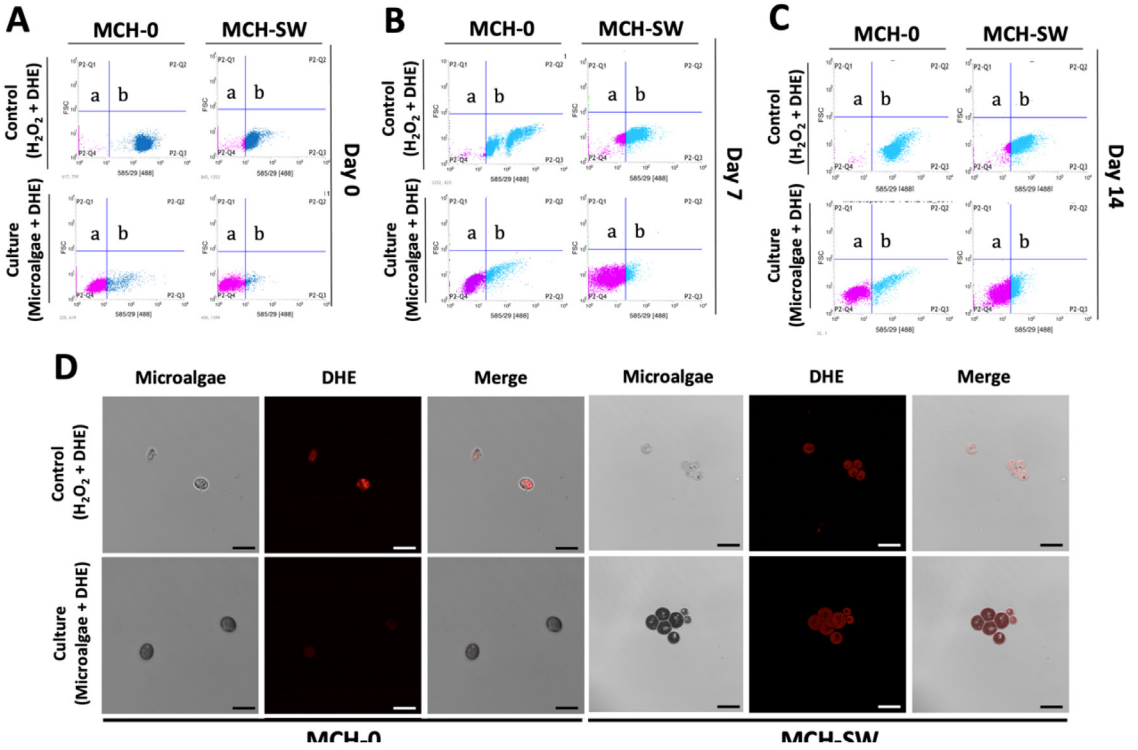

Fig. 5. Production of reactive oxygen species (ROS) in MCH *Muriellopsis* sp., outdoor by Flow Cytometry. (A) day 0, (B) day 7, (C) day 14, where the non-ROS producing cells are evident (a) and those that are producing it (b). (D) confocal microscopy to determine oxidative stress and cell morphology of MCH *Muriellopsis* sp., MCH-0 and MCH-SW (Scale bar: 25 μm). H_2O_2: oxygen peroxide, DHE: dihydroethidium, Merge: image overlay.

riensis, which has 0.53% lutein [55]. This pattern is maintained throughout the cultivation, which indicates that the high levels of salinity are responsible for causing changes in the conformation of MCH bioproducts, and it is also important to note that higher the availability of nutrients in the culture medium, greater will be the percentage of lutein produced [53,56,57]. Regarding the values in outdoor conditions (Fig. 6B), these amount to 0.4% of lutein in both salinities.

When analyzing lutein productivity, outdoor cultures (Fig. 6B) with high irradiance present higher values than indoor cultures (Fig. 6A). The culture MCH-0 outdoor had the highest lutein productivity throughout the culture, reaching 0.22 mg L^{-1} d^{-1} at day 14, while the equal value was reached by MCH-SW outdoor on the same day of cultivation.

This can occur because the environment for this microalgae is naturally freshwater and being in outdoor conditions could have led to a rapid photo-acclimatization, which resulted in high biomass production and achieving similar lutein productivity compared to MCH-SW, which despite the additional salinity stress (condition that causes the decrease in biomass), had a high productivity of lutein. This can be triggered because this carotenoid participates in photoprotection. Thus, it eliminates the photo-oxidative damage that excessive illumination could cause to a massive culture [19,57]. Blanco et al. [28] reported that *Muriellopsis* sp. had a high lutein productivity in summer reaching a value of 0.33 mg L^{-1} d^{-1} (100 mg m^{-2} d^{-1}), which is comparable to the values obtained in our study.

On the other hand, the low productivity in the other conditions may be due to the exposure conditions, causing a decrease in lutein levels and promoting the production of other carotenoids such as astaxanthin, β-carotene, or violaxanthin. Another factor that can affect lutein productivity is the instability that lutein has while being under stress conditions [28,56,58].

The carotenoid lutein, present in the MCH strain of *Muriellopsis* sp., was evaluated with HPLC to determine whether there is change in the content of microalgae when exposed to stress conditions (light and salinity) [57,59]. In Fig. 6C, it is observed that they all contain lutein, and a peak is observed after the 10th and 12th min of the retention time, which is the same as the peak observed in the lutein standard used as a control. The difference is in the purity of the lutein obtained, because the values increased with the course of the days, starting with 61.24% on day 6 of culture, reaching 95.79% for the case of MCH-0 and 59.50% increasing to 96.17% for MCH-SW. This accumulation of *Muriellopsis* sp. could be used to protect its cells from photodamage [60]. Another difference that can be observed is the absence of carotenoids on day 14, such as violaxanthin, which had 0.2% purity on day 6 in indoor culture.

3.5. Antioxidant activity

3.5.1. DPPH assay (2,2-diphenyl-1-picrilhydrazil)

The antioxidant effect was evaluated with DPPH which is a free radical that allows the evaluation of the elimination or neutralization of free radicals [22,61]. MCH of *Muriellopsis* sp. contains lutein that acts as an antioxidant, i.e., it can inhibit free radicals. Trolox, which is an analog of vitamin E, is used as a positive control. In Fig. 7, the antioxidant activity of the microalgae is observed in different culture conditions. Trolox with a concentration of 200 μg mL^{-1}, was used, which exceeded 98% of inhibition effect. The outdoor cultures showed lower values than those for the indoor cultures. The antioxidant activity is below 20% for both cultures. The highest antioxidant activity value of 54% was obtained on day 14 by MCH-SW with indoor conditions. These values do not seem to have a direct relationship with the productivity of lutein. Although in indoor conditions, both show an increase in this

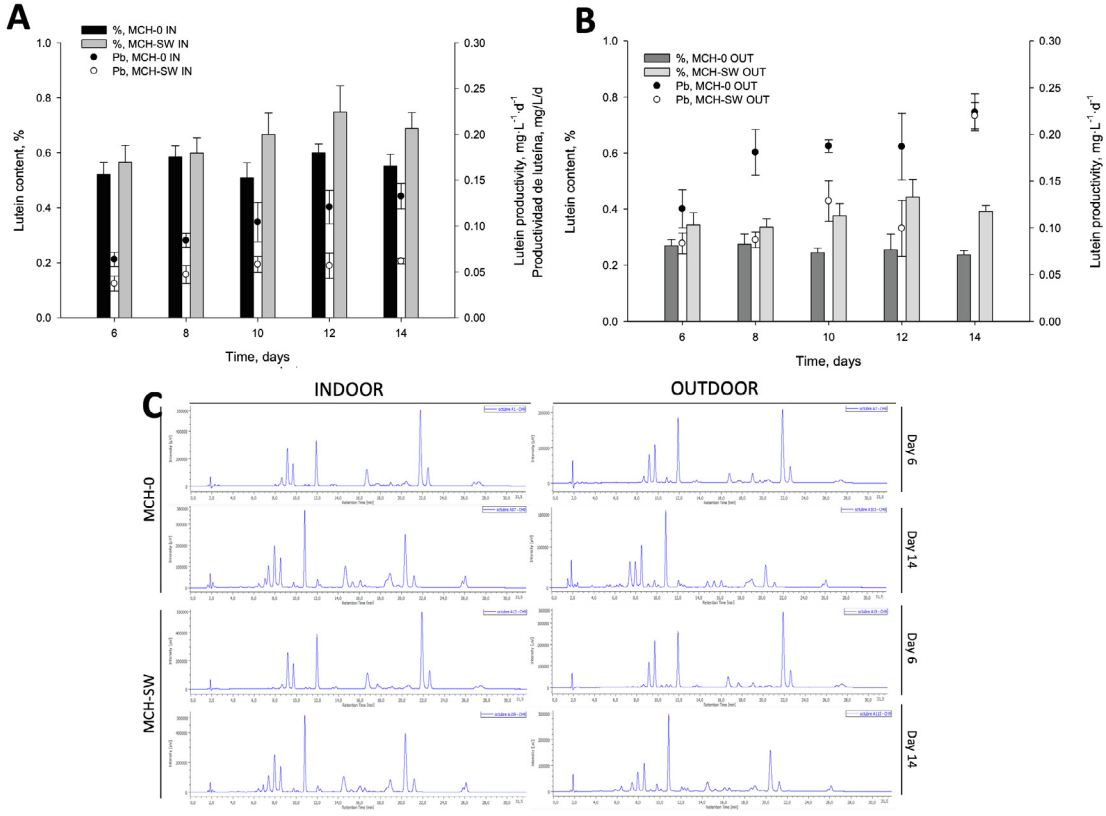

Fig. 6. Lutein content and productivity of MCH *Muriellopsis* sp. in indoor conditions (A), and in outdoor conditions (B) ($p \leq 0.05$). C) Chromatogram of microalgal extracts indoor and outdoor cultures. (a) violaxanthin, (b) lutein.

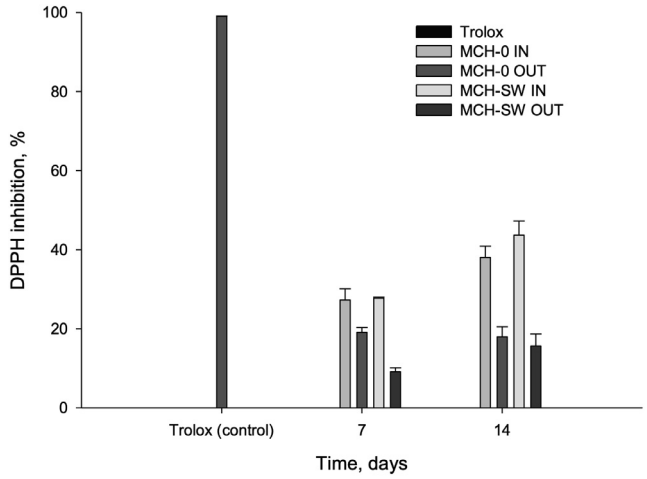

Fig. 7. Determination of the antioxidant activity of the lutein extracts of the microalgae MCH *Muriellopsis* sp. indoor and outdoor with the DPPH method ($p \leq 0.05$).

value, in outdoor cultures, this is not the case. This indicates that lutein not only acts as an antioxidant, but there must also be other compounds with antioxidant activity. When comparing the antioxidant activity with other microalgae of other species such as *Dunaliella* sp., *Chlorococcum* sp. and *Chlorella* sp., the strain MCH of *Muriellopsis* sp. continues to have higher antioxidant activity [62,63]. Unlike MCH of *Muriellopsis* sp., the main lutein producer in the world, calendula flower, has only 14% free radical inhibition, needless to mention the production time which is much longer and can be weeks or even months. Using the strain MCH, a large amount of biomass can be obtained only in a couple of days [64]. Exposing MCH to stress alters the balance between ROS production and its elimination, thus causing oxidative stress, i.e., damage through oxidation of cellular components. This disbalance also indicates a constant struggle to maintain the balance. In most microalgae, high salinity stress is beneficial for lipid accumulation. However, it generally leads to oxidative damage and decreased photosynthetic pigments [58].

4. Conclusions

In this study, *Muriellopsis* sp. MCH, when cultured in outdoor conditions of high irradiance, showed some changes such as an increase in cell number, biomass concentration, and carotenoid content. Physiological and biochemical behaviors were analyzed in MCH culture cultivated in seawater. These behaviors were significantly affected under this condition as shown by changes in growth, photosynthetic efficiency, lutein productivity, and antioxidant activity.

It is concluded that this study allowed us to identify the fact that in the conditions of outdoor cultivation, the strain MCH of *Muriellopsis* sp., increases the production of lutein content in both conditions, i.e., freshwater and seawater. The highest values obtained compared with that reported in the literature for other terrestrial microalgae and vegetables allowed us to conclude that *Muriellopsis* sp. is one of the largest producers of lutein. Although MCH is a freshwater microalga, it could manage to grow without difficulty in seawater. This attribute of MCH makes it feasible for us to reduce costs and carry out massive cultivation of MCH to enable us to extract a significant amount of lutein, a carotenoid that has well-known properties to prevent some diseases due to its high nutritional value.

Financial support

This research was supported by Fondef IDeA grant ID14I10110.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Dr. Fernando Silva for isolating the strain *Muriellopsis* sp. The data obtained in flow cytometry were supported by a Fondequip EQM120137 project.

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