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Extremophilic microorganisms for the biotechnological production of added-value molecules

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EXTREMOPHILIC MICROORGANISMS FOR THE BIOTECHNOLOGICAL PRODUCTION OF ADDED-VALUE MOLECULES

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EXTREMOPHILIC MICROORGANISMS FOR THE BIOTECHNOLOGICAL PRODUCTION OF ADDED-VALUE MOLECULES

MICROORGANISMOS EXTREMÓFILOS PARA LA OBTENCIÓN BIOTECNOLÓGICA DE MOLÉCULAS DE VALOR AÑADIDO

Memoria para optar al grado de doctora presentada por:
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Huelva, 2020

A todos los que hicieron esto posible,
Gracias

"What makes the desert beautiful, said the little prince, is that somewhere it hides a well..."

— Antoine de Saint-Exupéry, The Little Prince

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AB	alcian blue
APC	allophycocyanine
BABR	bisanhydrobacterioruberin
BR	bacterioruberin
C	carbon
CCD	central composite design
CrtB	phytoene synthase
Crtl	phytoene desaturase
DB	biomass
DNA	deoxyribonucleic acid
dw	dry weight
EPS	exopolysaccharides
EPS-S	sulfated exopolysaccharides
FA	fatty acid
FAMEs	fatty acid methyl ester
Fm	maximum fluorescence
Fo	basal fluorescence
Fv	dark-adapted fluorescence
GGPP	geranylgeranyl pyrophosphate
GS-GOGAT	glutamate synthase cycle
H mediterranei	*Haloferax mediterranei*
HPLC	high performance liquid chromatography
LyeJ	lycopene longase / lycopene 1,2-hydratase
MABR	monoanhydrobacterioruberin
ML	maximum likelihood
MP	maximum parsimony
N	nitrogen
NJ	neighbor joining
NPK	nitrogen:phosphorus:potassium
O.D.	optical density
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation (400-700nm)
PBP	phycobiliproteins
PBS	phycobilisome
PC	phycocyanin
PCR	polymerase chain reaction
PE	phycoerythrin

Abbreviations

PHA	polyhydroxyalkanoate
PSII	photosystem II
PUFA	polyunsaturated fatty acid
Qy	quantum yield
rDNA	ribosomal deoxyribonucleic acid
Rf	retention factor
ROS	reactive oxygen species
RSM	response surface methodology
SD	standard deviation
Std	standard order
TLC	thin layer chromatography
UV-A	ultraviolet A (315−400 nm)
UV-B	ultraviolet B (280−315 nm)
UVR	ultraviolet radiation
v/v	volume/volumen
w/v	weight/volumen
w/w	weight/ weight

Abstract

Extremophilic microorganisms are especially interesting from the biotechnological point of view. These microorganisms have developed mechanisms that lead to the production of valuable substances in order to survive in certain environments considered extreme for life. These substances can be used by the cosmetic, pharmaceutical industry and in human or animal feeding.

Compounds obtained from extremophilic microorganisms have a great added-value since they can have unique properties and their nature can be very diverse. In this doctoral Thesis, substances such as carotenoid pigments, phycobiliproteins and carbohydrates (exopolysaccharides) are studied. Pigments are especially interesting since in recent years there has been a growing demand, by consumers, towards natural food dyes. Carotenoid pigments, and also phycobiliproteins, are of great interest for their use as feed additives, especially as color enhancers. These pigments also have great value as antioxidants and, like exopolysaccharides, have been found to display anti-cancer and anti-inflammatory activity. In addition to the currently known applications, there are microorganisms from extreme environment that are yet to be discovered and whose potential has not been studied.

However, despite the biotechnological potential of extremophilic microorganisms, their use in biotechnology has certain limitations related to culture conditions, and the profitability of the compounds of interest produced. Therefore, it necessary to improve the production processes of these microorganisms in order to obtain competitive products in the market.

Based on this, this Thesis had as a main objective the isolation and the study of the biotechnological potential of microorganisms from extreme environment, and improving the production strategies of target compounds, with special emphasis on reducing production costs. For this, two extremophilic microorganisms were studied: a halophilic archaea, *Haloferax mediterranei* and a cyanobacterium from extreme arid environment, *Chroococcidiopsis* sp.

On the one hand, *H. mediterranei* stands out for its ability to produce a C_{50} carotenoid, particularly bacterioruberin, with high antioxidant capacity. Its possible medical application or in food industries, such as nutraceuticals, or obtaining functional foods makes bacterioruberin interesting. On the other

hand, *Chroococcidiopsis* sp. stands out for its versatility, since it is found in very diverse extreme environments. Due to the extreme radiation that affects the rocks of the Atacama Desert, this cyanobacterium colonizes the inside of the rocks, so that the light received by a part of the endolithic colonies can be meager and diffuse irradiation. In order to be more efficient in capturing light, these cyanobacteria produce phycobiliproteins, a pigment that can be used as a food coloring and also stands out for its antioxidant capacity. Furthermore, these cyanobacteria respond to desiccation by producing a shell of exopolysaccharides, which provides a moisture environment to the cyanobacterium and has a potential use for its antibacterial and anti-inflammatory characteristics.

Based on this, we optimized the growth and target metabolites production from both extremophiles. In the case of *H. mediterranei*, the effect of physicochemical parameters such as temperature, salinity and pH on the growth and carotenoids production was studied. In addition, the effect of nutritional factors such as glucose and yeast extract was analyzed. It allowed us to propose possible production strategies. After the isolation and identification of *Chroococcidiopsis* sp., the study of parameters such as the source and concentration of nitrogen or agitation was carried out to improve the cyanobacterial growth. Furthermore, the effect of other parameters such as the light irradiance on growth and the phycobiliproteins accumulation were studied. Finally, with the idea of reducing production costs, agricultural fertilizers were used as culture media, which means several advantages in large-scale cultures.

The results obtained in this Thesis have contributed to improve the productivity of the biomass of these microorganisms and to the improvement in the environmental and nutritional conditions of cultivation for an optimum production of high-added value compounds. In addition, our results allow us to predict the best conditions that might contribute to address the biotechnological process on a large scale.

Los microorganismos extremófilos son especialmente interesantes desde el punto de vista biotecnológico, ya que para poder sobrevivir en determinados ambientes considerados extremos para la vida, estos microorganismos han desarrollado mecanismos que conllevan la producción de sustancias de valor que pueden ser utilizadas por la industria cosmética, farmacéutica o en alimentación humana o animal.

Los compuestos obtenidos a partir de microrganismos extremófilos tienen un gran valor añadido ya que pueden tener propiedades únicas y la naturaleza de estos compuestos puede ser muy diversa. En esta Tesis doctoral, se estudiaron sustancias tales como pigmentos, carotenoides, ficobiliproteinas y carbohidratos (exopolisacáridos). Estos pigmentos son especialmente interesantes ya que en los últimos años ha crecido el interés, por parte de los consumidores, hacia colorantes alimentarios de origen natural (no sintéticos). Los pigmentos carotenoides, y también las ficobiliproteinas, son de gran interés por su uso como aditivos en alimentación, especialmente como potenciadores del color. Estos pigmentos tienen, además, un gran potencial como antioxidantes y, al igual que los exopolisacáridos, presentan actividad anticancerígena y antiinflamatoria. Además de las aplicaciones actualmente conocidas, existen microorganismos de ambiente extremo que están aún por descubrir y cuyo potencial no ha sido estudiado.

Sin embargo, a pesar del potencial biotecnológico de los microorganismos extremófilos, su uso en biotecnología presenta ciertas limitaciones relacionadas con el cultivo, y la rentabilidad de los compuestos de interés producido. Por lo tanto, existe la necesidad de mejorar los procesos de producción de estos microorganismos para obtener así productos competitivos en el mercado.

En base a esto, esta tesis tuvo como objetivo principal aislar y estudiar el potencial biotecnológico de microrganismos de ambiente extremo, y mejorar las estrategias de producción de los compuestos de interés haciendo especial hincapié en la reducción de los costes de producción. Para ello se estudiaron dos microorganismos extremófilos: una archaea halófila, *Haloferax mediterranei* y *Chroococcidiopsis* sp., una cianobacteria de ambiente árido hiperextremo.

Resumen

Por un lado, *H. mediterranei* destaca por su capacidad para producir un carotenoides C_{50}, particularmente bacterioruberina, con alta capacidad antioxidante, cuyo interés principal es su posible aplicación médica o en industrias alimentarias, como nutracéutico, en la obtención de alimentos funcionales. Por otro lado, *Chroococcidiopsis* destaca por su versatilidad, ya que se encuentra en ambientes extremos muy diversos. Debido a la radiación extrema que afecta a las rocas del desierto de Atacama, esta cianobacteria coloniza el interior de las rocas, por lo que la luz que recibe una parte de las colonias endolíticas puede ser difusa y de baja irradiación. Para poder ser más eficientes en la captación de luz, estas cianobacterias producen ficobiliproteinas, pigmento que puede ser utilizado como colorante en alimentación y también destaca por su capacidad antioxidante. Por otro lado, estas cianobacterias responden a la desecación mediante la producción de una envoltura de exopolisacáridos, la cual, proporciona un ambiente de humedad a la cianobacteria y tiene un potencial uso por sus características antibacteriana y antiinflamatoria.

En base a esto, nos propusimos optimizar el crecimiento y la producción de metabolitos de interés de ambos extremófilos. En el caso de *H. mediterranei*, se estudió el efecto de parámetros físico-químicos como la temperatura, la salinidad y el pH sobre el crecimiento y la producción de carotenoides. Además, se estudió el efecto de factores nutricionales como la glucosa y el extracto de levadura lo cual permitió proponer posibles estrategias de producción. En el caso de *Chroococcidiopsis* sp., tras su aislamiento e identificación, se llevó a cabo el estudio de parámetros relacionados con la mejora en el crecimiento como la fuente y concentración de nitrógeno o la agitación. Además se estudiaron otros parámetros como el efecto de la irradiancia de luz sobre el crecimiento y la acumulación de ficobiliproteinas. Por último, con idea de reducir costes de producción, se utilizaron como medios de cultivo fertilizantes agrícolas lo cual supone varias ventajas en cultivos a gran escala.

Los resultados obtenidos en esta Tesis han contribuido a mejorar la productividad de la biomasa de estos microorganismos y a la mejora en las condiciones ambientales y nutricionales de cultivo para una óptima producción de compuestos de alto valor añadido. Además, nuestros resultados

permiten sugerir las mejores condiciones que podrían contribuir a abordar el proceso biotecnológico a gran escala.

CHAPTER I

GENERAL INTRODUCTION

Introduction

Extremophiles and the limits of life

Microorganisms have been used since ancient times for food processing and bioproducts, and nowadays they are in addition used for biotechnological purposes in the chemical, pharmaceutical and food industries (Soares et al. 2012). An example is the use of *Lactobacillus* for the production of yoghurt and cheese. Most of the microorganisms live under moderate temperature, pH, salinity and pressure, such as 37 ºC, pH around 7, salinity between 0.9% and 3%, and atmospheric pressure. These parameters values are considered adequate for the proliferation of life. However, some microorganisms, called **extremophiles**, are able to live under extreme conditions by means of the expression of adaptation mechanisms (Canganella and Wiegel 2011).

The diversity in morphology, biochemistry, genomic, and adaptation mechanisms of extremophiles is remarkable (Durvasula and Rao 2018). According to the main extreme parameter in the habitat, extremophilic microorganisms can be classified in different groups (Merino et al. 2019), such as:

a) **Acidophiles**. These microorganisms grow optimally at pH below 5, characteristic of volcanic springs or acid mine drainage. Bacteria like *Bacillus* and *Chlostridium paradoxum* are typically found in these habitats, and an acidophilic eukaryotic microalga, named *Coccomyxa onubensis*, has been isolated from the acidic waters of Tinto river.

b) **Alkaliphiles**. These microorganisms show an optimal growth at pH of 9 or above and have been found in natural lakes, such as Texcoco, near Mexico city, in which the cyanobacteria *Spirulina (Arthrospira) platensis* proliferates spontaneously.

c) **Halophiles**. These microorganisms have the optimal growth over 1.5 M concentrations of salt (NaCl) and the main groups are *Halobacteriaceae* and the eukaryotic microalga *Dunaliella salina*.

d) **Metallotolerants**. They are able to tolerate high levels of dissolved heavy metals in the medium. *Ferroplasma* sp. and the above referred *Coccomyxa onubensis*, belongs to this group.

e) **Piezophiles or Barophiles**. These microorganisms adapted to life under conditions of high pressure in underground or in the deep ocean. They have been found at depths of 6.7 km in the earth's crust, and at more than 10 km below the ocean´s surface, at pressures of 110 MPa.

f) **Psychrophiles or Cryophiles**. These microorganisms grow optimally at temperatures below 20 ºC, typical of frozen soils, polar ice and cold ocean water. *Synechococcus lividus* and *Chlamydomonas nivali* are typical microalgae commonly in cold environments.

g) **Thermophiles**. These microorganisms require of temperatures above 45 ºC and can be present in thermal springs, like the bacteria *Thermus aquaticus*, that serves to Kary Mullis (Nobel price of Chemistry in 1993) as source of a thermostable DNA polymerase required for the Polymerase Chain Reaction (PCR).

h) **Xerophiles (mostly Xerotolerant).** While liquid water is absolutely essential for the growth and reproduction of all terrestrial life, certain microorganisms can tolerate periods of extreme desiccation. The study of this capacity provides valuable information about the origin of life on earth.

Many extremophiles fall under multiple categories and are classified as polyextremophiles because they may live in environments with different extreme conditions. For example, many hot springs are also acid or alkaline, and rich in heavy metals; the deep ocean is generally cold; or hypersaline lakes are also very alkaline. The bacterium *Thermococcus barophilus* can live from pH 0 to pH 12.8, in hydrothermal vents at 122 °C or in frozen sea water, at −20 °C (Rampelotto 2013).

As another example, the case of the bacterium GFAJ-1 (Figure 1.1), isolated from Mono Lake (California), is of great relevance. This bacterium is able to substitute arsenic for phosphorus in molecules such as lipids and nucleic acids (Wolfe-Simon et al. 2011). This provides exciting scientific information about the existence of molecular life patterns other than those

considered chemically unalterable on earth, suggesting that life existence on other planets might also be possible based on the chemical flexibility of the life molecules.

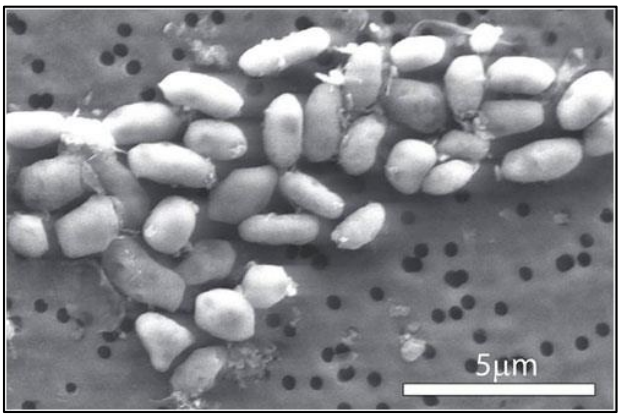

Figure 1.1. Scanning electron microscopy of strain GFAJ-1 (Wolfe-Simon et al. 2011).

Some fungi or bacteria can live in the pores of rocks, tolerating long periods of draught, high UV exposure and wide thermal fluctuations (Onofri et al. 2004). Photosynthetic microorganisms can also live in rock fragments, forming endolithic colonies (Wierzchos et al. 2012). With respect to this, some cyanobacteria can survive long periods of time almost without water (Cockell et al. 2011). These data support the hypothesis of Panspermia, which explains that the origin of life arose elsewhere in the universe, but seeds of life can travel across galaxies as "life spores" in comets, protected from ultraviolet radiation, seeding planets in the solar system and elsewhere in the universe (Lage et al. 2012; Lal 2008).

Studying these extremophilic microorganisms allows understanding the limits of life, and therefore provides answers to the fundamental questions about the origin, distribution and evolution of life (Varshney et al. 2015). Understanding the limits of life on earth gives relevant information about the search for life on other planets, allowing expand the search range of planets with the possibility of housing life. Since most of the planetary bodies in our solar system are frozen worlds, knowledge about survival at sub-zero temperatures is of great importance. For

example, the ability of some microorganisms to survive for long periods of time in frozen permafrost suggests possibilities for life in the layers of permafrost known in Mars (Bass et al. 2000) or in the moons of Jupiter or Saturn (Mann 2017).

Biotechnological use of extremophiles

Extremophilic microorganisms have developed adaptation mechanisms that lead to the production of substances of high biotechnological interest (Raddadi et al. 2015). However, it is estimated that less than 1% of the extremophiles of the earth have been identified and the percentage is even less for microorganisms whose biotechnological potential has been analyzed (Babu et al. 2015).

For biotechnological purposes, extremophiles have an important advantage with respect to mesophiles in terms of robust growth at industrial scale since they can easily adapt to environmental fluctuations, such as radiation intensity and/or seasonal temperature. Moreover, the risk of contamination by other microorganisms in outdoor cultures is low (Harun et al. 2010) due to the optimal extreme growth conditions stated for extremophiles that do not favor the growth of non-extremophilic microorganism. Therefore, the interest for extremophiles has increased in recent years (Forján et al. 2015; Varshney et al. 2015).

The growth rate and productivity are relevant parameters to approach economic feasibility of a cultivation process and derived applications. Therefore it is necessary to identify and analyze parameters related to the extremophile´s growth, in order to obtain large biomass and/or target metabolites productivities and in this way to make the product profitable from the economic point of view. The complete research process of the biotechnological production of extremophiles must reach the pilot-scale stage in open systems (Figure 1.2), which can lead to cost savings in the investment.

Figure 1.2. Cyanobacterial cultures in open raceway systems. The picture shows a raceway pond with paddle wheel. The main advantages of open systems are their low cost and ease of construction and operation.

Furthermore, the substitution of chemical components in the culture medium by a combination of agricultural fertilizers is a way to reduce costs during the production at large scale of an extremophilic microorganism (Nayak et al. 2016; Silva-Benavides 2016). This can be a way to make photosynthetic the cultivation easier and cheaper (Scardoelli-Truzzi and Sipaúba-Tavares 2017).

On the current market, we can only find few noticeable applications of extremophiles: (a) the use of enzymes in the production of biofuels (Barnard et al. 2010) and the well-known use of the thermostable DNA polymerases used in the polymerase chain reaction (PCR) (Ishino and Ishino 2014); (b) the use of acidophilic bacteria and archaea in the mining process, specifically for bioremediation or recovery of metals of commercial value from process waters and waste streams (Johnson 2014); and (c) the carotenoids which are currently marketed in the food and cosmetic industries (Oren 2010). Specific examples of these applications are described in the following subsections.

a) *Extremozymes*

An example of the biotechnological potential of extreme environment microorganisms is the use of their *extremozymes*. For instance, the adaptation of a thermophilic microorganism resulted in the expression of heat-resistant enzymes, which are of great interest due to many industrial processes are carried out at high temperatures (Dumorné et al. 2017; Zhong et al. 2009). It should be noted that the discovery of DNA polymerase from the thermophiles *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis* has an important impact in biotechnology (Lundberg et al. 1991). Moreover, *extremozymes* are also used in some steps of the biofuel production process, since it requires high temperatures and extreme pH (Coker 2016).

It is also interesting to highlight the use of *extremozymes* in depuration of industrial wastewaters, when normal enzymes require a pre-cooling step for optimal work, thus resulting an additional cost. The use of heat-adapted enzymes skips the pre-cooling step, which supposes an economic benefit through energy savings (Gavrilescu 2010). The special resistance of enzymes to high temperature is due to a combination of unique structural features which prevent protein degradation (Gomez-Pastor et al. 2018; Imanaka 2008).

The biotechnological potential of extremozymes also arises from those organisms that live in the deepest part of the ocean and are subjected to extremely high pressure (1,100 atm of pressure). These microorganisms can produce enzymes capable of withstanding higher pressures than the enzymes of their non-extremophilic counterparts. This is of great value for food industry applications, for instance in food pasteurization under high pressure. Also some applications related to the treatment of pressure-induced pathologies in humans are being investigated (Huang 2018; Babu et al. 2015).

b) *Bioremediation*

Bioremediation is an important technology for the clean-up of environments contaminated with persistent organic pollutants (e.g., pesticides, polychlorobiphenyls (PCBs), petroleum hydrocarbons) and

heavy metals (e.g., Hg, Cd) (Orellana et al. 2018). Specifically, biosorption of heavy metals from aqueous solutions has been proved to be very promising, offering significant advantages such as low cost, profitability, ease of operation, and high efficiency, especially when dealing with low metal concentrations (Javanbakht et al. 2014). Extremophiles are especially useful for bioremediation due to their unique metabolic plasticity and tolerance to extreme physic-chemical conditions. In addition, halophiles isolated from halite (rock salt) of the Atacama Desert have also been found to remove metals from the media, which means a possible strategy for bioremediation of contaminated soils (Popescu and Dumitru 2009). In addition, halophiles are of great importance in the bioremediation of wastewaters from industries, which frequently possess high salt concentration or high levels of organic compounds (Orellana et al. 2018).

c) *Carotenoids*

Carotenoids production with microorganisms at large scale is still economically challenging. However, there are three exceptions to this: bacteriorhodopsin, canthaxanthin, and β-carotene (Chandi and Gill 2011). These carotenoids are synthesized by microorganisms of extreme environments, haloarchaea (bacteriorhodopsin and canthaxanthin) and the halophilic alga *Dunaliella salina* which is the major source for β-carotene (Coker 2016). In addition, the number of synthetic colorants additives approved by regulatory food agencies has been reduced in the last years (Torres et al. 2016). In parallel, the consumer's mind is changing concerning the artificial food additives, driving them to choice natural colorants which may be healthier than synthetic ones. Consequently, marketed natural carotenoids are already replacing the use of artificial colorants in many foods (Mussagy et al. 2019).

Biotechnological production of bioactive compounds by extremophiles

Adaptation strategies to extreme environments can lead to develop production process of specific substances which can have interesting properties for industrial application. However, the optimal production of any metabolite by extremophiles requires optimization of processes and/or conditions, in order to make their biotechnological use economically

feasible (Coker 2016). There are several examples of production of bioactive compounds with extremophiles as real protagonists.

The halophiles have mechanisms that prevent the loss of water by maintaining the concentration of intracellular osmolytes and ions at levels that are similar to those of the environment. This entails the adaptation of enzymes and intracellular proteins. Some of them accumulate organic compatible solutes such as betaine and ectoine with the aim of reducing the osmotic pressure (Roberts 2005). Ectoine is hypothesized to provide protection to DNA from damage, hence reducing cancer risk. Furthermore, ectoine has been found to reduce or prevent dehydration of dry atopic and aging skin (Babu et al. 2015).

Figure 1.3. Picture of hypersaline environment in Santa Pola, Alicante (Spain).

Halophilic archaea can also produce two main types of biopolymers as an adaptation strategy to high salinity: extracellular polysaccharides (EPS) and the endocellular polyhydroxyalkanoate (PHA). EPS function as an outer protection layer against desiccation and have remarkable biotechnological applications in the food and pharmaceutical industries (Kazak et al. 2011), and the PHA provides an internal reserve of carbon and energy for cells under nutrient starvation and has been utilized in the production of biodegradable plastics (Poli et al. 2011). Furthermore, it is important to highlight that certain carotenoids, such as salinixanthin or

bacterioruberin, produced solely by some halophiles, are highly demanded products nowadays (Torregrosa-Crespo et al. 2018).

The Atacama Desert is one of the driest places on Earth, with a mean rainfall less than 5 to 2.4 mm per year (Orellana et al. 2018; Azua-Bustos et al. 2015; Warren-Rhodes et al. 2006). Although no higher organisms are prevalent in such extreme environments, important colonies of microorganisms are widespread in this habitat. Xerophiles have developed several strategies, like accumulation of osmoprotective metabolites such as trehalose, L-glutamate, glycine or betaine, which prevents them from water loss. Some of these substances are used in the cosmetic industry as thickeners and foam boosters in hair and skin care formulations, such as shampoos and soaps (Flick 1995). Xerophilic organisms can also modify the lipid profile composition of membranes in response to desiccation, specifically by reinforcing the membrane to maintain its integrity under conditions of anhydrobiosis. Another strategy to survive in the dry environment is the EPS production that regulates the loss and uptake of water by the cells and it serves as a matrix for the immobilization of other components (Potts 1999).

Besides the aforementioned applications, the potential of extremophiles for production of antibiotics, anticancer compounds, antifungal compounds (Herbert 1992) and other pigments (Coker 2016) is currently investigated. It has been recognized that the feature that helps extremophiles to survive in extreme environmental conditions could be effectively used in medical processes to develop applications that benefit human health (Babu et al. 2015). A summarized list of valuable compounds from extremophiles is shown in Table 1.1.

Table 1.1. Valuable compounds from extremophilic microorganisms.

Extremophile	Valuable compounds	Application	Ref.
Thermophiles	Enzymes	Plant material degradation	Gomes et al. (2016)
Piezophiles	Enzymes	Food pasteurization and medicine	Huang (2018); Babu et al. (2015)
Halophiles	Betaine and ectoine solutes Biopolymers C_{50} carotenoids (Bacterioruberin)	Stabilizers, pigments, antioxidants	Waditee-Sirisattha et al. (2016)
Xerophiles	Exopolysaccharides	Probiotic activity in food industry; anticancer, and antiinflammatory properties	Singh et al. (2019); Oleksy and Klewicka (2018)

As described above, the exploitation of extremophiles and the study of their properties are of great utility nowadays as provide opportunities for industrial and medical resources production and improvement.

Study groups of extremophiles in this Thesis

There is wide diversity of extremophilic microorganisms present in the trunks of the tree of life: Bacteria, Archaea, and Eukarya. Archaea is the main group to thrive in extreme environments, and although members of this group are generally less versatile than bacteria and eukaryotes, they are quite skilled in adapting to different extreme conditions, frequently holding records (Rampelotto 2013). Some haloarchaea have optimum growth under extreme conditions which makes them true (obligate) extremophiles.

a) Haloarchaea

Most members of the families *Haloferacaceae* and *Halobacteriaceae* can synthesize C_{50} carotenoids, including bacterioruberin as the most abundant C_{50} in most of the analyzed haloarchaea species (Rodrigo-Baños et al. 2015). Bacterioruberin has great potential as an antioxidant, because

it contains 13 pairs of conjugated double bonds versus the 9 pairs found in β-carotene, which makes bacterioruberin a more powerful radical scavenger (Yatsunami et al. 2014; Saito et al. 1997). Bacterioruberin, as part of the haloarchaea´s membrane, acts as a barrier to water and allows permeability to oxygen and other molecules, also giving fluidity to membranes, thus protecting the haloarchaea (Rodrigo-Baños et al. 2015; Fang et al. 2010; Lazrak et al. 1988).

The interest in bacterioruberin has increased in recent years (Rodrigo-Baños et al. 2015). Besides, carotenoid production by haloarchaea could be improved by genetic modification or by modifying several cultivation conditions such as nutrition, pH or temperature (Torregrosa-Crespo et al. 2017). The study of the effect of main cultivation parameters on the growth and accumulation of carotenoids in haloarchaea species would allow developing production strategies at several scales, establishing the process stages towards the production at industrial scale. In this sense, *Haloferax mediterranei*, a haloarchaea isolated from saline water of Santa Pola (Alicante), has been suggested as a potential source of bacterioruberin (Rodrigo-Baños et al. 2015).

b) Cyanobacteria

Cyanobacteria are a highly versatile group of photosynthetic organisms which are found in many different extreme environments (Rampelotto 2013). Particularly, the genus *Chroococcidiopsis* is known for its ability to survive under extreme environmental conditions, including both high and low temperatures, ionizing radiation, extreme arid environment and high salinity. Strains of *Chroococcidiopsis* are able to withstand years of desiccation (Billi 2009), surviving to ionizing radiation up to 15 kGy (Billi et al. 2000), and UVC doses as high as 13 kJ/m^2 (Baqué et al. 2013).

Cyanobacteria from arid environments have gained interest in recent years in relation to the search for hyperextreme life model microorganisms (Singh et al. 2016). In this respect, the Atacama Desert has in recent years been proven to be the habitat of hyperextreme life cyanobacteria (Wierzchos et al. 2012). This environment is characterized for being one of

the driest deserts in the world. Particularly, the permanent absence of clouds and the low concentration of ozone make the solar radiation extremely high and harmful, greatly increasing the possibility of a lethal photoinhibition and photooxidative damage for photoautotrophic microorganisms (Solovchenko and Merzlyak 2008).

Due to the extreme radiation that affects the rocks of the Atacama Desert, some photosynthetic microorganisms live in endolithic colonies (Figure 1.4.), so the received light can be diffuse and low in irradiance.

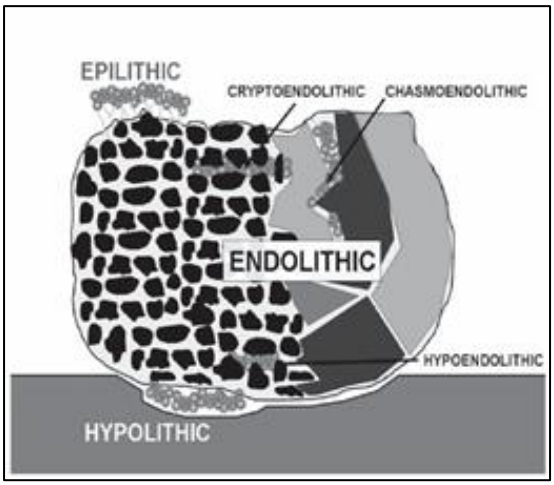

Figure 1.4. Microbial communities within rock. The figure shows the possible lithobiontic habitats of microorganisms. Epilithic (rock surface); hypolithic (rock underside in contact with the soil); endolithic (the main habitats of arid deserts), and further divided into cryptoendolithic, chasmoendolithic and hypoendolithic (Wierzchos et al. 2012).

This should probably enhance the expression of accessory pigments biosynthesis, including phycobiliproteins (PBP), of high economic value in the market. These pigments are used in food or cosmetics as a colorant. Furthermore, several bioactivities such as antioxidant, anticancer, neuroprotective, anti-inflammatory, hepatoprotective and hypocholesterolemic effects have been reported to be displayed by phycobiliproteins and other accessory pigments (Pagels et al. 2019; Sonani 2016), which in recent years attracted increasing interest. Cyanobacteria have the ability to

regulate the PBP production in response to nutrient availability, light intensity, and salinity (Kannaujiya et al. 2017).

Furthermore, these microorganisms have been reported to produce EPS since xerophiles microorganisms can have a layer of EPS that surrounds the cell in order to retain some water, thus providing a humid environment. Some microorganisms produce EPS, in response to drying, limiting nutrients or varying composition of the culture medium (Singh et al. 2019). These EPS offer a promising potential for application in industry based on their antimicrobial, anticancer, and anti-inflammatory activity (Moscovici 2015).

Thereby, the isolation, identification and study of the biotechnological potential of extremophilic microorganisms provide a new source of added-value compounds. However, the optimization of parameters related to growth and target-compounds accumulation is necessary in order to improve the profitability of the product.

Chapter I

Thesis outline and aims

The main aim of this Thesis is to enhance biomass productivity and evaluate the biotechnological potential of two extremophilic microorganisms.

First, we have studied a hypersaline environment archaeaon, *Halopherax mediterranei*, previously studied by other authors for its ability to produce polyhydroxybutirate (PHB), EPS and bacterioruberin, as described in the Introduction section.

In this Thesis, we analyzed the effect of different parameters such as temperature, salinity and pH on the growth of *H. mediterranei* and we also analyzed the effect of these parameters on the accumulation of bacterioruberin. We analyzed the effect of these parameters by using Response surface methodology (Chapter II), which allows studying the effect of the interaction of parameters on the response variables. This fact is of fundamental importance to address reduced production costs on a large scale and achieve profitable production. In addition to the aforementioned study, the effect of nutrients on the growth and production of bacterioruberin was also researched for the first time in *H. mediterranei* and described in Chapter III.

In Chapter IV of this Thesis, a new cyanobacterium from an arid environment was isolated and identified. In addition, the effect of different parameters on the growth of the cyanobacterium was analyzed in order to improve growth and productivity. Furthermore, its biotechnological potential was analyzed.

Phycobiliproteins from cyanobacteria are of great interest from a commercial point of view due to its antioxidant, anticancer and anti-inflammatory capacity, as described in the Introduction section. In Chapter IV the study of the production of this pigment by the isolated cyanobacterium (Chapter III) was carried out, paying special attention to the effect of light intensity.

Finally, it is well known that the cyanobacteria from extreme arid environment can produce valuable EPS as described in the Introduction section. For this reason, in Chapter VI the production of EPS from the cyanobacterium was analyzed and, in addition, the use of commercial fertilizers (NPKs) for its potential production at large scale was assessed in order to reduce production costs.

Chapter VII is a general discussion of the Thesis, aimed at highlighting the main achievements and challenges in the light of related research publishied. The potential of the studied extremophilic microorganisms for production of target and products is also discussed.

CHAPTER II

OPTIMIZATION OF ENVIRONMENTAL CONDITIONS FOR GROWTH AND CAROTENOID PRODUCTION BY *HALOFERAX MEDITERRANEI* USING RESPONSE SURFACE METHODOLOGY

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Abstract

Haloferax mediterranei produces C_{50} carotenoids that have strong antioxidant properties. The response surface methodology (RSM) tool helps to make accurate analysis of the most suitable conditions to maximize C_{50} carotenoids production by haloarchaea. The effects of temperature (15–50 °C), pH (4−10), and salinity (5–28% NaCl (w/v) on the growth and carotenoid content of *H. mediterranei* were analyzed using the RSM approach. Growth was determined by measuring the turbidity at 600 nm (an O.D. of 1.0 had a dry weight content of 1.60 $g \cdot L^{-1}$). In order to determine the carotenoid content, harvested cells were lysed by freeze/thawing, then re-suspended in acetone and the total carotenoid content determined by measuring in the obtained supernatant the absorbance at 494 nm [ε (1%) = 2.540]. The results indicated the theoretical optimal conditions of 36.51 or 36.81 °C, pH of 8.20 or 8.96, and 15.01% or 12.03% (w/v) salinity for the growth of haloarchaea (OD_{600} = 12.5 ± 0.64) and production of total carotenoids (3.34 ± 0.29 $mg \cdot L^{-1}$), respectively. These conditions were validated experimentally for growth (OD_{600} = 13.72 ± 0.98) and carotenoid production (3.74 ± 0.20 $mg \cdot L^{-1}$). Our findings suggest that the RSM approach is highly useful for determining optimal conditions for large-scale production of bacterioruberin by haloarchaea.

Keywords: Bacterioruberin; *Haloferax mediterranei*; Response surface methodology (RSM); Central composite design (CCD).

Chapter II

Introduction

Carotenoids (carotenes and xanthophylls) are pigments synthesized only by bacteria, algae, fungi and plants. They comprise a large family of over 700 naturally-occurring pigments characteristically present in leaves, flowers, and fruits, where they play a diversity of roles. In plants and algae, light energy is the driving force that supports the chlorophyll-dependent photosynthetic electron flow inside the chloroplasts. Carotenoids are secondary pigments which cooperate with chlorophylls in the light energy harvesting function, and also dissipate excess light energy. Owing to their antioxidant activity, carotenoids protect the photosynthetic machinery against photoinhibition caused by free oxygen radicals (Vílchez et al. 2011).

Humans cannot synthesize carotenoids, but can chemically modify them once taken from the diet. Carotenoids play an important role in human health by acting as provitamin A, which is required for vision and epithelial health. In addition, lutein is present in the eye macula where protects against degenerative oxidation (macula disease). These effects make carotenoids highly valuable for food, pharmaceutic and cosmetic industries. Although some commercial carotenoids are produced by chemical synthesis, microorganisms can also be important alternative sources of carotenoids and their active isomers. β-carotene, astaxanthin, lutein, and canthaxanthin are C_{40} carotenoids, which are highly valuable for biotechnological purposes (Torregrosa-Crespo et al. 2018; Jaswir 2012).

Halophilic archaea include microorganisms that grow optimally in culture media with high salt concentrations up to 4 M. The family *Haloferaceae* comprises non-photosynthetic and largely aerobic heterotrophs which produce carotenoids as components of their cytoplasmic membranes, especially under conditions of low salinity in the medium (Oren and Hallsworth 2014). Apart from carotenoids, haloarchaea also produce high added-value products of biotechnological interest, such as enzymes capable of being active at high temperature and high ionic strength, polysaccharides, polyalkanoates and polyhydroxybutyrate (Rodrigo-Baños et al. 2015). In addition, *Haloferax mediterranei* excretes halocins capable of killing other archaea. Halocin H4 is a protein of mass 34.9 kDa that targets the plasma

membrane of microorganisms, altering cell permeability and causing ionic imbalance (Meseguer and Rodriguez-Valera 1986).

Usually, the C_{50} carotenoid bacterioruberin and its derivatives monoanhydrobacterioruberin and bisanhydrobacterioruberin are the major components in the carotenoids fraction produced by halophilic archaea, and they can be found as *trans* and *cis* isomers (Calegari-Santos et al. 2016). C_{50} carotenoids improve the fluidity of the cell membrane (Chen et al. 2015) and, due to their strong antioxidant properties, protect the cells from the harmful effects of radiation energy as well as from the osmotic stress produced by low salinity in the medium (Yatsunami et al. 2014; Mandelli et al. 2012). Several halophilic bacteria also produce other carotenoids such as β-carotene, lycopene and canthaxanthin (Oren and Hallsworth 2014; Oren and Rodríguez-Valera 2001). C_{50} carotenoids produced by haloarchaea possess higher antioxidant capacity than C_{40} carotenoids produced by most photosynthetic organisms, due to the higher number of conjugated double bonds pairs. C_{50} carotenoids are therefore interesting in food applications and for the pharmaceutical industry. The relative proportion of bacterioruberin content in cells depends on the haloarchaeal strain and the culture conditions used, particularly temperature, pH and salinity. Other factors such as the addition of selected organic compounds to the culture medium also influence the carotenoid production of halophilic archaea (Oren et al. 2018). Culture conditions should be set beforehand to maximize biomass yield and carotenoid production, thereby improving yield and reducing costs (Rodrigo-Baños et al. 2015).

Studies on the biotechnological use of halophilic archaea are scarce, despite the widespread interest in C_{50} carotenoids. In this respect, *H. mediterranei* can be a good candidate of biotechnological value due to its ability to grow in a wide range of temperature, pH and salinity. Generally, the studies to find the optimal conditions for both growth and carotenoid production, particularly at large-scale, is experimentally complex (Calegari-Santos et al. 2016; Fang et al. 2010). Thus, statistical experimental methods such as central composite design (CCD) and response surface methodology (RSM) have been developed which can be used in microbial processes to determine conditions for optimal productivity (Hamidi 2014). In this Thesis we demonstrate that RSM is useful for optimizing growth conditions and

carotenoid production by *H. mediterranei* at lab scale. This approach should be valuable for carotenoid production at the industrial scale.

Materials and Methods

Microorganism

The highly halophilic archaeon *Haloferax mediterranei*, strain R4 (ATCC 33500T), used in this study, was provided by Dr. Rosa María Martínez from the Department of Agrochemistry and Biochemistry, University of Alicante, Spain. This archaeon (Figure 2.1) was first isolated and reported by Rodriguez-Valera et al. (1980).

Figure 2.1. Culture of *Haloferax mediterranei* grown on solid highly saline medium.

Growth conditions and biomass quantification

The haloarchaeon was grown in a basal culture medium as formulated by Fang et al. (2010) and containing (per liter): Glucose, 10 g; NaCl, 156 g; $MgCl_2 \cdot 6H_2O$, 13 g; $MgSO_4 \cdot 7H_2O$, 20 g; $CaCl_2 \cdot 6H_2O$, 1 g; KCl, 4 g; $NaHCO_3$, 0.2 g; NaBr, 0.5 g; yeast extract, 5 g. The pH was adjusted to 7.0 by addition of diluted KOH or HCl. The mother culture was prepared in 100 mL of liquid medium contained in a 250 mL flask and incubated at 37 °C and 100 rpm in an orbital shaker until the exponential phase of growth was achieved (standard conditions). This culture was used as inoculum source at 10% (v/v), in all the experiments.

The growth was determined by measuring the turbidity of the culture at 600 nm using a UV-Vis spectrophotometer (Evolution 201, Thermo Fisher Scientific, USA). The dry weight was determined using 1 mL sample of the corresponding culture, which was filtered through a pre-weighed membrane

(ϕ = 0.2 μm) and the retained cells were washed on the filter using 5 mL of 1% (w/v) NaCl solution. The membrane was then dried at 80 °C until a constant weight was reached. A control sample, 1 mL of basal culture medium, was run in parallel and dry weight obtained was later deducted from that of the culture sample. Culture with an O.D. of 1.0 at 600 nm had a dry weight of 1.60 $g \cdot L^{-1}$.

Extraction and analysis of pigments

For extraction of carotenoids, culture samples (10 mL) were centrifuged at 1600 × *g* for 45 min, and the harvested cells were lysed by freeze/thawing, and finally, the biological material was suspended in 1 mL of pure acetone and kept overnight at 4 °C. The suspension was then centrifuged at 3000 × *g* for 5 min, and the total carotenoid content of the supernatant was determined by measuring the absorbance at 494 nm and using an extinction coefficient, ε (1%), of 2540, according to the following expression:

$$mg \cdot L^{-1} = (OD_{494}/2540) \times 10^4$$

Response surface methodology experimental design

The one-factor-at-a-time approach to analyse a problem based on three or more variables overlooks the interactions between different parameters (Herney-Ramírez et al. 2008). To address these issues, RSM was used to identify the optimal value to be applied in order to determine the main effect as well as any significant interactions between factors that may exert important effects on response (Raheem et al. 2015; Hamidi, 2014; Song et al. 2012). A central and axial points design (CCD) approach was used to optimize the culture conditions for both cell growth (O.D. at 600 nm) and total carotenoid content ($mg \cdot L^{-1}$) of *H. mediterranei*. In this study, temperature, salinity and pH were considered for the CCD analysis and were investigated at five different levels within the following ranges: temperature (15–50 °C), pH (4−10) and NaCl concentration (5–28%, w/v) in order to deduce the optimum values of growth and carotenoid content. The code and actual values of the variables are presented in Table 2.1.

Table 2.1. The coded and actual values of experimental variables used in the central composite design (CCD).

Independent variables	Symbols	Levels				
		−1.68*	**−1**	**0**	**1**	**1.68***
Temperature (°C)	X1	17.8	23.8	32.5	41.3	47.2
pH	X2	4.5	5.5	7.0	8.5	9.5
NaCl (%, w/v)	X3	5.15	9.75	16.50	23.25	27.85

* alpha values used for axial points in this study.

A 2^3 full-factorial experimental design with six-axial points and six central points was chosen (Ferreira et al. 2007). The relationship between the response variables and the independent ones was fitted by a predictive quadratic polynomial equation. The quality of fit for the second-order model equation was expressed by the coefficient of determination (R^2) and its statistical significance was determined using the ρ-value. To provide an adequate degree of freedom (df = 5) for estimation of pure error, calculations at the central point were repeated six times. The regression equation used is described as follows:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i,j=1}^{3} \beta_{ij} x_i x_j \quad (1)$$

where y represents the predicted response variables (growth or total carotenoid); β_0 is a constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient of the model, respectively, and x_i and x_j (i= 1, 3; j = 1, 3; i≠j) represent the non-coded independent variables (temperature, pH, and salinity).

Statistical analysis

The data analysis for model construction was performed using Minitab 17.1.0.0 software (Minitab Inc., State College, USA), based on the response surface methodology. The model was statistically tested using analysis of variance (ANOVA) to test the significance and adequacy of the model.

Regression analysis was used to obtain the coefficients of a second polynomial order. Data are presented as the average of three independent experiments. Statistical significance was determined by ρ-value at $\rho<0.05$. The three-dimensional surface plot and contour plot performed by the regression model were drawn using the Statistica software package (version 11.0, StatSoft, USA) to highlight the effects on the independent variables and corresponding effects on the response variables.

Results and Discussion

Effect of the air volume fraction inside the culture flasks on the growth rate of *H. mediterranei*

Oxygen is required for an efficient and productive growth of *H. mediterranei* (El-Sayed et al. 2002). In order to define the most productive conditions for the haloarchaeon at lab scale, the effect of the air volume fraction with respect to the liquid phase in the flask was studied. All other cultivation conditions were kept constant. This study was not aimed at determining the optimal air volume fraction for production of *H. mediterranei* as it depends on the specific characteristics of the cultivation system. Three different air volume fractions inside the culture flasks were tested for their effect on the growth rate of *H. mediterranei*: 20%, 40% or 60% of the total flask volume. In all cases, the selected agitation rate of the orbital shaker was 100 rpm. The optimal growth rate for *H. mediterranei* was observed in the culture with 60% air phase in the flask, thus emphasizing the importance of oxygen for this haloarchaeon (Figure 2.2). Experiments with 80% air phase in the flask did not result in a significant improvement of the archaeal productivity when compared with the 60% culture (data not shown).

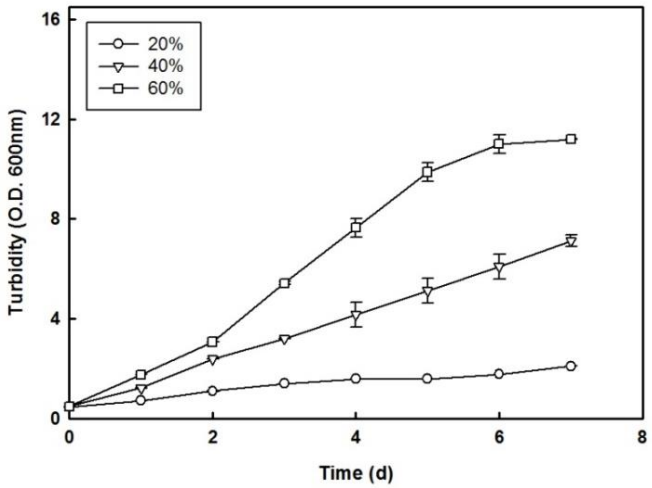

Figure 2.2. Effect of air volume fraction on the growth of *H. mediterranei*. Cells were grown under standard conditions, and using the indicated air phase, with a shaker speed of 100 rpm in all cases. The turbidity of the cultures was determined at 600 nm at the indicated times. More details of experimental conditions in the Materials and Methods section.

According to Figure 2.2, 60% of air volume fraction inside the cultivation flasks is adequate to get optimal growth rate for *H. mediterranei* cultures, which reached the stationary phase of growth after 6 days of cultivation. On the contrary, lower air volume fractions resulted in limited growth within the experiment time period due to the lower oxygen availability which lowered the haloarchaeal metabolism. Particularly, for those cultures with an air volume fraction of 60% the growth was approximately 1.5-fold and 6-fold higher than that of those cultures with air volume fractions of 40% and 20%, respectively. Thus, it unveils the limiting effect of the lower oxygen availability on the growth of *H. mediterranei*. However, in other works the air volume fraction roughly ranges from 10% to 30%, probably because they have not tried higher air volume fractions in the cultures (Hamidi 2014; Fang et al. 2010).

The oxygen level available by haloarchaea might also have an important impact on the production of carotenoids. Thus, the effect of the air volume fraction in the flasks on carotenoid production was analysed. The results are shown in Figure 2.3.

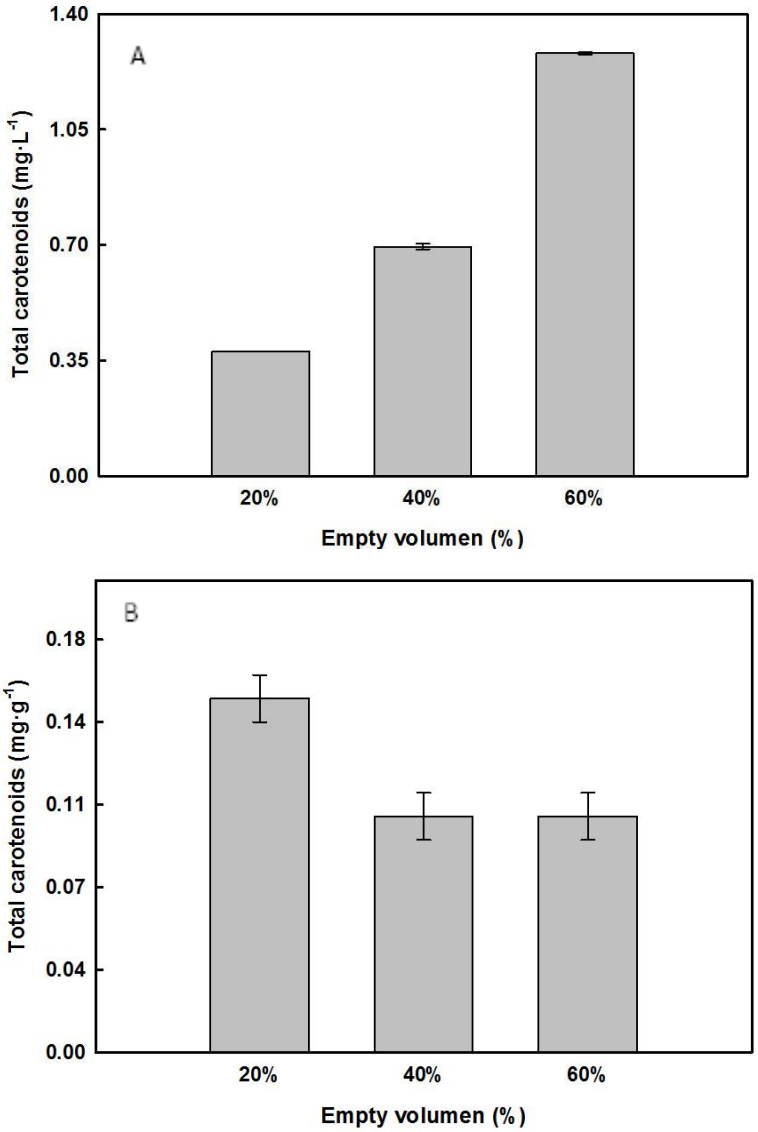

Figure 2.3. Effect of air volume fraction on carotenoid production by *H. mediterranei*. Cells were grown under standard conditions, with the indicated air volume fractions. In all cases, the cultures were shaken at 100 rpm. A, carotenoid concentration of the cultures; B, carotenoid concentration of the biomass. More details of experimental procedures are indicated in the Materials and Methods section.

The volumetric carotenoid accumulation increased with the air volume fraction (Figure 2.3A) due to the increased biomass density (Figure 2.2). However, the accumulation of carotenoids related to dry weight (Figure 2.3B) was higher in cultures with lower oxygen availability (20% air volume fraction). This effect might be coherent with the enhanced biosynthesis of bacterioruberin under limited oxygen availability as this C_{50} carotenoid has been reported to play a role in making the cells more permeable to oxygen under stress conditions (Rodrigo-Baños et al. 2015; Fang et al. 2010). According to the obtained results, an air fraction volume of 60% was selected for further experiments.

Effect of the shaker speed of flasks on the growth rate of *H. mediterranei*

Agitation is also a key factor influencing the productivity of haloarchaea cell cultures. Agitation is necessary for the following events: (i) keeping culture suspensions homogeneous, thus avoiding biomass stratification and nutrient gradients inside the cultures, and (ii) allowing the culture medium to get saturated in oxygen required for the bacterial growth. Excess of agitation might result in mechanical stress of the cultured cells, besides technical cultivation difficulties. Conversely, insufficiently agitated cultures might result in non-homogeneous and oxygen limited cell suspensions. For the aforementioned reasons, agitation was analysed in order to define the most suitable conditions for a productive cultivation of *H. mediterranei* in our experimental design.

The influence of the agitation rate on the growth of *H. mediterranei* was evaluated while all other cultivation conditions were kept constant. For these experiments, an air volume fraction of 60% of the total volume of the cultivation flasks was selected according to the results previously shown in Figure 2.2. The growth was tested under the following conditions regarding culture agitation by an orbital shaker: no agitation (0 rpm), 100 rpm or 150 rpm. Figure 2.4 shows that agitation speed of the orbital shaker greatly impacts in *H. mediterranei* growth. The optimal growth was reached at 150 rpm, though the haloarchaeon can also grow at 100 rpm. No growth was observed in non-agitated cultures (0 rpm), thus indicating that aeration is required for haloarchaeal growth suggesting extremely low oxygen availability below the non-agitated cultures surface which seems to affect most of cultured cells, then limiting or inhibiting growth. As observed in

Figure 2.4, the two agitation speeds tested resulted in growth curves that reached stationary phase within the experiment time (7 days), the 150 rpm culture reached the stationary phase a bit more rapidly –approximately a day earlier- than the 100 rpm culture. The 150 rpm culture reached a maximum O.D. at 600 nm of approximately 16, which is 45% higher than the maximum reached by the 100 rpm culture. These findings are consistent with those reported for *H. alexandrinus*, which showed optimal growth in 100 mL of culture in 500 mL flasks (80% air volume fraction) (Asker and Ohta 1999), and *Halorubrum* sp. SHI, which required agitation at 550 rpm for optimal growth in 50 mL of culture in 250 mL flasks (80% air volume fraction) (de la Vega et al. 2016).

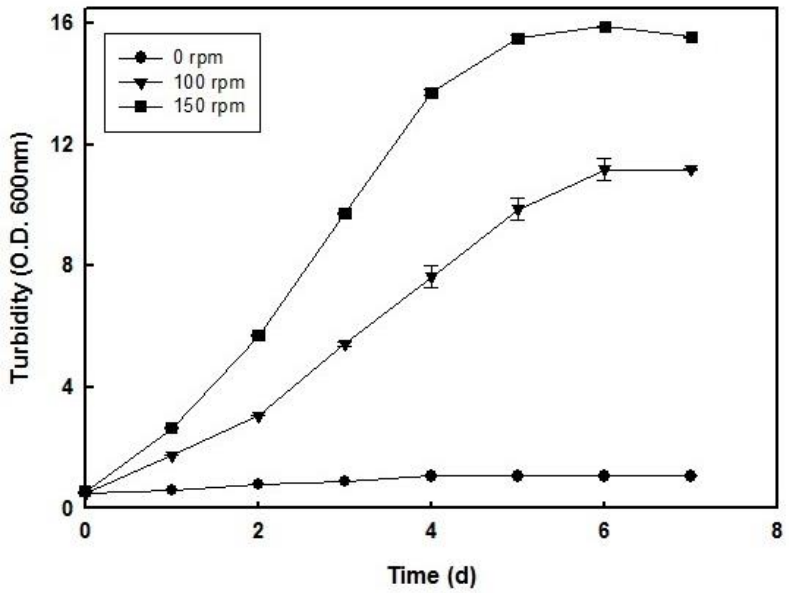

Figure 2.4. Effect of the shaking speed on the growth of *H. mediterranei* cultures. Cells were grown under standard conditions and using the indicated shaker speed, with air fraction of 60% in all cases. When indicated, the turbidity of the culture was determined at 600 nm. More details in the Materials and Methods section.

According to the obtained results, an agitation speed of 150 rpm and an air volume fraction of 60% in the culture flasks were selected for further experiments in this Thesis. Under these conditions, *H. mediterranei* showed a

generation time of 33.6 h and maximum productivity of 22.16 g dw·L^{-1} . This achieved productivity is significantly higher than that obtained previously, not only for *H. mediterranei* (Fang et al. 2010), but also for other aerobic haloarchaea (Hamidi 2014). Based on the obtained results, it can be concluded that the agitation speed has to be considered in order to select adequate conditions for the production of haloarchaea. In addition to the effect of agitation speed on *H. mediterranei* growth the impact of agitation speed on carotenoid accumulation was also assessed, and no significant effect was observed (data not shown).

Beyond oxygen availability from the air volume fraction inside the flask, oxygen is actually available to the haloarchaea cells according to its solubility in the culture medium. Salinity, temperature and pH affect oxygen solubility and therefore the growth of haloarchaea (D'Souza et al. 1997). According to Schneegurt (2012), an increase of 10% (w/v) in salinity reduces oxygen solubility by 50% approximately, which might have an impact on oxygen availability and thus cell growth. Concerning the effect of temperature on oxygen availability, the higher the temperature the lower the oxygen solubility (Truesdale et al. 1955). Consequently, combined conditions of salinity, temperature, and pH might have a complex influence on the growth rate of *H. mediterranei* cultures depending on the oxygen solubility in the culture medium under the established conditions.

Use of RSM to optimize culture conditions for growth and carotenoid production by *H. mediterranei*

Together with oxygen availability, temperature, pH and salinity are the environmental parameters that most influence the growth of haloarchaeal cultures (Hamidi 2014). Central composite design (CCD) was used to define the experimental growth conditions, which should led to obtain the predictive model for optimal growth and carotenoids production by *H. mediterranei*. As shown in Figure 2.2, the culture with 60% air volume fraction and an agitation speed of 150 rpm (Figure 2.4) reached the mid logarithmic phase on day 4, which seems adequate for the growth optimization experiments designed by the CCD tool. The central composite design and the yields reported by the model are shown in Table 2.2. Accordingly, 20 cultivation experiments were run and the haloarchaeal growth and carotenoid production were followed. The results led to obtain the response models in three dimensional surfaces for

the response variable growth and total carotenoid of *H. mediterranei* (Figure 2.5). According to the model, optimal growth of *H. mediterranei* should be obtained at 36.51 °C, pH of 8.20, and 15.01% (w/v) NaCl.

Table 2.2. Central composite design (CCD) matrix and the responses of growth and total carotenoid content at different temperature, pH, and salinity levels. Std Order: Standard Order. *Central point values contributing to the degree of freedom for pure error calculation.

	Independent variables coded levels						Responses	
Std Order	**Temperature (°C)**		**pH**		**Salinity NaCl (%, w/v)**		**Turbidity (O.D.600 nm)**	**Total carotenoids ($mg \cdot L^{-1}$)**
1	−1	23.8	−1	5.5	−1	9.8	2.53	0.28
2	1	23.8	−1	8.5	−1	9.8	3.09	1.61
3	−1	41.3	1	5.5	−1	9.8	7.88	0.80
4	1	41.3	1	8.5	−1	9.8	11.79	3.12
5	−1	23.8	−1	5.5	1	23.3	0.88	1.34
6	1	23.8	−1	8.5	1	23.3	5.31	1.05
7	−1	41.3	1	5.5	1	23.3	2.04	0.24
8	1	41.3	1	8.5	1	23.3	7.25	0.78
9	−1.68	32.5	0	4.5	0	16.5	1.01	0.19
10	1.68	32.5	0	9.5	0	16.5	11.51	2.85
11	0	17.8	1.68	7.0	0	16.5	0.29	0.13
12	0	47.2	1.68	7.0	0	16.5	5.14	0.85
13	0	32.5	0	7.0	−1.68	5.1	5.22	0.66
14	0	32.5	0	7.0	1.68	27.9	3.82	0.37
15*	0	32.5	0	7.0	0	16.5	10.93	3.34
16*	0	32.5	0	7.0	0	16.5	11.25	3.13
17*	0	32.5	0	7.0	0	16.5	10.78	2.40
18*	0	32.5	0	7.0	0	16.5	10.62	2.71
19*	0	32.5	0	7.0	0	16.5	11.21	2.50
20*	0	32.5	0	7.0	0	16.5	12.34	3.07

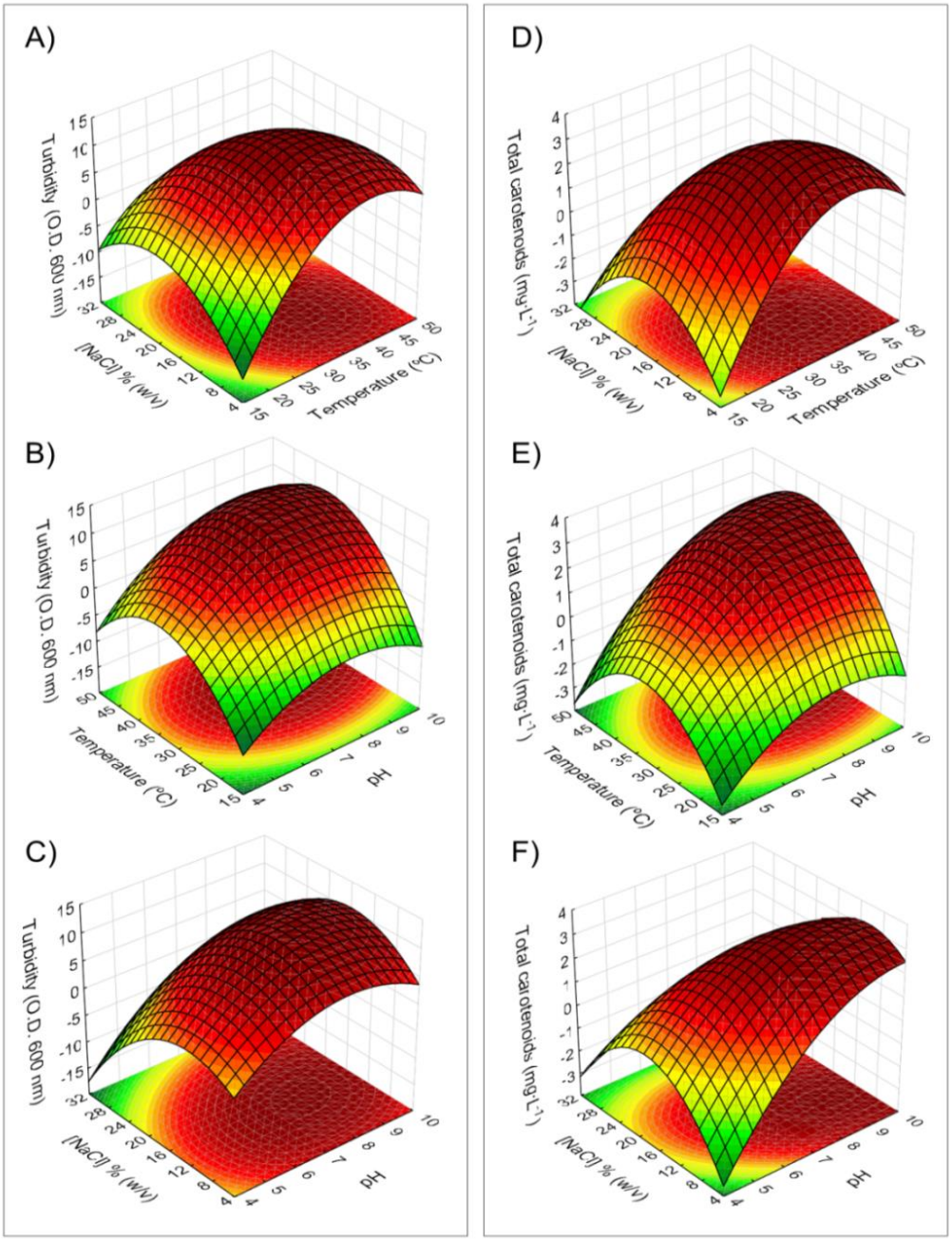

Figure 2.5. The 3-D-surface and contour response plots generated from a quadratic model representing the combined effects of temperature, pH and salinity on the growth rate (**A**–**C**) and carotenoids content (**D**–**F**) by liquid cultures of *H. mediterranei*. The interactions between salinity and temperature (A) and (D); pH and temperature (B) and (E), and pH and salinity (C) and (F) were analyzed. Other details of experimental conditions are stated in the Materials and Methods section.

The following equation could be used to predict the O.D. at 600 nm under different conditions:

$$O.D.600\,nm = -85.1 + 8.74 \cdot X_2 + 2.681 \cdot X_1 + 1.729 \cdot X_3 - 0.681 \cdot X_2^2 - 0.03635 \cdot X_1^2 - 0.04710\,X_3^2 + 0.0394\,X_2 \cdot X_1 + 0.0639\,X_2 \cdot X_3 - 0.02318\,X_1 \cdot X_3 \quad (2)$$

where X_1, X_2, X_3 denote temperature, pH, and salinity, respectively (see Table 2.2).

On the other hand, the maximum total carotenoid content in cultures of *H. mediterranei* cells should be observed at 36.81 °C, pH of 8.96, and 12.03% of NaCl. The carotenoid content at any point during the culture under different conditions could be predicted according to the following equation:

$$Carotenoids(mg \cdot L^{-1}) = -27.78 + 2.913 \cdot X_2 + 0.647 \cdot X_1 + 1.027 \cdot X_3 - 0.1692 \cdot X_2^2 - 0.00974 \cdot X_1^2 - 0.01612 \cdot X_3^2 + 0.0171\,X_2 \cdot X_1 - 0.0419\,X_2 - X_3 - 0.00718\,X_1 - X_3 \quad (3)$$

Using the one-factor-at-a-time approach, optimal conditions to produce carotenoids (2.06 mg·g^{-1} dw of cells) by *H. alexandrinus* were 37 °C, pH of 7.2, and 25% NaCl (Asker and Ohta 1999), which are considerably different to those obtained in this study, thus reiterating the importance of the haloarchaea species used and the interactions between factors.

Validation of the optimal conditions for growth and total carotenoid production by *H. mediterranei*

The accuracy of the model was verified by analyzing the predicted and observed experimental results. Three experiments were carried out to determine the reliability of optimal conditions predicted by the models of Equations (1) and (2), using the data obtained for biomass and total carotenoid content, respectively. Figure 2.6 shows that high values of R^2 (93.1%) and adjusted R^2 (92.7%) highlight the coherence between predicted and observed experimental values. Hence, an acceptable relationship between independent variables (temperature, pH and salinity) and response variables (growth and total carotenoids) was proven. The highest biomass production (21.95 ± 1.57 g dw ·L^{-1}) and total carotenoid content (3.74 ± 0.20 mg·L^{-1}) were very close to the values estimated by RSM at the optimal conditions (20.18 ± 1.02 g dw.L^{-1}, and 3.34 ± 0.29 mg·L^{-1}, respectively) indicating that RSM is effective in

determining culture conditions where several variables could influence the final result. It can also predict results for other potential culture conditions of the haloarchaea, as reported by Hamidi et al. (2014), in similar studies on *Halorubrum* sp. TZB126.

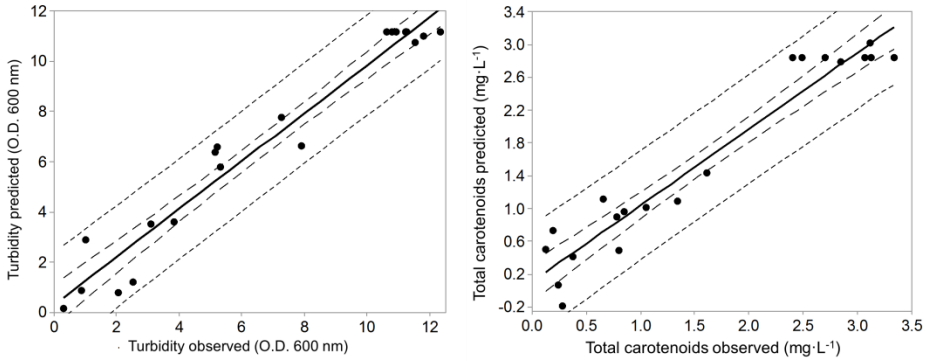

Figure 2.6. Theoretical values of response variables predicted from the respective models and observed values of the experimental design with a *p*-value<0.05 for the growth rate and total intracellular carotenoids by *H. mediterranei*. The growth and carotenoids content are measured as described in Materials and Methods. CI = reliable interval and PI = predicted interval.

The data demonstrates for the first time in *H. mediterranei* that the RSM approach might be used to predict optimal conditions for large scale carotenoid production. As shown above, the model predicted the growth pattern accurately. In addition, as shown in Figure 2.5, the haloarchaea growth is negatively affected by extremes of the analyzed parameters. Such negative impact on growth was also accurately predicted by the model. It is well known that salinity affects cell viability of most of microorganisms growing in liquid cultures, particularly those non-tolerant ones. High salinity produces oxidative stress and very low salinity may lead to cell disruption of halophiles as a major consequence of the low osmotic pressure outside the cell (D'Souza et al. 1997). This type of osmotic effect was extensively described for halotolerant microorganisms including halobacteria (Müller et al. 2005) and microalgae (Chen and Jiang 2009). In laboratory experiments we observed massive cell lysis in cultures of *H. mediterranei* incubated under very low NaCl concentrations (lower than 5% w/v, data not shown).

Besides the optimal conditions above referred, the predictive model also allows to propose ranges of conditions under which haloarchaea growth still remains at high rates, for instance above 90% of the maximum values. This can be of great importance for cost reduction, at pilot scale. For instance, temperature control has a great influence on the cost of the final product (Béchet et al. 2014). Thus, decreasing the operational temperature of the growth process would definitely result in energy cost savings, if the growth rate is kept over 90% or higher. From the formula 2, we determined that temperature for growth of *H. mediterranei* can range between 36.65 to 42.40 °C while the growth parameter remained above 90% of its maximum value. Therefore, the model showed to be a flexible tool for making decisions on setting operational process parameters.

Carotenoids production by *H. mediterranei*

The maximum carotenoid yield in our experiments was 3.74 $mg \cdot L^{-1}$ (equivalent to 23.51 $mg \cdot g^{-1}$ dw), which is different from that reported in other haloarchaea. This yield mainly depended on the haloarchaea strain and on the culture conditions used. *H. alexandrinus* accumulates 2.6 $mg \cdot g^{-1}$ dw (Asker and Ohta 1999); *Halobacterium salinarum*, 45 $\mu g \cdot g^{-1}$ dw and *Halococcus morrhuae*, 89 $\mu g \cdot g^{-1}$ dw (Mandelli et al. 2012), *Halobacterium halobium*, 7.63 $mg \cdot L^{-1}$ (Abbes et al. 2013), *Halorubrum* sp., 10.78 $mg \cdot L^{-1}$ (Hamidi 2014), *H. mediterranei*, 125 $mg \cdot L^{-1}$ (Chen et al. 2015), *Haloarcula japonica*, 335 $\mu g \cdot g$ dw^{-1} (Yatsunami et al. 2014); *Halorubrum* sp. SH1, 25 $mg \cdot L^{-1}$ (de la Vega et al. 2016), and *Haloterrigena turkmenica*, 32 $\mu g \cdot g^{-1}$ dw (Squillaci et al. 2017). However, in most cases, no information had been published regarding biomass production and/or cell viability under the conditions used for carotenoid production of the aforementioned microorganisms, which makes it difficult to select one strain of haloarchaea for large-scale production of carotenoids.

The chosen strategy for carotenoid production also affects the final costs. We have two options: (i) one-step production under optimal growth conditions, in which the carotenoid production is directly linked to the biomass production of the cultures, or (ii) a two-step system, the first for optimal biomass production, and the second for optimal carotenoids accumulation in the biomass. In our study, low salt content in the culture medium is required for optimal production of carotenoids; however,

haloarchaea requires high salt concentration for optimal growth. Chen et al. (2015) showed that *H. mediterranei* growing at 40 S/m conductivity (an indirect measurement of salt concentration) in saline medium accumulated 125 $mg \cdot L^{-1}$ of total carotenoids; however, if the conductivity of the medium was reduced to 25 S/m, the pigments increased up to a maximum value of 555.6 $mg \cdot L^{-1}$. From Equations (2) and (3), we estimated that *H. mediterranei* can produce 3.34 $mg \cdot L^{-1}$ of carotenoids, while the theoretical value for the growth of haloarchaea under such conditions is 18.51 g $dw \cdot L^{-1}$, which corresponds to a loss of about 7.5% of biomass productivity. Thus, the option of one-step process is adequate for the high-scale bacterioruberin production by *H. mediterranei*.

Calegari-Santos et al. (2016) reviewed the effect of different stress conditions on carotenoids production in halophilic archaea. In addition to the variables considered in this work, the C-source and metals presence were also relevant. However, the effect of N-starvation and other nutritional stress factors remain to be examined.

CHAPTER III

EFFECT OF NUTRIENTS ON GROWTH AND CAROTENOIDS PRODUCTION BY *HALOFERAX MEDITERRANEI*

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Abstract

The C_{50} carotenoids produced by *Haloarchaea* are of biotechnological value. The aim of this Chapter is to analyze the carotenoid accumulation dynamics in *Haloferax mediterranei*, as well as the carotenoid profile, under the optimal environmental conditions obtained in Chapter II for carotenoids accumulation. The effect of carbon and/or nitrogen starvation and the amount of magnesium sulphate on the growth and carotenoid accumulation were studied. Optimum production of carotenoids was obtained under deficiency of carbon and nitrogen in the culture media, conditions in which the growth of *H. mediterranei* was very poor, thus a two-stage process seems to be a required strategy to get optimal carotenoids yield by *H. mediterrenai*. In the first stage, nutrients replete culture media enabled the haloarchaea to grow and accumulate a considerable amount of cells, and in the second-stage incubating the haloarchaea biomass under carbon and nitrogen deficiency led to carotenoid accumulation. Besides, no correlation was found between the concentration of magnesium sulphate in the culture medium and the growth rate or carotenoid production by *H. mediterranei*. The results show a profile formed by four isomers of the C_{50} carotenoid bacterioruberin (89.13%).

Keywords: Bacterioruberin; *Haloferax mediterranei*; Nutrient starvation; Haloarchaea; Magnesium.

Chapter III

Introduction

Haloferax mediterranei produces bacterioruberin which is the major carotenoid component in the *Halobateriaceae* family, where it has an important role by reinforcing the cellular membrane, improving its fluidity, and acting as a barrier for oxygen and other molecules (Rodrigo-Baños et al. 2015), thus protecting the DNA against damage induced by UV-radiation and reactive oxygen species (ROS) (Shahmohammadi et al. 2008). This carotenoid has a wide biotechnological interest due to its antioxidant potential. It is well known that bacterioruberin contains 13 pairs of conjugated double bonds versus the 9 pairs of the β-carotene what makes it more effective than β-carotene as a radical scavenger (Rodrigo-Baños et al. 2015). Additionally, it has a potential use in humans to repair damaged DNA strands caused by ionizing radiation such as ultraviolet radiation (UVR) and hence might prevent skin cancer (Vatsa et al. 2014).

The commercial interest of carotenoids with high antioxidant activity increased in the last decades, but its shadows come from the high production costs. Searching for optimal conditions of carotenoids production is an important strategy to improve economic conditions. In *H. mediterranei* carotenoids yield is strongly dependent of the culture conditions (Calegari-Santos et al. 2016; D'Souza et al. 1997). Therefore it is highly relevant to analyze the effect of nutritional and environmental factors on carotenoid accumulation by halophilic archaea such as *H. mediterranei* in order to assess bacterioruberin production feasibility.

Montero-Lobato et al. (2018), Fang et al. (2010) and D´Souza et al. (1997) reported the effect of environmental parameters on the production of carotenoids in *H. mediterranei*, being the salt concentration in the culture medium the key factor to reach optimal yield. However, no reports have been published concerning the effect of nutritional parameters on the growth and pigment production in *H. mediterranei*. Nitrogen is supplied to the haloarchaea by the amino acids present in the yeast extract, which is also the most common carbon source in halophiles culture medium along with glucose (Schneegurt 2012). The effect of glucose on carotenoid production in haloarchaea has been investigated by Gochnauer et al. (1972). This study reports that the production of carotenoids is inhibited by a high concentration

of glucose (tested 4%) but the glucose requirement depends on the haloarchaea species. Besides this, the requirement of magnesium for growth depends on the haloarchaea and it has an effect on the production of carotenoids (Calegari-Santos et al. 2016). The demand for magnesium sulphate may be due to its presence in the cell wall of halophilic archaea.

According our previous studies about the effect of temperature, pH and salinity on growth and carotenoid production (Chapter II), in this Chapter, we analyze the effect of nutritional conditions of *H. mediterranei* on the time course of carotenoid production. In addition we established the best strategy to achieve minimal costs and maximum yields of carotenoids production. The growth of the microorganism and the metabolites accumulation may arise at different cultivation conditions which can even be opposed to each other. It would require the production of a given metabolite to be developed in a two-phase process: (i) growth phase and (ii) metabolite accumulation phase. This can be the case for production of bacterioruberin by *H. mediterranei*, and accordingly, in this Thesis, we analyzed the effect of different nutritional factors on both growth and carotenoids accumulation. In the first stage, the haloarchaeon was grown under non-limiting nutritional conditions, and in the second stage, the carotenoids biosynthesis was analyzed under nutrient starvation. The results allow discussing adequate conditions for producing bacterioruberin with *H. mediterranei.*

Chapter III

Materials and Methods

Microorganism and growth conditions

The halophilic archaeon *Haloferax mediterranei* was used in this study. Figure 3.1.shows a standard culture of the haloarchaeon used in this Thesis.

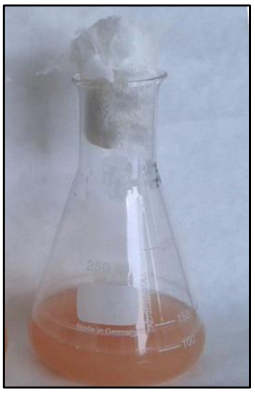

Figure 3.1. Picture of a laboratory culture of *H. mediterranei*.

According to Chapter II, the main environmental parameters affecting growth and carotenoid accumulation in cultures, including temperature, pH and salinity were established as 36.81 ºC, pH 8.96 and 12.02% (w/v) NaCl for biomass production and carotenoid accumulation by *H. mediterranei.* In this Chapter we study the effect of nutritional conditions on growth and carotenoid accumulation processes, using cultures in 250 mL erlenmeyer flasks. The culture broth was inoculated from standard cultures, at the end of the linear phase, when the absorbance at 600 mn was around 0.5. The biomass production was analyzed daily by measuring turbidity at 600 nm in order to determine changes.

Cultivation in the presence or absence of glucose and/or yeast extracts

The experiments carried out to evaluate the effect of the glucose and yeast extract starvation on the growth and carotenoid accumulation by *H. mediterranei* were performed in two stage cultivation process. Two different culture media were used for stages 1 and 2, respectively. In the first cultivation stage, *H. mediterranei* was cultivated in full nutrient standard

culture medium (basal medium, first stage culture medium) which contained 10 $g \cdot L^{-1}$ of glucose and 5 $g \cdot L^{-1}$ of yeast extract. The first stage was run until the turbidity at 600 nm was 6. For the second stage, aimed at triggering carotenoid production, the cells were harvested from the cultures of step 1 by centrifugation, and then were resuspended with an equal volume of fresh medium lacking glucose and/or yeast extract.

Cultivation at different magnesium sulphate concentration

In order to determine the influence of the magnesium sulphate concentration on the growth and carotenoid accumulation of *H. mediterranei*, batch cultures with different concentrations of magnesium sulphate were studied. The magnesium sulphate concentrations were 1, 5, 10, 20, 40, 60 and 80 $g \cdot L^{-1}$, and the cultures were incubated under optimal conditions growth as described in Chapter II.

Extraction and analysis of pigments

We used the procedure described in Chapter II and the ratio of pigments per biomass was calculated by dividing the absorbance at 494 nm by the turbidity of the culture broth ($OD_{494} \cdot OD_{600}^{-1}$).

Figure 3.2. Carotenoid extraction in acetone from *H. mediterranei*.

Analysis of pigments by thin layer chromatography (TLC)

Pigments in acetone extract were analyzed by thin layer chromatography (TLC) as described by Strand et al. (1997). A TLC plate of

silica Merck 5553 was used, with 50% acetone in n-heptane (v/v) as the development liquid. The Rf value of each individual spot after chromatography development was calculated, and then each spot was scraped off, and extracted with acetone. The supernatant was then scanned under 300–600 nm to obtain the UV-VIS absorbance spectrum.

Quantification and analysis of pigments by HPLC

The HPLC analysis of carotenoids in acetone was performed using a Poroshell 120-C18 column of 3 × 50 mm and 2.7 μm (Agilent, USA) on an Agilent 1200 series system equipped with a diode array detector scanning from 400 to 690 nm. To determine the mass spectra of the different compounds, a 6410 Triple Quad LC/MS system (Agilent, USA) was used equipped with an electrospray ionization source (ESI) operating in positive scan mode (m/z range of 300–900), with ± 0.1 u.m.a. precision, and controlled by MassHunter Workstation Software (Agilent, B.05.00, USA). The following specific working conditions were used: capillary voltage 4000 V, gas flow rate 10 L min^{-1}, gas temperature 300°C, and nebulizer pressure 35 psi (de la Vega et al. 2016).

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

Results and Discussion

Carotenoid production under optimal conditions by *H. mediterranei*

This section of the work aimed at investigating the carotenoid accumulation dynamics in *H. mediterranei*. To do this, cells were incubated in standard medium (first stage), and cells from these cultures in the 4th day of growth, were harvested, resuspended in fresh medium, and cultured under conditions which were previously identified in Chapter II as optimal for carotenoids production (at 36.81 ºC, pH 8.96 and 12.02% (w/v) NaCl).

Figure 3.3A shows the typical growth curve of *H. mediterranei* under the referred condition in standard medium, with the growth increasing during the first 3 days of cultivation, the exponential phase observed later during days 3 and 5, with a generation time of cell growth around 32 h, and the stationary phase reached after 5 days of growth. The maximum turbidity at 600 nm under these conditions was 14.5.

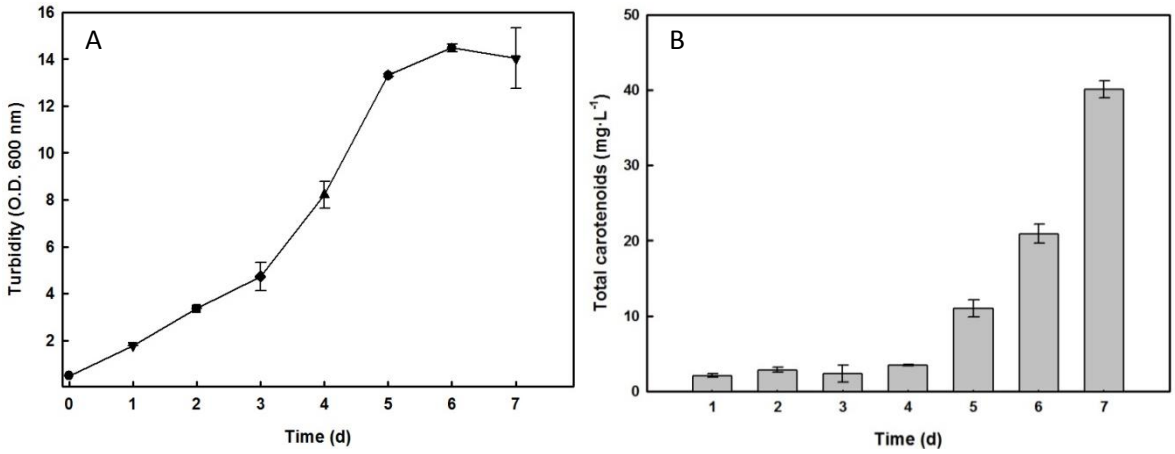

Figure 3.3. Time course of *H. mediterranei* growth (A) and carotenoids production (B). Cultures of *H. mediterranei* were grown under optimal condition established for carotenoid production in Chapter II. At the indicated times, turbidity and carotenoid production were determined. More details in the Materials and Methods section.

Figure 3.3B shows the time course evolution of intracellular carotenoids content of the *H. mediterranei* culture, under optimal conditions. Cells increased the accumulated carotenoids after 4 days and after 5 days of

growth, the total carotenoid content was up to 3-fold higher than the average content of 1-4 days cultures. On day 7, the carotenoid production per volume of culture accounted for about 10 to 15-fold higher than that average.

These results should be carefully considered since a high volumetric carotenoid accumulation (total carotenoid content per unit volume of culture) does not necessarily suppose an intracellular increase of pigment biosynthesis rate. For clarifying this aspect, Figure 3.4 represents the carotenoids content in the haloarchaeal biomass. The efficiency of pigment production was calculated by dividing the pigments absorbance by the turbidity of culture, expressed as A494 nm divided by A600 nm ratio, as described in Materials and Methods section.

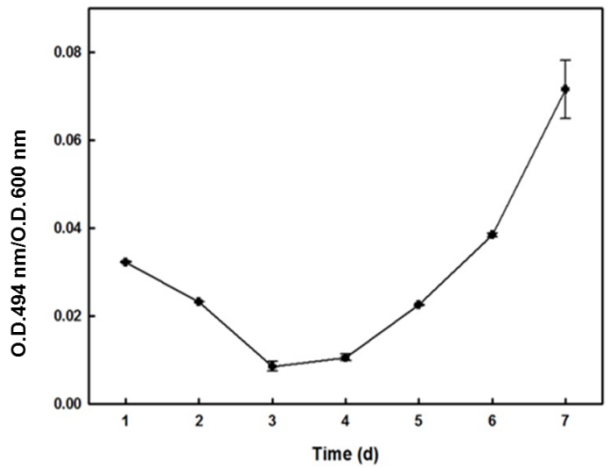

Figure 3.4. Carotenoid per biomass ratio in *H. mediterranei* cultures growing under optimal conditions for carotenoid production. The ratio was calculated at different times during growth, by dividing the O.D. at 494 nm versus O.D. at 600 nm.

The time course variation of this ratio indicates that carotenoids intracellular accumulation rate increases with time, except during the lag phase (first 3 days), when cells adapted to the new culture conditions; because the biomass used as inoculum for the experiment had been previously grown under optimal conditions for biomass production which included a higher concentration of NaCl (15% v/w). According to Figure 3.4, the highest carotenoids biosynthesis rate was obtained in *H. mediterranei* at the stationary

phase, when biomass production in the culture has ceased, probably due to nutrient limitation. During the first 3 days of cultivation, in this way, the inoculated cells had to adapt to the new culture conditions previously determined in Chapter II as most suitable to improve carotenoid accumulation in growing cells. Further, the carotenoid content per cell biomass decreased during the exponential phase, the maximum values being obtained at the stationary phase. The latter suggests that nutrient limitation might be triggering the rapid biosynthesis and intracellular accumulation of carotenoids.

In order to take advantage from the highly active metabolism of exponentially growing cultures of *H. mediterranei*, key nutrients can be starved at that phase by eliminating any of them from the culture medium. Indeed, nutrient starvation might become a strategy for inducing carotenoid production in *H. mediterranei* cultures, and it was also studied in this Thesis.

Carotenoid profile obtained from *H. mediterranei*

The carotenoid profile obtained from *H. mediterranei* under optimal conditions for carotenoids production was analyzed using HPLC and the results are shown in Figure 3.5. The chromatogram (Figure 3.5A) shows 10 well defined peaks where bacterioruberin (89.13%) was the major compound produced. Figure 3.5B shows a similar 3-finger type absorption spectrum for these carotenoid fractions with typical bacterioruberin absorption maxima at 468, 494, and 530 nm. In addition, peaks 1–4 had the same molecular weight (Table 3.1), thereby indicating that they were isomers of the main carotenoid. They are probably 13-cis-bacterioruberin, and 9-cis-bacterioruberin, respectively (Mandelli et al. 2012). The fourth isomer remains to be identified.

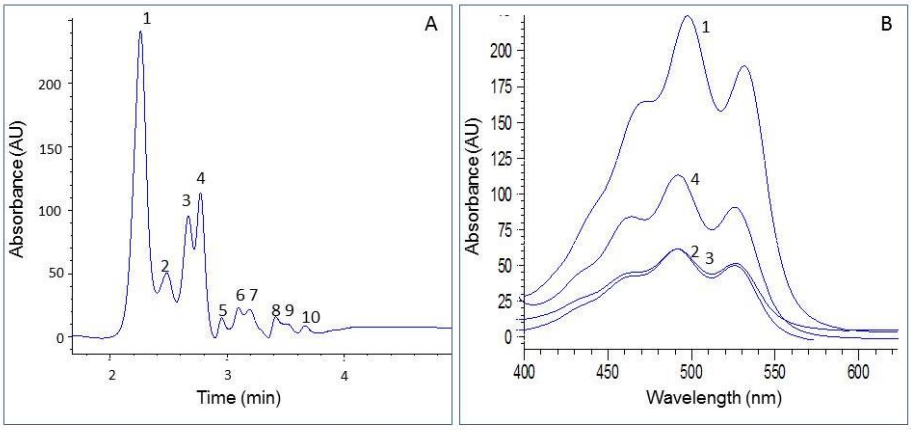

Figure 3.5. HPLC analysis of the carotenoids present in *H. Mediterranei* (A) and the absorption spectrum of the obtained four main peaks (B). Carotenoids were extracted from culture samples after 4 days of growth under optimal conditions for carotenoids production. More details are described in the Materials and Methods section.

Figure 3.5A also reveals other minor peaks corresponding to chemically modified bacterioruberin-derived compounds such as monoanhydrobacterioruberin and bisanhydrobacterioruberin (Fang et al. 2010), with different molecular weights than bacterioruberin (Table 3.1). The other observed peak in the chromatogram corresponds to unknown carotenoids (Calo et al. 1995). Other studies on *H. mediterranei* reported 70% and 52.4% bacterioruberin in the carotenoid fraction (Chen et al. 2015; Fang et al. 2010), respectively, indicating the influence of the culture conditions on the yields of carotenoids and composition of the haloarchaea. The bacterioruberin content obtained from other haloarchaea is highly variable, as indicated by 98.1% in *Halorubrum* sp. (Hamidi 2014), 68.1% in *Haloarcula japonica* (Yatsunami et al. 2014); and 49.2% in *Halobacterium* SP–2 and 55.3% in *Halorubrum* SP–4 (Liying et al. 2014). These data show that *H. mediterranei* grown under the conditions stated in this Thesis contains high levels of bacterioruberin as compared with other haloarchaea.

Table 3.1. Identification of carotenoids present in *H. mediterranei*. BR: bacterioruberin; MABR: monoanhydrobacterioruberin; BABR: bisanhydrobacterioruberin.

Peak	Carotenoid	Retention Time (min)	λmax (nm)	Molecular ion (m/z) M+	Fragments profile
1	BR	2.325	468, 496, 530	740.7	723.7, 705.7, 687.7, 666.7, 561.5, 515,1
2	BR	2.553	468, 494, 528	740.7	723.7, 705.7, 681,6, 666.8, 655.6, 627.6
3	BR	2.740	468, 496, 528	740.7	723.7, 705.7, 682.6, 669.6, 665.6
4	BR	2.816	464, 494, 524	740.7	723.7, 705.7, 682.6, 665.6
5	MABR	3.021	470, 500, 534	737.7	725.6, 709.6, 699.7
6	BABR	3.168	460, 488, 520	705.7	681.6, 669.7, 579.7, 522.7
7	BABR	3.233	456, 485, 526	705.7	699.7, 671.7, 668.7, 647.6, 579.6
8	BABR	3.508	472, 498, 532	705.7	699.7, 687.7, 671.7, 653.8, 607.6
9	BABR	3.620	468, 490, 520	705.7	699.7, 671.7, 653.8, 550.6

Analysis of pigment composition by Thin Layer Chromatography (TLC)

As shown in Fig. 3.6, TLC revealed three major red spots (spots 1–3) and an orange-reddish spot (spot 4). Spot 1 showed the most intense red color, and the Rf for the four spots was 0.19 (spot 1), 0.40 (spot 2), 0.58 (spot 3), and 0.50 (spot 4). Each individual spot was analyzed by HPLC, and measured the corresponding absorption spectrum (400-600 nm) was also measured which revealed the typical 3-finger shape of C_{50} carotenoids, like those in Figure 3.5B, with maximum absorption around 500 nm for spots 1-3, and other absorption maxima around 528, 494, 470, and 388 nm, which were consistent with the absorption peaks of the C_{50} carotenoid bacterioruberin (Fang et al. 2010; Fong et al. 2001; Pfander 1994). From the above results spot 1 was considered bacterioruberin, spot 2 monoanhydrobacterioruberin, and spot 3 bisanhydrobacterioruberin.

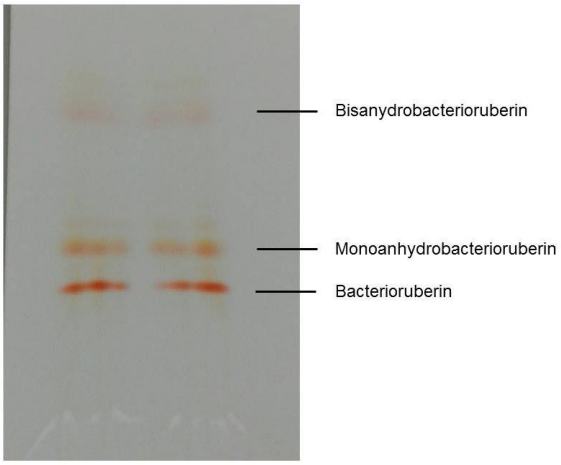

Figure 3.6. Pigment profile of *H. mediterranei* using thin layer chromatography (TLC). Culture samples growing under the above referred conditions were harvested (day 4), extracted, and the pigments separated through TLC according to the procedures described in the Materials and Methods section.

Effects of nutrient-starvation on growth and synthesis of C_{50} carotenoids by *H. mediterranei*

Nutrient starvation-induction of carotenoid accumulation has been reported to occur in microalgal cultures, as stated in *Dunaliella salina* (Lamers et al. 2012), *Nannochloropsis gaditana* (Simionato et al. 2013), *Haematococcus pluvialis* (Orosa et al. 2001) and other especies (Menegol et al. 2017; Couso et al. 2012). However, this strategy was not investigated yet in haloarchaea cultures, including *H. mediterranei*.

Carbon and nitrogen are part of major macromolecules such as lipids, proteins, complex carbohydrates and nucleic acids. Halophilic archaea are also able to use carbohydrates as carbon sources, including glucose, fructose, and ribose (Hechler 2008) and nitrogen compounds, like amino acids, from yeast extracts. The high or low availability of carbon and/or nitrogen in the culture medium obviously affects microbial growth. To investigate whether a similar effect might also take place in haloarchaea related to the accumulation

of C_{50} carotenoids, in this Thesis the effect of carbon and/or nitrogen starvation on the growth of *H. mediterranei*, and accumulation of C_{50} carotenoids was studied. In a first stage, *H. mediterranei* was cultivated in a culture media plenty of nutrients until the absorbance of the culture at 600 nm reached a value of 6. After that, the cells were centrifuged and used as the inoculum for the second stage, when the cultures were incubated under optimal conditions for carotenoids production, but with glucose and/or yeast extract deficiency. Figure 3.7 shows the time course evolution of *H. mediterranei* in glucose and/or yeast extract-starved culture medium, during the second stage.

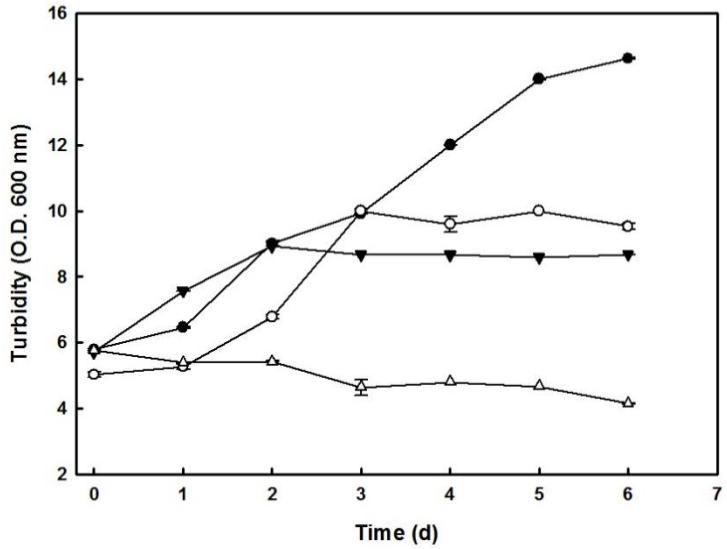

Figure 3.7. Effect of glucose and/or yeast extract starvation on the growth of *H. mediterranei* during the second cultivation stage. Cultures under optimal growth conditions (day 4 of growth) were harvested and suspended in fresh culture medium plenty of nutrients (-●-) or lacking glucose (-▼-), yeast extract (-○-), or both (-Δ-). More details in the Materials and Methods section.

In spite of nutrients deficiency, cultures still showed growth until the third day, which could be possibly due to the carbon and nitrogen intracellular reserves that have been mobilized to support some cell growth and energy-demanding maintenance cellular functions (Rabouille et al. 2006). In this sense, *H. mediterranei* produces poly-ß-hydroxybutyrate (PHB) which is one of the major

reserve materials found in eubacteria and that could be used by the archaea as a carbon source (Garcia Lillo and Rodriguez-Valera 1990). After 2 days no further growth was observed in glucose and/or yeast extract starved cultures.

According to Figure 3.7, after 6 days of incubation, glucose or yeast extract starvation resulted in a 40% and 35% reduction of biomass concentration respectively, compared to the control culture which enters stationary phase at that time. In order to assess the effect of glucose and/or yeast extract starvation on carotenoids production, the total production of carotenoids in *H. mediterranei* cultures was analyzed during the second cultivation stage (Figure 3.8).

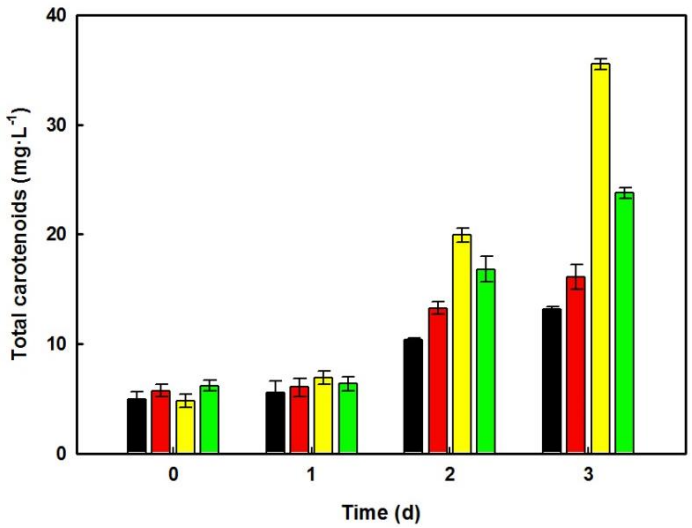

Figure 3.8. Effect of glucose and/or yeast extract starvation on the carotenoid production by *H. mediterranei* during the second cultivation stage. Samples of the cultures corresponding to days 1, 2 and 3, in Fig. 3.7 were used as source of carotenoids, which were extracted and quantified. Dark bars represent control cultures plenty of nutrients, red bars represent yeast extract-lacking cultures, yellow bars represent glucose-lacking cultures and green bars represent both yeast extract and glucose-lacking cultures. More details of experimental procedures as in Fig. 3.7 and the Materials and Methods section.

As shown in Figure 3.8, after 3 days of growth cells of the glucose-starved culture present 2.68-fold higher carotenoids content than standard

cultures. Yeast extract-lacking cultures shows a carotenoid content 1.77-fold higher than control, and the cells of culture-lacking in both yeast extract and glucose only increase in 1.23-fold. Accordingly, glucose-starved cultures led to the maximum C_{50} carotenoids productivity among the several conditions tested. The relative content of carotenoids versus the biomass accumulated, represented by the O.D. 494 nm/O.D. 600 nm ratio of the studied cultures in Fig. 3.8, is shown in Fig. 3.9. The results suggest that the biosynthesis of C_{50} carotenoids increase under glucose starvation, while yeast extract starvation does not seem to have a significant effect on the carotenoids production in *H. mediterranei*.

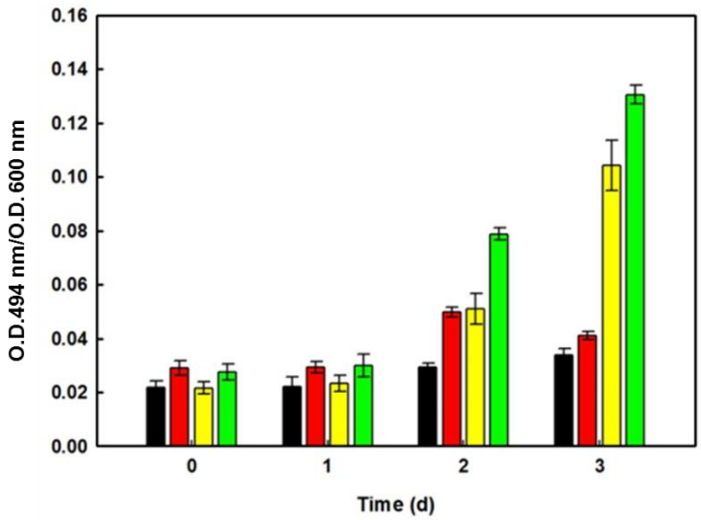

Figure 3.9. Effect of glucose and/or yeast extract starvation on carotenoid accumulation in *H. mediterranei* biomass. The color bars significance is similar to that stated in Fig. 3.8. The O.D. 494 nm/O.D. 600 nm ratios were obtained from carotenoids data of Fig. 3.8 and biomass data from Fig. 3.7. More details of experimental procedures are described in the Materials and Methods section.

Glucose can contribute to maintain the osmotic pressure in cultures of *H. mediterranei* (El-Enshasy et al. 2007). The glucose concentration in the culture medium (10 $g \cdot L^{-1}$) accounts for approximately 2.3% of the total solutes concentration in mmol per L. C_{50} carotenoids may be synthesized to supply the role of osmolites and cell membrane stabilizer in order to reduce the cell lysis due to the osmotic pressure (Calegari-Santos et al. 2016). In

addition, the results suggest that glucose is required for *H. mediterranei* growth but is not necessary for C_{50} carotenoids biosynthesis. Thus, the carbon required for C_{50} carotenoids biosynthesis under both glucose and yeast extract starvation should necessarily come from stored carbon in poly-ß-hydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA) (Poli et al. 2011; Hermann-Krauss al. 2013; Rodriguez-Valera 1992). These results suggest that the production of bacterioruberin by *H. mediterranei* could be designed in a two-stage process, consisting of growth in culture medium plenty of nutrients followed by a glucose starvation induction phase.

Effects of magnesium sulphate on the growth and synthesis of pigments by *H. mediterranei*

The concentration of the chemical components of the culture medium has been reported to greatly influence the biomass production (Schneegurt 2012) and therefore determine the conditions leading to achieve the highest biomass productivity. Magnesium is a common component of microbiological media. Haloarchaea are generally considered to require more Mg ion for growth than other microorganisms (Schneegurt 2012). Indeed, magnesium and sulfate ions are required by haloarquaea for growth and carotenoid production (Calegari-Santos et al. 2016). The magnesium requirement may be explained by its use for cell division. Besides, the demand for sulphate ions may be due to their presence in the cell wall of halophilic archaea (Calegari-Santos et al. 2016). In this Chapter, the effect of magnesium sulphate on the growth and C_{50} carotenoids accumulation of *H. mediterranei* was investigated.

H. mediterranei was grown under different concentrations of magnesium sulphate in order to find the most appropriate concentration for biomass production. The results are shown in Figure 3.10. *H. mediterranei* was able to grow in a wide range of $MgSO_4$ concentration, and according to the results, no major differences were found in growth and carotenoid production in cultures of *H. mediterranei* grown under different magnesium sulphate concentrations. Moreover, concentrations above 20 $g \cdot L^{-1}$ magnesium sulphate in the culture medium resulted in growth and carotenoid production data slightly lower than those obtained at lower magnesium sulphate levels (Figure 3.10). Interestingly, the lowest concentrations of $MgSO_4$ allowed growth until stationary phase. Thus, the growth of *H. mediterranei* in the presence of the lowest $MgSO_4 \cdot 7H_2O$ concentration would be economically

more suitable for potential applications of the archaea. The haloarchaea was also able to grow until stationary phase in presence of high magnesium sulphate concentration (80 $g \cdot L^{-1}$) without any sign of toxicity.

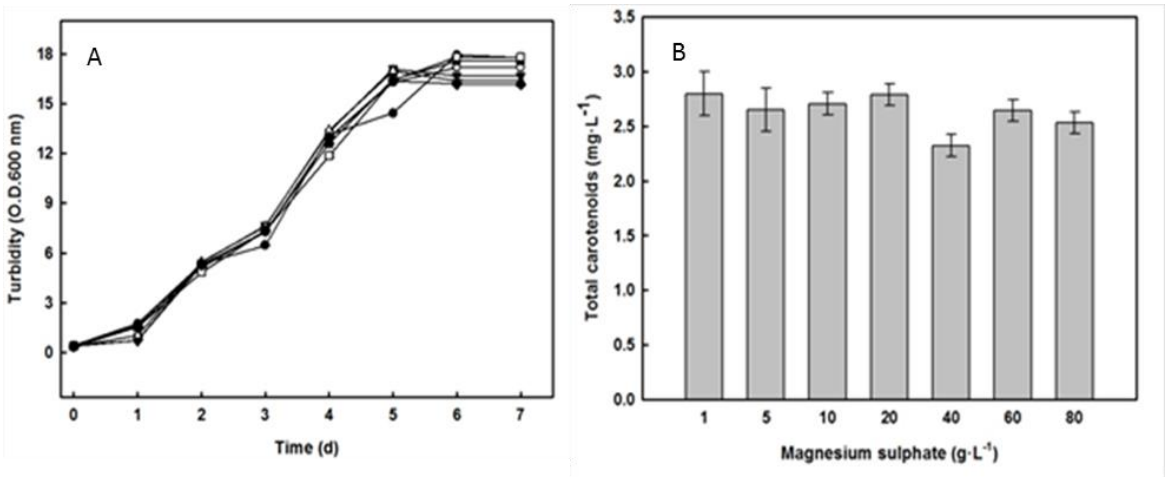

Figure 3.10. Effects of magnesium sulphate concentration on the growth (A) and carotenoid production (B) of *H. mediterranei*. Cells were grown in standard medium, supplemented with the following concentrations of $MgSO_4 \cdot 7H_2O$; 1g·L^{-1} (-o-); 5g·L^{-1} (-●-); 10g·L^{-1} (Δ); 20 g·L^{-1} (-▼-); 40 g·L^{-1} (-■-); 60 g·L^{-1} (-□-); and 80 g·L^{-1} (-♦-). Turbidity was determined daily, and carotenoids were determined after 6 days of growth. More details of experimental procedures in the Materials and Methods section.

According to results obtained by Fang et al. (2010), the production of pigment by *H. mediterranei* in a two-stage process increased under high concentrations of magnesium sulphate, 80 $g \cdot L^{-1}$, in cultures grown under 5% (w/v) of NaCl. This study reported reduced cell lysis and increased pigment synthesis which may be due to the role of magnesium sulphate as a modulator of the environmental osmosis (Calegari-Santos et al. 2016). This may partly explain the large tolerance of *H. mediterranei* to magnesium sulphate, also shown in this Thesis.

The response of halotolerant microorganisms to increased levels of $MgSO_4 \cdot 7H_2O$ may vary noticeably depending on specific genus and species. For instance, according to Soliman and Trüper (1982), addition of $MgSO_4 \cdot 7H_2O$ above 0.25% w/v to cultures of other *Halobacterium pharaonic* inhibits its growth. Conversely, *H. volcanii* not only requires high magnesium

concentrations (0.1 M) but also shows tolerance toward extremely high concentrations being able to grow at 1.4 M Mg^{+2} (Mullakhanbhai and Larsen 1975). Similarly, *Halobacterium sodomense* isolated from the Dead Sea also shows an optimal growth between 0.6 to 1.2 M Mg^{+2} (Oren 1983). Thus, the tolerance to magnesium sulphate by haloarchaea is species dependent, *H. mediterranei* being highly tolerant although not showing any direct relation between increased levels of magnesium sulphate and carotenoid accumulation. In terms of potential applications at large scale, cultivating *H. mediterranei* with low magnesium sulphate levels would be economically positive. Increasing the amount of magnesium sulphate in the medium, the relative ratio of bacterioruberin was increased, reaching a constant level at 8% (w/v) of magnesium (Fang et al. 2010). In our study, 2% magnesium sulfate was used in the culture medium for *H. mediterranei*.

CHAPTER IV

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *CHROOCOCCIDIOPSIS* SP., A NOVEL CYANOBACTERIUM FROM EXTREME ARID ENVIRONMENT

Abstract

Many cyanobacteria stand out for their ability to thrive in extreme environments, and in order to survive under these conditions, they are able to produce bioactive compounds with several biotechnological, industrial, pharmaceutical, and cosmetic uses. The aim of this work was the isolation and the identification of a new cyanobacterium from extreme arid environment. The optimal conditions for biomass production and its potential as biotechnological source of high added value products were also analyzed. The results obtained showed the isolation of a cyanobacterium, identified as belonging to the genus *Chroococcidiopsis*, which was able to grow stably and rapidly (0.21 $g \cdot L \cdot d^{-1}$) in liquid medium on high nitrate and urea concentrations (6-9 mM of nitrogen), Its major biochemical profile indicate an interesting potential for biotechnological applications.

Keywords: Antioxidant; *Chroococcidiopsis* sp.; Cyanobacterium; High-added value products; Phycocyanin.

Chapter IV

Introduction

Cyanobacteria have been reported to be valuable as raw material both for obtaining high-added value products and for the use of biomass itself as human food or animal feed. Cyanobacteria are the source of a large number of compounds of industrial interest, such as carotenoids, phycocyanin, phycoerythrin and fatty acids, exopolysaccharides (EPS), cyanotoxins and scytonemin (Kumar et al. 2019; Morone et al. 2019). These compounds find applications mainly as nutraceuticals. Cyanobacteria are also used in wastewater treatment, and for fertilizers production (Abed et al. 2019).

The biotechnological production of these compounds is not always economically feasible (Johnson et al. 2016), and thus it should be necessary to study the parameters involved in optimal cyanobacterial growth and high-added value products accumulation. In this context, it is very important to explore for new species with the ability to produce substances of industrial interest (Vitorino and Bessa 2017).

Key parameters involved in the cultivation of the cyanobacteria are culture agitation, type and amount of nutrients, light intensity and quality, temperature and salinity, among the most influencing factors (Sharma et al. 2014). With the optimization of the culture conditions, a greater quantity of microorganism biomass is achieved, which can be translated into higher productivities of target compounds with biotechnological applications (Forján et al. 2015).

The agitation of the culture has a fundamental role since it produces the homogenization of biomass distribution in the culture medium and prevents the biomass from flocculating which would limit light accessibility to part of the biomass, therefore decreasing the growth rate of the microorganism. On the other hand, after carbon, nitrogen is the most abundant element in the cyanobacterial biomass, accounting for about 5-10% of the dry weight (Grobbelaar 2013). Nitrogen is an essential component of many biomolecules, as nucleic acids (DNA, RNA), amino acids (proteins) and other pigments such as chlorophyll or phycobiliproteins. Nitrogen can be uptake from the inorganic form (NO_3^-, NO_2^-, NH_4^+) and can also be assimilated into organic form (urea and amino acids) (Flores and Herrero 2005), the optimization of

concentration and source of this nutrient is essential for optimum biomass production.

In this Chapter, the isolation and identification of a cyanobacterium obtained from Atacama desert (Chile), which is characterized by being the most arid nonpolar environment on Earth (Bull et al. 2016) was carried out. In addition to extreme desiccation, this area has a temperature range between -5 ºC and 50 ºC, and a high incidence of UV radiation, what makes it an inhospitable place for life (Meslier et al. 2018). To survive this environment, cyanobacteria produce substances that may be interesting from a biotechnological point of view (Hu et al. 2012). For example, cyanobacteria produce abundant exopolysaccharide-rich (EPS) envelopes to survive the severe anhydrobiosis, because it provides both a repository for water and a matrix which stabilizes desiccation-related enzymes and molecules (Billi et al. 2017; Wierzchos et al. 2015). EPS have industrial interest due to its immunomodulatory effect, in addition to antitumor and antiviral activities among others, recently described for these compounds (Gugliandolo et al. 2014). Besides, maintenance of membrane integrity in anhydrobiotic organisms represents a central mechanism of desiccation tolerance (Carpenter and Crowe 1989, Crowe and Crowe 1992). Accordingly, the role of membrane fluidity and lipid composition on survival of bacteria at extreme temperatures, salinity and drying has been reported to be crucial (Singh et al. 2002).

Chrooccocidiopsis sp. is a cyanobacterium of endolythic life, which means it thrives inside rocks thus avoiding the accessibility of the high UV radiation of the environment. In addition, it produces pigments to increase the efficiency in harvesting PAR light of specific wavelengths. These pigments, which include phycocyanin, have interest for their use in the food and cosmetic industry and also for their medical applications (Stanic-Vucinic et al. 2018).

Thus, analyzing the biotechnological potential of the cyanobacterium isolated from the extreme arid environment, *Chroococcidiopsis* sp., was taken as mandatory scientific task in this Thesis work.

Materials and Methods

Isolation of microorganisms from rocks

The methodology followed for the isolation of the microorganism used in this work consisted in a four steps process: (1) Treatment of gypsum samples from Atacama Desert in Chile which were kindly provided by collages from CSIC (National Museum of Natural Science) (Fig. 4.1), (2) the green part of the rocks were scratched away and transferred in liquid mineral medium, (3) then, liquid samples were grown in Petri plates that contained solid culture medium in agar, and (4) the liquid culture medium was inoculated with colonies isolated in former step.

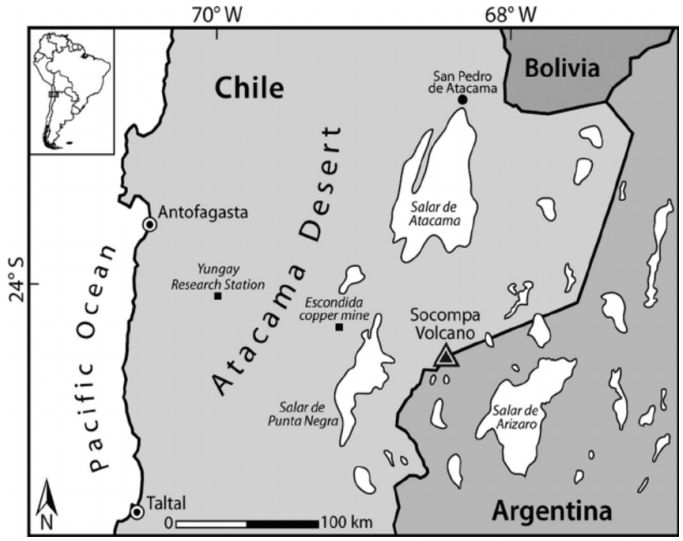

Figure 4.1. Map of Northern Chile showing the region of the Atacama Desert. Source: Costello et al. 2009.

(1) Treatment of gypsum samples: The biological material was obtained from gypsum samples selected by the expedition team (Museum of Natural Sciences, CSIC, Madrid, Spain, 2014) in the Atacama Desert (Chile) and supplied to Ciderta for scientific research purposes only within a project in collaboration with the University of Huelva. The *on-site* selection of the gypsum samples was made based on the presence of greenish and blueish coloured endolithic layers that revealed the possible presence of photosynthetic microorganisms (Figure 4.2A y 4.2B).

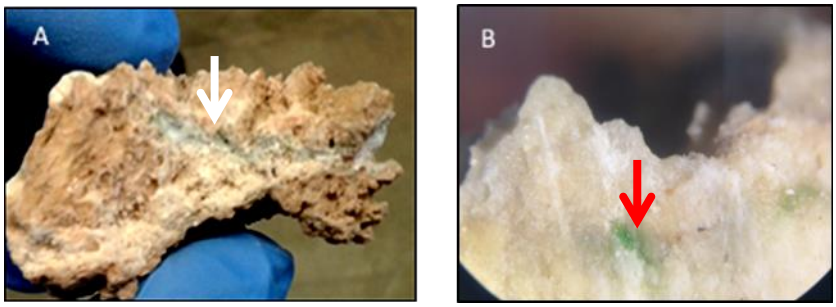

Figure 4.2. Sample of gypsum fraction obtained in the arid zone of the Atacama Desert. A blue-green colour band corresponding to endolithic colonisations by photosynthetic organisms is observed. (A) A cross fractures of the rock reveals a distinct blue-green layer representing the zone colonized by cyanobacteria. This layer appears 3–7 mm below the rock surface (white arrows). (B) Close-up of the gypsum rock showing a green-blue layer parallel to the surface (red arrows).

The biological material was obtained by scraping the coloured bands corresponding to endolithic colonisations. The first material obtained from the areas in direct contact with the external medium was always discarded.

(2) Transfer to mineral liquid medium: The biological material from the scraping of the gypsum samples and physically adhered to them, was introduced in 1.5 mL of Eppendorf tubes containing 1 mL of several culture media (Figure 4.3). The culture media used were the BBM (Bischoff and Bold 1963; Bold 1949), BG11 and M8a. The chemical compositions of such culture media are collected in Tables 4.1, 4.2 y 4.3, respectively. The volume of 1 mL of culture medium is less than the total volume of the Eppendorf tube, so that a small air volume was always preserved which allowed the availability of carbon dioxide for its fixation by photosynthesis of microalgae or cyanobacteria.

Table 4.1. Chemical composition for a liter of BBM medium.

Components	$g \cdot L^{-1}$
$NaNO_3$	0.25
$Co(NO_3) \cdot 6H_2O$	$9.8 \cdot 10^{-4}$
KH_2PO_4	0.35
K_2HPO_4	0.15
$CaCl_2 \cdot 2H_2O$	0.05
$MgSO_4$	0.15
$FeSO_4 \cdot 7H_2O$	$9.96 \cdot 10^{-3}$
$ZnSO_4 \cdot 7H_2O$	0.018
$CuSO_4 \cdot 5H_2O$	$3.14 \cdot 10^{-3}$
$MnCl_2 \cdot 4H_2O$	$2.88 \cdot 10^{-3}$
EDTA (acid)	0.10
$Na_2MoO_4 \cdot 2H_2O$	$1.42 \cdot 10^{-3}$
H_3BO_3	$2.284 \cdot 10^{-3}$

Table 4.2. Chemical composition for a liter of BG11 medium.

Components	g·L-1
$NaNO_3$	1.50
$K_2HPO_4 \cdot 3H_2O$	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
Na_2EDTA	0.001
Na_2CO_3	0.02
H_3BO_4 ($\mu g \cdot L^{-1)}$	2.86
$MnCl_2 \cdot 4H_2O(\mu g \cdot L^{-1})$	1.81
$ZnSO_4 \cdot 7H_2O(\mu g \cdot L^{-1})$	0.222
$Na_2MoO_4 \cdot 2H_2O(\mu g \cdot L^{-1})$	0.391
$CuSO_4 \cdot 5H_2O(\mu g \cdot L^{-1})$	0.079
$Co(NO_3)2 \cdot 6H_2O(\mu g \cdot L^{-1})$	0.049

Table 4.3. Chemical composition for a liter of M8a medium.

Components	g·L-1
KH_2PO_4	0.74
$Na_2HPO_4 \cdot 2H_2O$	0.26
$MgSO_4.7H_2O$	0.40
$CaCl_2.2H_2O$	0.013
KNO_3	3
EDTA ferric sodium salt	0.116
$Na_2EDTA \cdot 2H_2O$	0.0372
H_3BO_3	0.00618
$MnCl_2.4H_2O$	0.00130
$ZnSO_4.7H_2O$	0.00320
$CuSO_4 \cdot 5H_2O$	0.00183

The addition of scraped material from the gypsum to the different liquid culture media contained in the Eppendorf tubes was carried out preserving the sterility of the operation (Figure 4.3). The samples were kept under indirect natural light, by placing them inside the laboratory close to a window, shaking them gently once every 24-48 h. The samples were kept under such conditions until proliferation of active cells in the culture medium was observed under the optical microscope. As cited in Results and Discussion, the BBM medium yielded the best results.

Figure 4.3. Material from the scraping of gypsum rock and incubated in various liquid mineral culture media, whose chemical composition is detailed in Tables 4.1, 4.2 y 4.3.

(3) Growing in Petri dishes containing solid medium: Photosynthetic growth was observed in several gypsum samples in the Eppendorf tubes, and such tubes were used as a source of samples to seeding in solid medium in Petri dishes. The plating medium was prepared by the addition of 0.75% (w/v) bacteriological agar to the BBM medium, described above. The spreading was carried out both in surface and in depth. The samples were grown with PAR light of low irradiance, in microalgae culture room, until green or bluish-green colonies appeared (Figure 4.4).

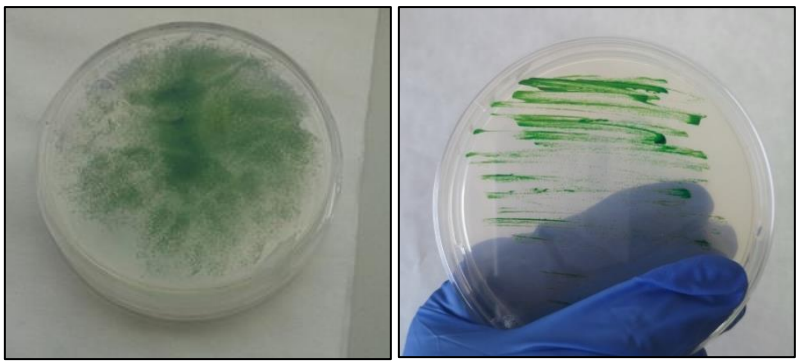

Figure 4.4. Isolation of the microorganism from the solid medium. Cyanobacteria colonies were seeded in Petri dishes on solid medium with agar by following the procedures described in the Materials and Methods section.

(4) Growing isolated organisms in liquid mineral medium: The cultivation in solid medium in Petri dishes produced colonies that could be taken for sowing in a liquid mineral culture medium, specifically in BBM, as described earlier in this section of the Thesis. The growth in liquid medium was initially carried out in 10 mL of culture medium, under very mild gassing with air enriched in CO_2 (5%, v/v) and moderate illumination. These cultures produced enough biomass to address the task of identifying isolated species.

Fluorescence and electron microscopy

Microscopy techniques were realized at the laboratories of National Museum of Natural Sciences (CSIC, Madrid), making use of available microscopy equipment under the kind supervision of Dr. Jacek Wierzchos. Isolated cyanobacterial cells from the liquid cultures were observed in differential interference contrast mode (DIC) and in fluorescence mode (FM)

using a D1 Zeiss fluorescence microscope (AxioImager M2, Carl Zeiss, Germany) with Apochrome oil immersion objective x64 n=1.4, according to methods described by (Wierzchos et al. 2011).The Rhodamine filter set (Zeiss Filter Set 20; Ex/Em: 540-552/567-647 nm) was used for the acquisition of single section images of autofluorescence red signal potentially proceeding from chlorophyll. The same fluorescence microscopy with Apochrome oil immersion objective x64 n=1.4 was used for detection of fluorescence signal proceeded from the cyanobacteria stained with SYBR Green I (SBI) dye. For this purpose the Multichannel Image Acquisition (MIA) system was used with a combination of the following filter sets: filter set for eGFP (Zeiss Filter Set 38; Ex/Em: 450-490/500-550 nm) for SBI green fluorescence, and Rhodamine (Zeiss Filter Set 20; Ex/Em: 540-552/567-647 nm) for chlorophyll red autofluorescence signal (Crits-Christoph et al. 2016).

For the electron microscopy studies, cells obtained from liquid cultures were harvested by centrifugation at 3000 x *g* and resuspended in 3% glutaraldehyde in 0.1M cacodylate buffer and incubated at 4°C for 3 hours. The cells were then washed three times in cacodylate buffer, postfixed in 1% osmium tetroxide for 5 hours before being dehydrated in a graded series of ethanol and embedded in LR White resin. Ultrathin sections were stained with lead citrate and observed with an EM910 (Leo, Germany) and CM200 Philips (Philips, The Netherlands) transmission electron microscopes at 80 kV acceleration potential.

DNA extraction, PCR amplification and sequencing

The extraction of DNA was done by using the Power Soil kit (Mo Bio Laboratories Inc.). For amplification of the 16S rDNA, two conserved prokaryote-specific primers (forward primer, 27F: AGA GTT TGA TCC TGG CTC AG (Neilan et al. 1997) and reverse primer, 23S30R: CTT CGC CTC TGT GTG CCT AGG T (Taton et al. 2003) were used to amplified almost complete16S rRNA and 16S–23S ITS regions. Amplifications were achieved under the following conditions: a first denaturation step of 95 °C was carried out for 5 min, followed by 35 cycles composed of a first step of denaturation at 95 °C for 10 seconds, a second step of binding the primers at 58 °C during 30 seconds and a third extension step at 72 °C for 30 seconds, a final extension step of 5 min was applied at 72 °C. The PCR product was

purified by the protocol of E.Z.N.A. Cycle-Pure (Omega Bio-tek) Genetic sequencing was done at external service (Secugen S.L, Spain).

The sequences obtained were compared with the sequences of the database of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Blast/) to assess homology with organisms already deposited in the database. In this method, the comparison is based on the percentage of similarity between paired sequences and the percentage of aligned sequences.

Phylogenetic analyses

Phylogenetic analyses were performed with the sequences of our strain and other sequences of the same genus retrieved from the public database (NCBI). For phylogenetic analysis, multiple alignments of sequences were performed using ClustalX (Thompson et al. 1997) and then manually arranged with SeaView (Gouy et al. 2010). Aligned sequences were calculated on nucleotide alignments by using three methods: maximum likelihood (ML) (Guindon et al. 2010), maximum parsimony (MP) (Farris 1970) and neighbor joining (NJ) (Saitou and Nei 1987). Reconstruction of phylogenetic trees by the Maximum-Likelihood algorithm (ML) (Stamatakis et al. 2008) was carried out with SeaView (Gouy et al. 2010) which was tested using 1000 bootstrapping replicates.

Optimal culture conditions for *Chroococcidiopsis* sp. in liquid medium.

Prior to inoculation, the liquid BBM medium was buffered at pH = 7 by addition of 10% (v/v) NaOH solution, and sterilized in autoclave for 25 min at a temperature of 121 °C and under a pressure of 1 atmosphere. The liquid medium was then inoculated from sterile samples of the cyanobacterium grown in solid medium with agar, and standard growing conditions were performed in a culture room under controlled temperature at 25 ºC, using Erlenmeyer flasks of different volumes depending on the case. The carbon source was supplied by bubbling air enriched in CO_2 at 5% (v/v) through a glass rod immersed in the medium containing the cyanobacterial culture. The light source used to provide light energy to the cultures in the culture room was generated by Philips TL-D 30W/54-765 1SL lamps, (Philips Ibérica SAU, Spain), which supplied 70 μE m^{-2} s^{-1} on the surface of the culture flasks.

For experiments of incubation with different nitrogen sources ($NaNO_3$, $NaNO_2$, NH_4^+ and urea) at different concentrations (3, 6 and 9 mM), the original BBM medium of *Chroococcidiopsis* sp. was modified, preparing a BBM medium devoid of nitrogen and adding on it different nitrogen sources of each experimental condition. Other growing conditions are as indicated previously.

Biomass productivity measurement

The optical density of the sample (triplicate) was measured at 750 nm (O.D. 750 nm) in a spectrophotometer (Evolution 201, Thermo Fisher Scientific, USA). As previously reported for microalgal cultures (Griffiths et al. 2011), O.D. at 750 nm correlates with biomass concentration, in healthy cultures in the linear growth phase. If necessary, samples were previously diluted, if necessary, to obtain absorbance values between 0.2 and 0.8. The culture medium used in each experiment was taken as blank for the measurement.

Alternatively, dry weight in the cultures was also used for biomass determination. We proceeded using Whatman glass microfiber filters of Ø 47 mm, pore size 0.7 μm (MFV-5, AnoiaFilterlab, Spain), which were dried at 95 °C overnight and placed in a desiccator at room temperature. The empty filters were weighed. Filtration was carried out with culture samples using 10 mL samples of each culture, by triplicate. The filter was rinsed twice with demineralized water to remove adhering inorganic salts, and the wet filters containing wet biomass were dried at 95 °C overnight, allowed to cool at room temperature in a desiccator, and weighed (Vaquero et al. 2014).

Maximal photosynthetic efficiency determination

The maximum photosynthetic efficiency of Photosystem II (PSII) or maximum quantum yield (Qy) can be used as an indirect indicator of cell viability (Maxwell and Johnson 2000). The maximum quantum yield (Fv/Fm) was determined using a PAM device (Pulse Amplitude Modulation, model AquaPEN AP100, Photon System Instruments, Czech Republic) in accordance with Cuaresma et al. (2012). The determinations were carried out by placing an aliquot of the culture broth in the cuvette of the PAM and keeping it in darkness for 15 min, to oxidize the reaction centers of the photosystems and to obtain the level of basal fluorescence (F0). Subsequently,

a pulse of saturated light of 1,500 µmol photons.m^{-2}·s^{-1} (50% of the total intensity) is emitted, which quickly saturates the reaction centers of the photosystems and induces the emission of maximum fluorescence (Fm). The variable fluorescence of dark-adapted sample (Fv) can be determined from the difference between the above parameters (Fm-F_0). From these data, the value of Fv/Fm was calculated according to Cosgrove and Borowitzka (2011) as:

$$\left({}^{F_v}/_{F_m}\right) = \frac{(F_m - F_0)}{F_m}$$

Analysis of cyanobacterial biomass

Carbohydrates content determination

The quantification of carbohydrates was carried out following the method reported by Dubois et al. (1956). Firstly, acid hydrolysis was carried out by adding 2.5 M HCl in a proportion of 0.5 mL of HCl per mg of biomass, and its subsequent incubation at 100 °C in a water bath during 1.5 h. The neutralization of the samples was carried out with 2.5 M NaOH in the same proportion as the acid. After that, phenol and H_2SO_4 were added to the samples and these were incubated in a water bath at 35 °C during 30 min was carried out. This last step resulted in an orange-yellow colour which was measured at 483 nm in a spectrophotometrically (Evolution 201, Thermo Fisher Scientific, USA). The method requires the prior preparation of a calibration curve with glucose at concentrations located in the range of 0 – 0.1 mg·mL^{-1}.

Analysis of fatty acids

The analysis of fatty acids by gas chromatography requires them to be volatile at the working temperatures used in the chromatographer. To do this, it is necessary to transform the fatty acids, esterified or free, in their respective methyl esters through a reaction of esterification with alcohol. This procedure, called transesterification, was performed following the modified method described by various authors (Halim et al. 2011; Prabakaran and Ravindran 2011; Gouveia and Oliveira 2009; Widjaja et al. 2009).

The transesterification was acidic and approximately 10 mg of the oil residue (triglycerides), previously extracted by Soxhlet, was combined with an alcohol containing a catalyst (methanol enriched at 5% v/v in H_2SO_4) to produce glycerol and alkyl esters of fatty acids. The transesterification reaction was carried out at 70 °C during 3 hours under continuous agitation in a shaker incubator (model MaxQ 4450, ThermoScientific, United States) to favor the conversion. Subsequently, the samples were refrigerated at 4 ºC and distilled water was added to wash them. Finally, hexane was added, which is the matrix that drags the methylated fatty acids obtained in the transesterification reaction. The hexane solution was preserved at -20 ºC for further analysis by gas chromatography.

For the later separation and quantification of the main microalgae fatty acids, a gas chromatographer (6890N, Agilent, China) was used. It included an automatic sampler (7683 B, Agilent, China) and a flame ionization detector (FID), with a capillary column (Omegawax 320, Supelco, USA) 30 m long, 0.32 mm wide and 0.25 μm internal diameter. The carrier gas was He and the flow rate selected was of 20 $mL \cdot min^{-1}$. Table 4.5 shows the temperature gradient of the gas chromatographer (GC) used to obtain defined peaks in the resulting chromatograms. A FID detector temperature of 260 °C, split injector mode and injection volume of 1 μL were used.

The identification and quantification of the specific fatty acids present in each sample was based on the comparison of their retention times with those of a mix of commercial standards with known concentration (Supelco 37 FAME Mix, Supelco, USA). The used fatty acids standard purity ranged from 98.7 to 99.9% and was diluted in hexane as well as the tested samples. The appropriate conversion factors for the fatty acids quantification were calculated from the resulting chromatogram of the standards.

Table 4.5. Temperature gradient used for fatty acids determination.

Oven ramp	Slope (ºC·min^{-1})	Temperature (ºC)	Retention time (min)	Runtime (min)
0	---	90	0	0
1	10	140	0	5
2	5	165	2	12
3	1	190	2	39
4	5	220	15	60

Proteins determination

The protein content of the microalgal biomass was estimated from the nitrogen content measured by elemental composition analysis, using 4.5 as nitrogen-to-protein conversion factor (NTP) (López et al. 2010).

Elemental CHN analysis

The elemental analysis of carbon (C), hydrogen (H), nitrogen (N) and sulfur (S) was performed by a calorimetric method by the General Research Services of University of Huelva and the amount of N in the samples was determined according to Gnaiger and Bitterlich (1984).

Pigments extraction and analysis

For pigment extraction from biomass, 2 mL samples of cyanobacterial cultures were placed into Eppendorf tubes were harvested by centrifugation at 14000 x *g* for 5 min and the supernatant was discarded. Glass beads of 0.25-0.5 mm and 1 mL of methanol were added to the samples and the cells were disrupted in a bead miller (Restch M400, Restsh GmbH, Germany) during 5 cycles (5 min max speed and 30 s interval, each cycle). Then, the suspension was centrifuged at 14000 x *g* for 10 min, and the supernatant was carefully transferred into a clean Eppendorf tube. 1 mL of methanol was added again to the resulting pellet and the mixture was vortexed for 10 s and centrifuged (14000 x *g* during 10 min). A blue pellet was obtained, which was kept at 4 ºC until use for the phycobiliproteins content determination.

The phycobiliproteins content was extracted with 1mL of phosphate buffer (0.1 M; pH 7) and put into a water bath for ultrasound during 2 h at 30 ºC. Then, the samples were centrifuged at 14000 x *g* for 10 min, and the

supernatant was transferred into a clean Eppendorf tube to measure the phycobiliproteins content. The absorbance was measured at 565 nm, 620 nm and 650 nm. After that, the concentration of phycobiliproteins in the biomass pellets was calculated by Bryant equations and given in mg·mL^{-1} extract, as described by Lobban (1988):

$$Phycocianin[PC; mg/mL] = [A_{620} - (0.72\ x\ A_{650})]/6.29$$

$$Allophycocianin\ [AC; mg/mL] = \ [A_{650} - (0.191\ x\ A_{620})]/5.79$$

$$Phycoerythrin\ [mg/mL] = \ \left[A_{565} - \left((2.41\ x\ CPC) - (1.41\ x\ CAPC)\right)\right]/13.02$$

Statistics

Unless otherwise indicated, the presented data are the means of three independent experiments. The standard deviations of each set of experiments are represented in the corresponding figure (bars). The data groups were analyzed with a one-way analysis of variance using the SPSS version 19 statistical analysis package (IBM, USA). Differences were accepted as significant when $P < 0.05$.

Chapter IV

Results and Discussion

Identification of the microalga obtained from gypsum rocks of Atacama Desert

Morphological studies

The main objective was to isolate photosynthetically active microorganisms from gypsum rocks samples (described in the Materials and Methods section), and to identify those microorganisms that showed rapid growth in appropriate culture media. According to Wierzchos et al. (2015) and our own observations (data not shown), the thin colored lines visible in gypsum rocks cross sections are not necessarily indicative of the viability of the microorganisms of endolithic colonies, since such coloration -particularly from yellow to red colors- may also be due to pigments (carotenoids) of non-active photosynthetic cells, or even to pigments released from lysed photosynthetic cells. Thus, the samples obtained from the gypsum rocks were taken from a number of like-photosynthetic endolithic colonies (colored spots) differently located inside the rocks. The first growth step in the process of isolating microalgae from the biological material contained in the Atacama gypsum rocks resulted in the production of bluish-greenish liquid cultures in Eppendorf tubes containing BBM medium, specially suitable for the growth of cyanobacteria (Figure 4.5). Blue-green colonies obtained under photoautotrophic conditions typically correspond to cyanobacterial cells which therefore were expected to grow in BBM medium. Once growth was visible in the Eppendorf tubes, the samples were seeded in Petri dishes prepared with BBM medium in agar, as explained in the corresponding section of Materials and Methods.

Figure 4.5. Eppendorf tube containing Atacama gypsum rocks samples and blue-green aggregates. Growth was observed in Eppendorf tubes containing gypsum fragments and BBM culture medium. The appearance of biomass can be observed adhered to the rock fragments.

Several scientific studies published in recent years reported the occurrence of photosynthesis under the extremely arid conditions of halite (salt rock) and gypsum rocks in the Atacama Desert (Wierzchos et al. 2015; Vítek et al. 2013; Wierzchos et al. 2011). In some of these studies, photosynthesis is reported to occur inside translucent gypsum which provides protection against excess solar radiation, thus allowing photosynthetically active microorganisms survival. Moreover, based on taxonomic assignment, cyanobacteria were revealed to be the dominant phyla of the endolithic microbial diversity found in gypsum rocks samples, representing between 67% and 83% of the DNA read sequences. Among the dominant cyanobacterial members, Chroococcales, mostly belonging to the *Chroococcidiopsis* genus, represents the 64% of total (Wierzchos et al. 2015).

Optical microscopic observations of culture samples revealed the presence of an apparently photosynthetic microorganism, characterized by fluorescent green coloration of chlorophylls and without flagella (Figure 4.6). The ultrastructure was determined by transmission electron microscopy (Figure 4.7). Solitary cells, or cells agglutinated in like-spherical or irregular groups, with thin, firm, colourless sheaths were also observed. These features are characteristic of the order *Chroococcidiopsidaceae* (Komárek et al. 2014).

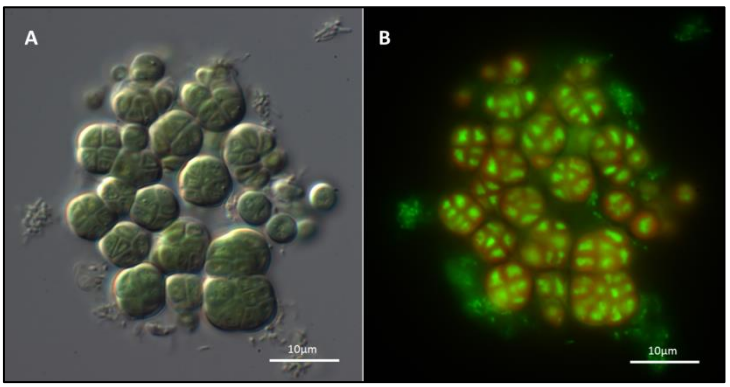

Figure 4.6. Morphology of the cyanobacterium isolated from gypsum rocks. Colonies of different sizes are observed. (A) Images of cell aggregates of the cyanobacterium isolated from gypsum rock samples. (B) Cells stained with SYBR. This compound binds to DNA showing green coloration. Scale bars: (A) and (B) 10 µm. Details are described in the Materials and Methods section.

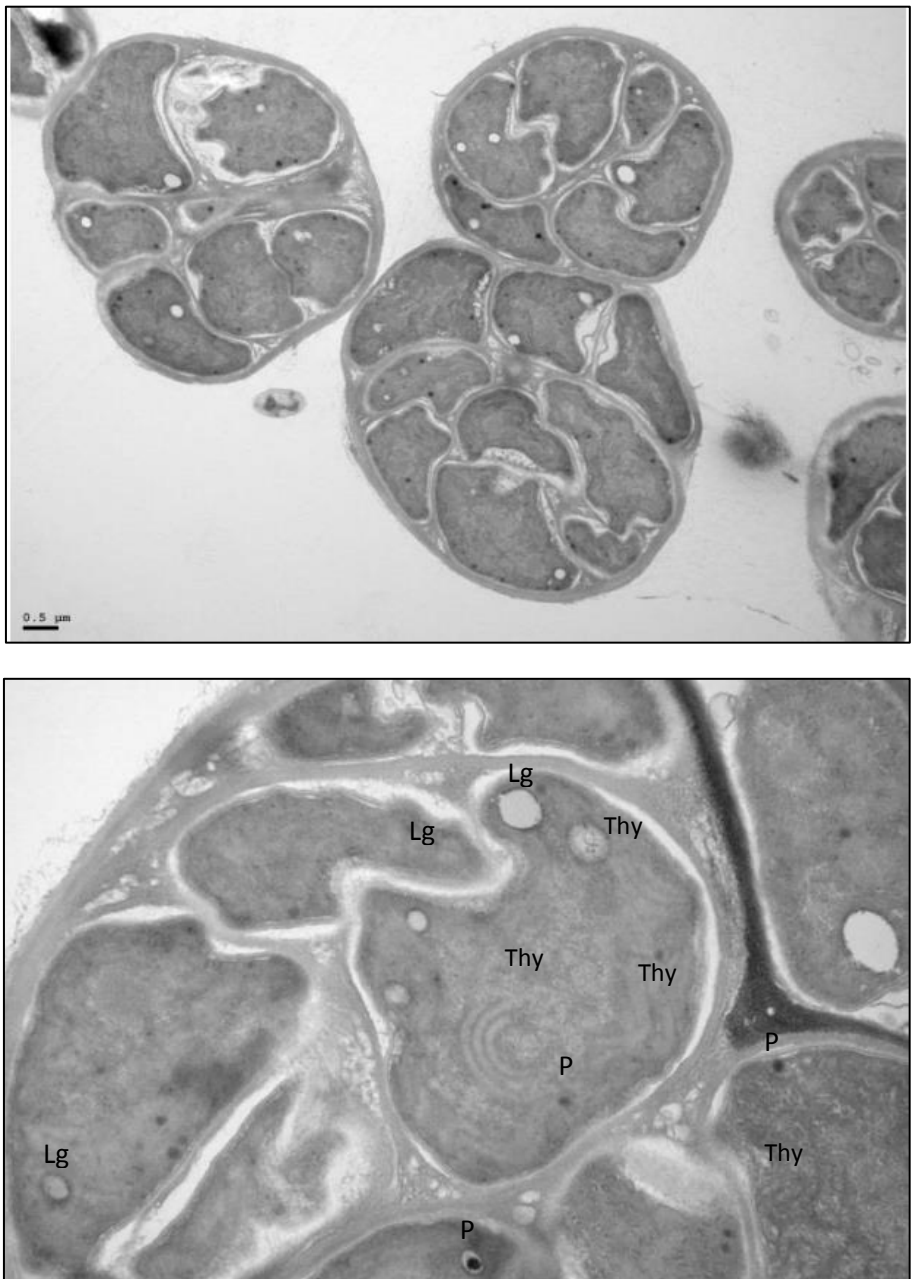

Figure 4.7. Transmission electron microscopy pictures of *Chroococcidiopsis* sp. Scale bars: (A) 0.5µm (x 6,000); (B) 0.2µm (x 30,000). Abbreviations: EPS, exopolysaccharide. Lg, lipoidal globules. Thy, thylakoids, P, polyphosphate bodies.

The internal structure of the isolated *Chroococcidiopsis* does not differ from other cyanobacteria, and within the cell, the cytosol contains storage structures such as cyanophycin granules, carboxysomes, ribosomes, DNA, and thylakoids, which contain the light harvesting complexes of photosystems I and II (Donner 2013). The thylakoid arrangement is thought to be the most important feature of the inner cell structures which is usable for taxonomic studies (Komárek and Anagnostidis 1999). For *Chroococcidiopsis* PCC 7203, thylakoids are reported to form fascicles (short parts of the membranes) usually with a radial position within the cell (Komárek and Kastovsky 2003). *Chroococcidiopsis* strains PCC 7432 and PCC 7436 show a parietal (orientated along the cell wall) to stacked (small fragments packed together) arrangement (Waterbury and Stanier 1978), visualized in transmission electron microscopy pictures.

Although the aforementioned morphological features are initially consistent with the inclusion of the isolated cyanobacterium in the order *Chroococcidiopsidaceae*, this assignment it must be assessed through an accurate phylogenetic approach based on the use of molecular taxonomy techniques.

Molecular studies

Prior to the molecular characterization of the isolated photosynthetic microorganism, which according to the optic and electron microscope pictures seemed to be a cyanobacterium, the liquid cultures purity was first assessed by optical microscopy techniques to verify that the cultures contained only one cell type. The identification of the genus was made by molecular techniques, as described in the Materials and Methods section of this work. Identification was performed by amplifying and sequencing DNA encoding ribosomal RNA corresponding to the small 16S subunit. The sequence obtained was compared with other similar sequences published in the database of the National Center for Biotechnology Information (NCBI). The percentage of alignment and homology of the DNA sequences was used as a criterion for said comparison.

The results obtained allow us to conclude that the isolated microorganism belongs, with high probability, to the cyanobacterial genus *Chroococcidiopsis*, which is one of the most primitive one on the Earth (Friedmann and Ocampo-Friedmann 1995). It is a photosynthetic and

coccoidal bacterium. Many of the species identified within this genus have the ability to express adaptive responses to conditions of high or low temperature, salinity or ionizing radiation, all these being conditions specific in the extreme environments (Seckbach and Rampelotto 2015). In fact, numerous species belonging to the genus *Chroococcidiopsis* are considered extremophilic organisms, and the American Space Agency (NASA) has proposed these cyanobacterial genus as the most appropriate type of photosynthetic microorganism for studies of oxygen and biomass production in conditions equivalent to those of the surface of Mars, suggesting this genus as a candidate to provide the human species with an adequate atmosphere on the red planet, in a future still apparently distant (Billi et al. 2000).

From the sequence alignment above referred, a maximum likelihood phylogenetic tree was inferred (Figure 4.8).

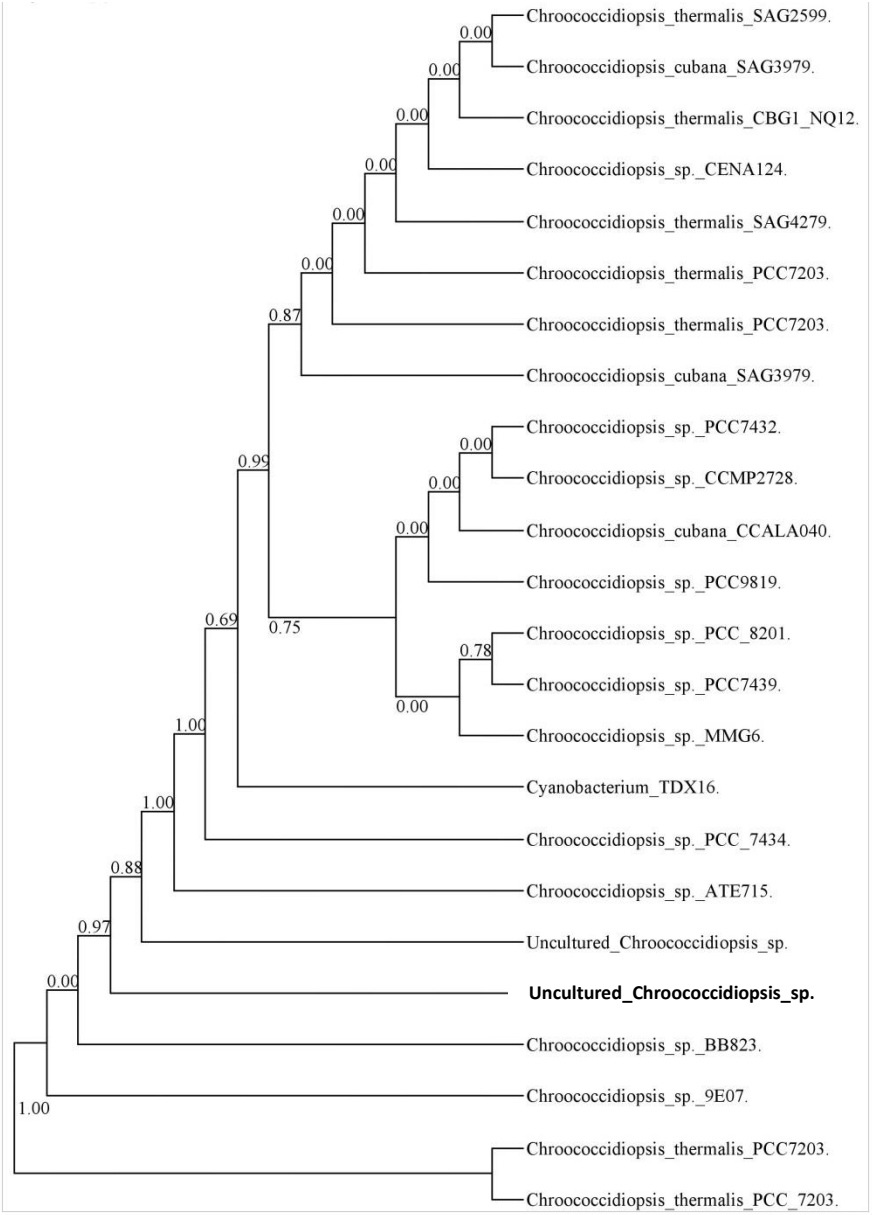

Figure 4.8. Phylogenetic tree based on gene sequences of the genus *Chroococcidiopsis* sp. Distances within the tree were constructed using neighbor joining method with Clustal W. Horizontal lengths are proportional to the evolutionary distance. Bar = 0.01 substitutions per nucleotide position.

Optimal growing conditions for *Chroococcidiopsis* sp.

Culture agitation

Chroococcidiopsis sp. divides giving rise to large colonies, which may produce biomass deposits in liquid medium. Thus culture agitation is required in order to have the cells suspended in the culture medium, since sedimentation has a negative effect on the growth rate of the cyanobacterium due to limited of nutrients availability to deposited cells (Gupta et al. 2015; Doran 2013; Feng et al. 2003).

Prior to carry out systematic assessment of the cell growth under different cultivation conditions, the effect of mechanical agitation on the growth of the cyanobacterium was evaluated and compared with cultures without agitation. Differences were found between cultures of the cyanobacterium under constant agitation at 500 rpm versus non-agitated cultures. During the first 24 h, no effect of agitation on the cultures growth was observed; however, after 48 h the biomass of the cultures under agitation increased 170% with respect to the control cultures (Figure 4.9).

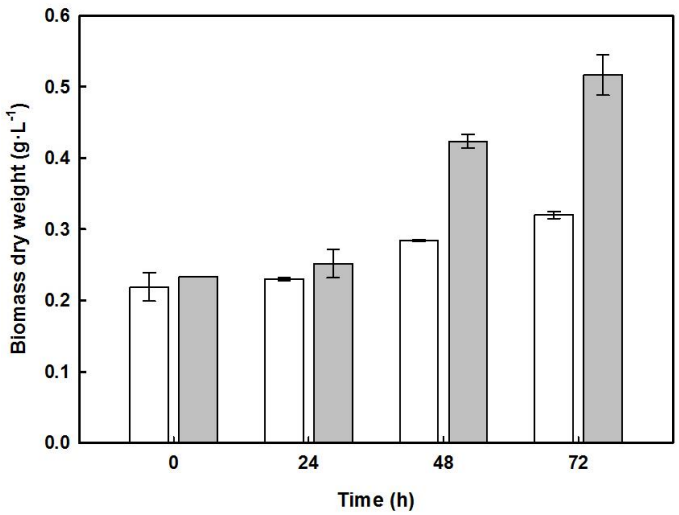

Figure 4.9. Effect of agitation speed on the growth of *Chroococcidiopsis* sp. Cells were grown in liquid BBM culture medium with 3 mM $NaNO_3$. Details of growth conditions are described in the Materials and Methods section. White bars represent cultures without agitation and grey bars represent cultures grown with 500 rpm agitation speed.

Several authors investigated the effect of agitation on the growth of cyanobacteria and microalgae in liquid cultures. For instance, Hu et al. (2019) showed that the agitation speed could improve the growth of *Porphyridium cruentum*. However, agitation can also be harmful to algal cultures; previous studies demonstrated that agitation was associated with damage to microalgal cell viability and indicated that hydrodynamic effects need to be studied carefully (Benavente-Valdes et al. 2017; Camacho et al. 2000). In our experiments, samples of *Chroococcidiopsis* sp. cultures subjected to agitation were observed under the microscope and no cellular damage was observed (data not shown). This suggests high resistance of *Chroococcidiopsis* sp. cell cover to mechanical stress, in good agreement with studies reporting similar behavior of other microalgal strains in response to strong culture agitation (Hanslmeier et al. 2012).

Figure 4.10 shows the complete growth curve at the standard culture in liquid BBM medium with constant agitation speed (500 rpm) and 3 mM of nitrate BBM medium. Under these conditions, the cultures of *Chroococcidiopsis* sp. showed an adaptation phase of 24 h. After this, the exponential phase reached its maximum on day 3-4. From this moment on, the decreased cell growth means the beginning of the stationary phase. This culture showed a productivity of 0.15 $g \cdot L \cdot d^{-1}$, which can be considered low as compared with the productivity value of 0.72 $g \cdot L \cdot d^{-1}$ reported for other cyanobacteria such as *Spirulina* (*Arthrospira platensis*). In this sense, no previous reports on the productivity of *Chroococcidiopsis* sp. in liquid cultures have been published. Thus, further characterization of the influence of cultivation factors on the productivity of the cyanobacterium is required to analyze its biotechnological potential and the culture implementation at large scale. In this Chapter, the study of the effect of the nitrogen source and its concentration on the culture medium was carried out.

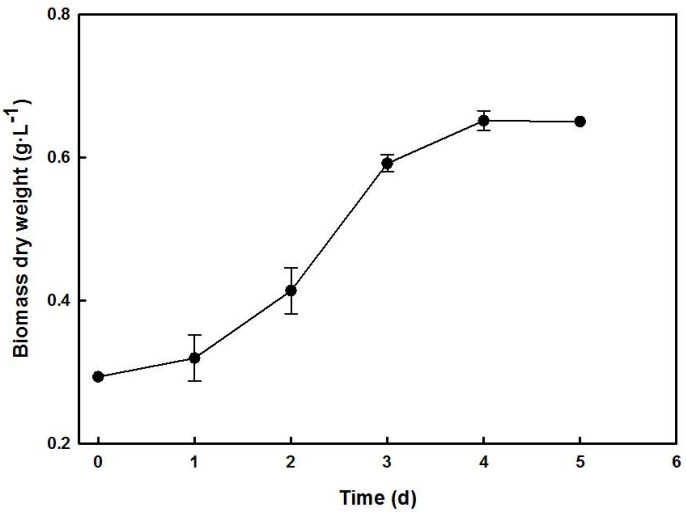

Figure 4.10. Time course evolution of dry weight in cultures of *Chroococcidiopsis* sp. Cells were grown in liquid BBM culture medium with 3 mM $NaNO_3$. Details of growth conditions are described in the Materials and Methods section.

Optimal nitrogen source for cultures

Nitrate (NO_3^-) is the most commonly used inorganic nitrogen form for cyanobacteria cultivation on synthetic media (Markou et al. 2014), with $NaNO_3$ being the most frequently used, followed by KNO_3 (Markou et al. 2014). This form of nitrogen did not produce toxicity to growing cells of *Chroococcidiopsis* sp. in liquid medium even at much higher concentration than that of standard culture media (3 mM). However, previous experiments have shown that an excessive increase in nitrate concentration affects culture growth negatively (Jeanfils et al. 1993) possibly due to a high intracellular levels of nitrite (NO_2^-) and/or ammonium (NH_4^+) produced from reduction of nitrate, which are toxic for the cells (Dai et al. 2014; Markou et al. 2014; Chen et al. 2009). Nitrite is frequently found in natural environments as an intermediate product of the nitrification process (oxidation of ammonium to nitrate), and also it is an intracellular intermediate of the nitrate assimilatory pathway, being the product of nitrate reduction catalyzed by the enzyme nitrate reductase.

In cyanobacteria, nitrate is reduced to nitrite, in a 2-electrons dependent reaction, catalyzed by ferredoxin-nitrate reductase (EC.1.7.7.2) and nitrite is reduced to ammonium in a 6-electrons dependent reaction catalyzed by ferredoxin-nitrite reductase (EC 1.7.7.1). The ammonium is further incorporated to the carbon skeleton of 2-oxoglutarate, by the catalytic action of glutamine synthetase and ferredoxin- glutamate synthase (GS-GOGAT cycle) to give rise a net molecule of L-glutamate. The required redox power and energy is provided by the photosynthetic chain (Vega 2018).

Although we initially used nitrate as nitrogen source to produce dense cultures of *Chroococcidiopsis* sp., the efficiency of other nitrogen sources and concentrations on the cell growth was assessed in liquid cultures of the cyanobacterium in order to select the most suitable nitrogen conditions. The nitrogen sources tested were $NaNO_3$, $NaNO_2$, NH_4^+ and urea, in addition to a nitrogen-free medium in which atmospheric N_2 (supplied as air) was the only nitrogen source available to the cyanobacterium. Three nitrogen levels of each nitrogen source were assayed: 3, 6 and 9 mM. Figure 4.11 shows the maximum cyanobacterial biomass concentration reached after 10 days of cultivation. The results obtained show that the culture medium with urea and the culture medium with nitrate achieved the highest biomass productivity, while culture media with nitrite, ammonium or air, as the only nitrogen source showed the lowest biomass yields. Regarding nitrogen concentration, in all cases 3 mM seems insufficient regardless the nitrogen source, while 6 and 9 mM supply enough, roughly, nitrogen to reach a 4-5-fold higher productivity, than the cultures with 3 mM of nitrogen (Figure 4.11).

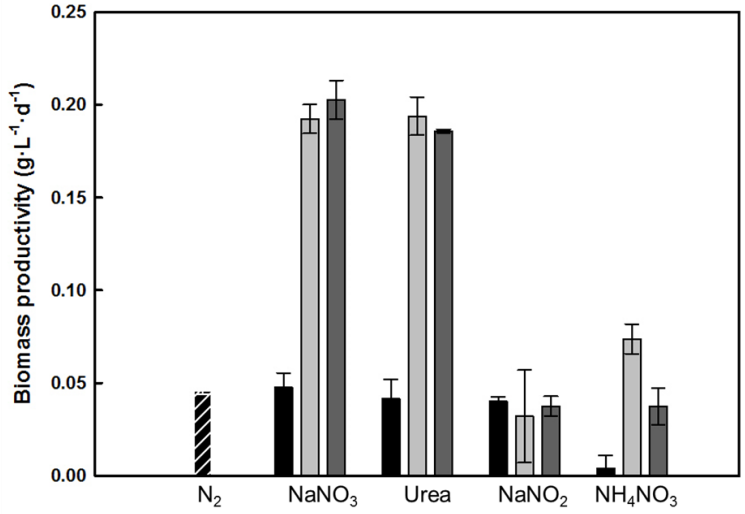

Figure 4.11. Effect of nitrogen source and nitrogen concentration on the growth of *Chroococcidiopsis* sp. Maximal productivity obtained from cultures with different nitrogen sources, at 0 (cross-hatched bar), 3 (black bars), 6 (light grey bars) or 9 (dark grey bars) mM of nitrogen availability. Other conditions are indicated in the Materials and Method section.

The cell viability under the assayed conditions was measured to evaluate the adequacy in the use of the different nitrogen sources for cell growth. The efficiency of PSII (Figure 4.12), measured in the form of fluorescence emitted upon light excitation of chlorophylls according to the procedure described in the Materials and Methods section, provides information on the photosynthetic capacity of growing cells. Any harmful effect of a given nitrogen source (or the lack of it) in the photosynthesis apparatus or process should be evidenced by the lowered efficiency of light collectors, the chlorophyll molecules. Incubating cyanobacteria cultures in nitrogen deficiency affects PSII reaction centers, thus decreasing the efficiency of the photosystem, due to both a dramatic decrease in chlorophyll antenna size and the photosynthetic dysfunction derived from the deficiencies in PSII protein structure (Cetner et al. 2017). For instance, the fact that the cyanobacterium is unable to grow on nitrite or ammonium, as only nitrogen source, may be due to the low photosynthetic efficiency shown by the cells in these cultures (Figure 4.12), derived from toxic effect of both nitrogen sources

to the cells as unveiled for instance, by the direct relationship between Qy and ammonium concentration.

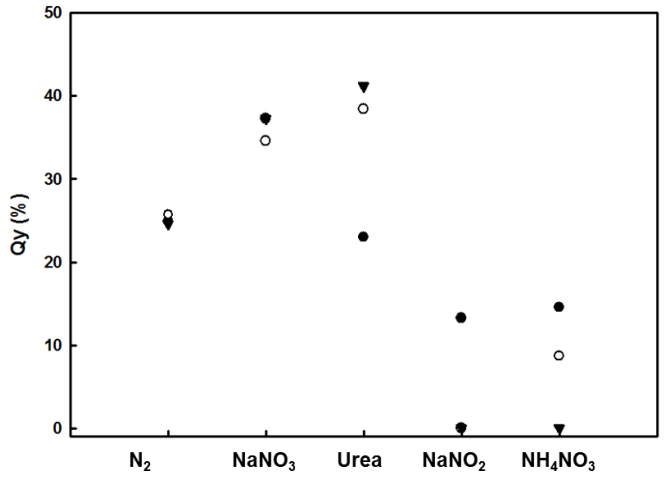

Figure 4.12. Maximum quantum yield (QY) of PSII (Fv/Fm) of *Chroococcidiopsis* sp. cells cultured under different nitrogen concentration and sources. Average Qy data during the exponential phase of growth. Symbols: (-●-) represents cultures with 3 mM of nitrogen, (-○-) 6 mM of nitrogen and (-▼-) 9 mM of nitrogen.

Overall, the biomass productivity of *Chroococcidiopsis* sp. cultures was improved until reaching a maximum value of 0.21 $g \cdot L^{-1} \cdot d^{-1}$, which is similar to that obtained with other photosynthetic microorganisms of extreme environment such as *Coccomyxa onubensis* (Fuentes et al. 2016) and suggests that *Chroococcidiopsis* sp. could be produced at larger scale with moderate nitrate concentration in the culture medium.

Analysis of *Chroococcidiopsis* sp. biomass

The elemental composition of microalgae and cyanobacteria provides helpful information in order to define the suitable nutrient requirements for optimal growth. In this work, the elemental stoichiometric composition of *Chroococcidiopsis* sp. biomass was obtained. To do this, the previously selected cultivation conditions, 9 mM of nitrogen in the form of nitrate and constant agitation, were applied to produce cyanobacterial biomass whose elemental composition (C,H,O,N) was analyzed (Table 4.6).

The results showed that the elemental stoichiometric composition of *Chroococcidiopsis* sp. under the above conditions was $\mathbf{CH_{1.67}\ O_{0.50}\ N_{0.15}}$. Compared to the elemental composition of a marketed microalgal genus, *Chlorella*, $CH_{1.78}O_{0.36}N_{0.12}$ (Kemp 2000), both have very similar compositions.

According to the stoichiometric composition obtained for *Chroococcidiopsis* sp., 1 mol of biomass weights 23.77 g which is slightly higher than that obtained by Duboc et al. (1999) for *Chlorella* sp., 21.15 $g \cdot mol^{-1}$. The C to N ratio for *Chlorella* sp. (8.3) is a bit higher than that for *Chroococcidiopsis* sp. (6.7), and the content of oxygen is significantly higher in the *Chroococcidiopsis* sp. biomass. Regarding the latter, although the biochemical arguments to explain such higher oxygen content in *Chroococcidiopsis* sp. biomass could be numerous, and assigning accurately the most influential one would be difficult, the higher carbohydrate content of *Chroococcidiopsis* sp. (Table 4.7). might partly explain the higher oxygen presence in the elemental composition of the biomass.

Table 4.6. Elemental composition of *Chroococcidiopsis* sp. biomass. The values express the relative abundance of each chemical element per 100 g of biomass (%).

Element	Mass percentage (%)
Carbon, C	48.12
Hydrogen, H	6.70
Oxygen, O	31.85
Nitrogen, N	8.16

Composition of the cyanobacterial biomass

The culture that produced the highest biomass concentration is that grown on 0.9 mM of nitrate as only nitrogen source and was selected to determine the chemical composition of the biomass and to evaluate possible biotechnological applications. To perform these studies, the samples were

taken from the exponential growth phase, the cells were harvested and further used for proximate analysis corresponding biomass analyzed.

The analysis of *Chroococcidiopsis* sp. biomass (Table 4.7) showed similar composition to that from other cyanobacteria (Banerjee et al. 2011; Rajeshwari et al. 2011; Caudales et al. 2000). Carbohydrates of cyanobacteria are usually found in the form of glucogen, glucose and other related carbohydrates (Khan et al. 2018), which are easily digested, making cyanobacteria generally suitable as food and feed (Pulz and Gross 2004).

In the case of the *Chroococcidiopsis* sp. described in this Thesis work, the carbohydrate content is high compared to other cyanobacteria, such as *Spirulina platensis* (Shekharam 1987), commonly used as healthy food supplement. Under the growth conditions described in the Materials and Methods section, *Chroococcidiopsis* sp. contains 45% carbohydrates. Although the cultivation conditions might have an impact in the carbohydrate content (Cheng et al. 2017), the results obtained in *Chroococcidiopsis* sp. might be related to the polysaccharide sheath that protects this cyanobacterium against desiccation from its extreme natural environment (Billi et al. 2017). Although the precise chemical composition of the *Chroococcidiopsis* EPS remains unknown, previous studies showed that the EPS composition of different cyanobacteria consists mostly of glucose, galactose, mannose, xylose and rhamnose, with varying specific monosaccharide abundance depending on species and cultivation conditions (Kumar 2018). Assessing the potential bioactivity of the EPS produced by *Chroococcidiopsis* sp. would require further research to get insight knowledge of the EPS chemical composition.

The high protein content of cyanobacteria and microalgae makes them an interesting alternative as human food and animal feed. The protein potential as food and feed would be determined by the aminoacids profile and their bioavailability, which means the amount of aminoacids from cyanobacteria that can be absorbed by any organism (Caporgno and Mathys 2018). The protein content of the cyanobacteria *Chroococcidiopsis* sp. showed lower values than *Spirulina platensis* (Table 4.7).

Table 4.7. Analysis of biomass from *Chroococcidiopsis* sp.

	***Chroococcidiopsis* sp.**	***Spirulina platensis***	**References**
Ash	8.28	7.4-16	Tokuşoglu and Ünal (2003)
Carbohydrates	45.40	8-14	Rempel (2018)
Proteins	36.72	50-65	Roy and Pal (2015)
FA	5.60	2.70 - 6.80	Rajeshwari and Rajashekhar (2011).
Nucleic acids	<9.50*	4-6	Gutiérrez-Salmeán et al. (2015)

*According to the analysis performed by Finkel et al. (2016) by means of a statistic model applied to a number of microalgae and cyanobacteria. The values were expressed as percentage (g/100g of dw).

Fatty acids are interesting compounds from the industrial point of view. The content of the cyanobacterium in fatty acids is similar to that reported for *Spirulina platensis* (Rajeshwari and Rajashekhar 2011). The fatty acid content of nutrient-sufficient exponentially growing cells of *Chroococcidiopsis* sp. can be considered relatively low when compared to that of many microalgal species (Pratoomyot et al. 2005; Mata et al. 2010). Nevertheless, further studies specifically designed to address increased lipid content in *Chroococcidiopsis* sp. cultivated under suitable conditions, should be expected to improve the lipid productivity of the cyanobacterium. As a first step, knowing the fatty acids profile of *Chroococcidiopsis* sp. would allow to evaluate whether the cyanobacterium could have potential as PUFA producer.

Accordingly, the fatty acids profile of *Chroococcidiopsis* sp. was obtained following the procedure described in the Materials and Materials section. Table 4.8 shows the main fatty acids of the cyanobacterium and their relative abundance with respect to the total fatty acids content. The major fatty acids were palmitic (C16:0; 29.86%) and palmitoleic (C16.1; 12.75%) acids, as in *Spirulina platensis* (Cañizares-Villanueva et al. 1995). Linoleic (C18: 2n6c + C18: 2n6t), with a relative abundance of 18.2%, and stearic (C18:0; 9.64%) acids are also abundant in *Chroococcidiopsis* sp. grown under the aforementioned conditions. From the total fatty acids content, 40.65% was assigned to saturated fatty acids, 12.75% to monounsaturated fatty acids and

31.42% to PUFA. Interestingly, roughly 30% of the total fatty acids content of nutrient-sufficient exponentially growing cells of *Chroococcidiopsis* sp. corresponded to linoleic and linolenic acids, essential PUFAs for humans. Other PUFA with relevance in food and feeding applications such as EPA and DHA were not detected during the peak integration process, according to the fact widely reported that these PUFAs are not particularly abundant in cyanobacteria (Guedes et al. 2011).

Table 4.8. Fatty acid composition of *Chroococcidiopsis* sp. biomass. The values express the relative abundance (%) of each fatty acid with respect to the total fatty acids content of the biomass. The analysis was performed on biomass samples collected during the exponential phase of cultures grown on 9 mM of nitrate.

Fatty acid	Common name	Relative abundance (% Fatty acid)
C10:0	Capric acid	6,29
C11:0	Undecylic acid	1,62
C12:0	Lauric acid	1,13
C13:0	Tridecylic acid	0,99
C14:0	Myristic acid	0,01
C14:1	Myristoleic acid	0,98
C15:1	Pentadecylic acid	1,10
C16:0	Palmitic acid	29,86
C16:1	Palmitoleic acid	12,75
C16:2	Hexadecadienoic Acid	0,10
C17:0	Margaric acid	0,30
C17:1	Heptadecenoic acid	1,10
C18:0	Stearic acid	9,64
C18:1n9c + C18:1n9t	Oleic acid	2,30
C18:2n6c + C18:2n6t	Linoleic acid	18,20
C18:3n3	Alpha-linolenic acid (ALA)	10,92
Unknown		2,20
Saturated		40,65
Monounsaturated		12,75
Polyunsaturated		31,42

Biotechnological applications of *Chroococcidiopsis* sp.

Most of the compounds mentioned above have been reported to display bioactivities which have been described in the Introduction section and could have potential applications in biotechnology. In this Thesis work, some of the main valuable cyanobacterial molecules with potential biotechnological applications were selected and targeted for further analyzing their production by the isolated cyanobacterium. Results obtained from the first analysis of the content of these molecules in growing cells of *Chroococcidiopsis* sp. (data not shown) suggested the interest of carrying out deep studies on their accumulation. This was investigated in this work and the results are shown and discussed in Chapters 5 and 6. The major biochemical composition of *Chroococcidiopsis* sp. shows a valuable profiles of carbohydrates, proteins and the polyunsaturated fatty acids linoleic and linolenic (Table 4.7 and 4.8), all of them with commercial value, particularly to improve the nutritional quality of food and feed resulting in positive effects on human and animal health (Gouveia and Oliveira 2009). Table 4.9 compares the major biochemical composition of different cyanobacteria with that of various food sources. The results show that *Chroococcidiopsis* sp. has higher protein content than conventional foods such as rice and milk, similarly to *Spirulina platensis*. In addition, its carbohydrate content is higher than in products such as meat, milk and soybeans.

Table 4.9. Major biochemical composition of different foods and cyanobacteria.

	Proteins (%)	**Carbohydrates (%)**	**Lipids (%)**
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soya	37	30	20
Chroococcidiopsis sp.	36.72	45.4	5.6 (FA)
*Spirulina maxima**	50-65	8-14	4-9

*Roy and Pal (2015)

We also analyzed the potential of *Chroococcidiopsis* sp. to produce phycocyanin, a blue pigment present in cyanobacteria, which commercial value trades for at least 500 US$ per kg^{-1} for food-grade and 125 US$ per mg^{-1} of analytical grade (SigmaAldrich). As detailed in the Introduction section of Chapter V, this pigment is especially valuable in the cosmetic and pharmaceutical industry due to its wide array of bioactivities. Under the cultivation conditions detailed above in this Chapter, *Chroococcidiopsis* sp. produces 17 $mg \cdot g^{-1}$ of phycocyanin after 9 days of growth. This quantity is produced under non-optimal conditions of accumulation, thus an increase in the production of this pigment should be expected by adjusting culture parameters that might favor its production, such as the quality and quantity of light (Kumar et al. 2011). This was studied in this Thesis (Chapter V).

In short, the cyanobacterium isolated from hyper-arid environment, *Chroococcidiopsis* sp., was able to grow in liquid culture medium, under vigorous agitation and plenty of nutrients, achieving moderate biomass productivities. The harsh conditions the cyanobacterium has to cope with in nature –extreme water scarcity and relatively high UV radiation- make it attractive to screen for valuable compounds of biotechnological application. Biomass produced under replete nutrient condition is rich in carbohydrates and in linoleic and linolenic acids, as main valuable molecules of eventual application as food and feed. In addition, *Chroococcidiopsis* sp. seems to have potential for phycocyanin accumulation, valuable compound used as colorant marker. In addition, the cyanobacterium tendency to produce sediment at the bottom of the cultivation flasks should eventually be taken as an advantage to eventually produce the cyanobacterium at large scale.

CHAPTER V

PHYCOBILIPROTEINS ACCUMULATION IN *CHROOCOCCIDIOPSIS* SP.

Abstract

Chroococcidiopsis sp. is an endolithic cyanobacterium isolated from gypsum rocks of Atacama Desert (Chile). This cyanobacterium uses phycobiliproteins as auxiliary pigment to collect light energy under low light intensity. These compounds are widely used as a colorant in the cosmetic and food industry. In addition, phycocyanin has been reported to display anti-oxidative, anticancer and anti-inflammatory activities. Determining physicochemical cultivation conditions that trigger pigments accumulation in the cyanobacterium would help to develop competitive production processes at large scale. In this Chapter, the effect of low light intensity on phycobiliproteins accumulation by *Chroococcidiopsis* sp. has been investigated. The cyanobacterium was grown at 10, 50, 70, 100 and 150 μmol photons m^{-2} s^{-1} reaching the maximum total phycobiliprotein content, 204 $mg \cdot g^{-1}$, under the lower light intensity of 10 μmol photons m^{-2} s^{-1}. Phycobiliproteins profiling was also analyzed, unveiling that phycocyanin and phycoerythrin content increased as light intensity decreased, however, a decrease in allophycocyanin was observed in cyanobacterial cells cultivated under low light intensity of 10 μmol photons m^{-2} s^{-1}. In short, the results unveil that accumulation of specific phycobiliproteins of *Chroococcidiopsis* sp. is light intensity-dependent, which thus must be carefully addressed at large scale, because each targeted pigment production requires different conditions.

Keywords: Cyanobacterium; *Chroococcidiopsis* sp.; Phycobiliproteins; Phycocyanin; Allophycocyanin; Phycoerythrin

Introduction

In cyanobacteria, the photosynthetic apparatus consists of two photosystems (also found in eukaryotic microalgae and plants) and a characteristic phycobilisome (PBS) mainly composed of phycobiliproteins (PBP) and uncolored proteins, which contribute to attach the system to thylakoidal membranes (Pagels et al. 2019). However, the composition of the PBS varies among species, and even in cyanobacterial cells acclimated to environmental factors including nutrient level shifts (Guan et al. 2007).

PBS has a role as antenna pigments used by cyanobacteria to efficiently capture light, and transfer the light energy to chlorophyll molecules that drive it to the reaction centers where the light-transduction process into chemical energy takes place. Each phycobilisome contains a core consisting of allophycocyanin, which together with phycocyanin and phycoerythrin form a light harvesting panel, that which efficiently transfer the energy absorbed to chlorophyll pigments for their use in photosynthetic reactions (Figure 5.1). Besides the light-harvesting function, PBS also plays an important role in the photo-protection mechanisms expressed under high irradiances (Kirilovsky and Kerfeld 2013).This is because the antenna pigments also function as an energy dissipation mechanism moderating the energy that reaches the photosynthetic centers, thus protecting the reaction centers from light-induced damage under high light conditions (Gwizdala et al. 2011)

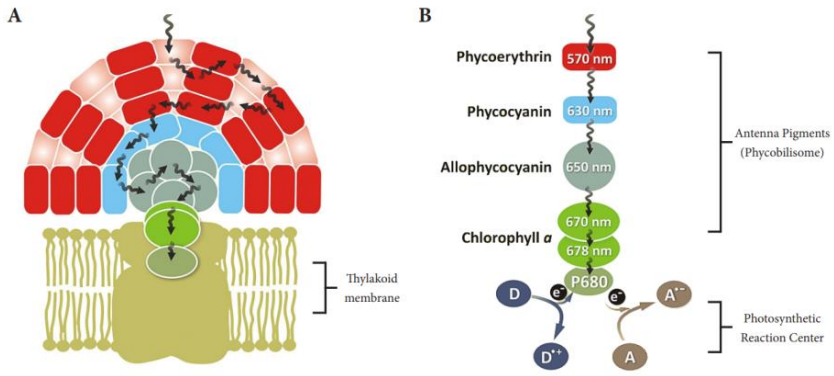

Figure 5.1. Schematic diagram of a phycobilisome (A) and the energy transfer steps (B). D, donor, A. acceptor. (Govindjee and Shevela 2011).

The most relevant applications of PBPs are listed in Table 5.1, and they are particularly important for human health (Singh et al. 2015). The PBPs are classified into three groups based on their absorption of PAR light. (1) phycoerythrin (PE), has a maximum absorbance peak at 540-570 nm. This pigment has been shows to display antitumor activity against human liver carcinoma cells SMC 7721 (Bei et al. 2002) and recently its anti-Alzheimer potential has been reported (Sonani et al. 2015). (2) The intermediate energy PBP known as phycocyanin (PC) has its maximum absorption peak at 610-620 nm. PC has been reported to display high antioxidant capacity (Sonani et al. 2016) and to inhibit cell proliferation of human leukemia K562 cells (Bermejo et al. 2008), lung cancer, liver cancer, breast cancer, ovarian cancer, colon cancer and malignant melanoma (Hao et al. 2019); and (3) the low energy allophycocyanin (APC) (λmax 650-655 nm) inhibits the enterovirus 71-induced cytopathic effects (Wollina et al. 2018).

Table 5.1. Application of phycobiliproteins in pharmaceutical and biomedical industries.

PBP	λmax	Medical applications	Other applications
Phycoerythrin (PE)	540-570 nm	Anti-Alzheimer and anti-liver cancer	Fluorescent biomarker (Martínez et al. 2019)
Phycocyanin (PC)	610-620 nm	Antioxidant and anti-cancer (lung, liver, breast, ovarian, colon and malignant melanoma)	Food colorant (Stanic-Vucinic et al. 2018)
Allophycocyanin (APC)	650-655 nm	Antiviral	Quantum yield assay (Jevremovic et al. 2019)

In addition to medical applications, PBP are used in food and cosmetic industry. Specifically, phycocyanin is currently present in everyday products such as biscuits, pasta, milk-based products and various breads (Stanic-Vucinic et al. 2018). In addition, due to their unique fluorescent properties, PBP is also used in flow cytometry, fluorescent immunoassays and

fluorescence microscopy and it can also be used as protein markers for electrophoretic techniques (Sonani et al. 2016). Due to this wide range of applications of phycobiliproteins, the biotechnological production of PBP gained scientific relevance in recent years (Khazi et al. 2018), and its production by cyanobacteria seems interesting for market. PBP intracellular accumulation is highly influenced by a number of parameters such as nutrient availability, high pH, light and salinity (Pandey et al. 2011) with light intensity being the most influencing one. Light triggers photoadaptation and photoinhibition processes in microalgae and cyanobacteria, in which photosynthetic cells modify their biochemical composition, often with alterations in their ultrastructure and physiological behavior (Juneja et al. 2013).

Light was reported to exert a major influence on the structure of the PBS. Particularly, relative abundance of phycobiliproteins was shown to shift upon changed intensity of incident light on cyanobacterial cultures (Stanic - Vucinic et al. 2018; Chenu et al. 2017; Lee et al. 2016; Ojit et al. 2015). For instance, Chenu et al. (2017) described the adaptation of the PC:APC ratio of PBS antennas as response to the light intensity, which results in shifted contents of these valuable pigments, thus having an impact on their biotechnological production.

In this Chapter, the production of phycobiliproteins during the growth of *Chroococcidiopsis* sp. and the influence of light intensity (10, 50, 70, 100 and 150 μmol photons m^{-2} s^{-1}) on cell growth and pigment production, especially phycobiliproteins, are analyzed. The results allowed us to determine suitable light conditions for producing cyanobacterial biomass with high intracellular PBP content. These results should be taken into account for any eventual production at large scale.

Materials and Methods

Microorganism and standard growth condition.

The cyanobacterium *Chroococcidiopsis* sp. was isolated from gypsum samples as it was described in Chapter IV of this Thesis. This cyanobacterium was cultured at 25ºC, in erlenmeyer flasks (<1L) containing sterile BBM (Bold's Basal Medium) liquid culture medium. According to our previous studies, nitrate 9 mM was used as the only nitrogen source and the pH adjusted was at 7.0. These conditions of cultivation are so-called "standard" in this Thesis. The cultures were continuously illuminated with white light fluorescent tubes (Philips 30W/33), and the distance of the different cultivation flasks to the lamps was used to control the incident light intensity on the culture surface. Under standard conditions, the light intensity was 70 μmol photons·m^2·s^{-1} of PAR light on the surface of the culture flasks.

Semicontinuous cultivation

Some of the experiments in this manuscript were performed in semicontinuous mode. For this, optical density of cyanobacterial cultures was kept within a short interval of optical density values. That was done by diluting the cultures with fresh culture medium on a daily basis. The semicontinuous cultures were performed in 500 mL Erlenmeyer flasks, and the cultures incubated in a culture room under controlled temperature (25 ºC) and continuous illumination of white fluorescent light at a fixed light intensity of 10, 50, 70, 100 and 150 μmol photons m^{-2} s^{-1}.

Biomass productivity measurement

The cyanobacterial growth evolution was daily assessed spectrophotometrically (Evolution 201, Thermo Fisher Scientific, USA) by following optical density (O.D.) at 750 nm. Alternatively, dry weight measurements were carried out using 10 mL samples of each culture which were filtered through a glass microfiber filters of 47 mm diameter and 0.7 μm pore size (MFV-5, AnoiaFilterlab, Spain). Biomass productivity was calculated as the increase in dry weight in a given culture volume during a specific period of time in the linear phase of the cyanobacterial growth. Similarly, the specific productivity of each relevant metabolite produced by

the cyanobacterium was calculated as the increase in the quantity of this product in a given culture volume, over a given period of time.

Maximal photosynthetic efficiency

The photosynthetic performance was evaluated by measuring the chlorophyll fluorescence in dark-acclimated cells, considered as maximum photosynthetic efficiency of photosystem II (Fv/Fm). This parameter was determined as indicated in Chapter 4.

Pigment extraction and PBP determination

The pigment extraction was performed in 2 steps. First, 2mL of cyanobacterial culture were placed into Eppendorf tubes (the amount of cell suspension required for analysis could vary according to the culture cell density). After that, the cells were centrifuged at 14000 x *g* for 5 min and supernatant was discarded. Glass beads of 0.25-0.5 mm and 1 mL of methanol were added to the different samples and the cells were disrupted in a bead miller (Restch M400, Restsh GmbH, Germany). Disruption procedure consisted of 5 cycles of 5 min at max speed followed by 30 seconds pause). Then, the cells were centrifuged again at 14000 x *g* for 10 min. After that, the supernatant was carefully removed with a glass Pasteur pipette and transferred into a new Eppendorf tube. Then, 1 mL of methanol was added again to the resulting pellet and the mixture was vortexed for 10 s and centrifuged (14000 x *g* during 10 min). After that, a blue pellet was obtained.

In order to analyze the phycobiliproteins content it was necessary to add 1mL of phosphate buffer (0.1 M; pH= 7) to cell pellet. Next, the samples were vortexed for 30 seconds and placed into water bath with ultrasound, at 27-30ºC. The samples were centrifuged at 14000 x *g* during 10 min. Then, supernatants were transferred into a clean Eppendorf tube by a pipette to measure the phycobiliproteins content.

To analyze the phycobiliproteins content, the absorbance of the obtained cyanobacterial extracts was measured at 565 nm, 620 nm and 650 nm. After that, the concentration of phycobiliproteins content was calculated by the following equations, as described by Round et al. (1989):

$$Phycocianin[PC; mg/mL] = [A_{620} - (0.72\ x\ A_{650})]/6.29$$

$$Allophycocianin\ [AC; mg/mL] = \ [A_{650} - (0.191\ x\ A_{620})]/5.79$$

$$Phycoerythrin\ [mg/mL] = \ \left[A_{565} - \left((2.41\ x\ CPC) - (1.41\ x\ CAPC)\right)\right]/13.02$$

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

Results and Discussion

Growth and phycobiliproteins production of *Chroococcidiopsis* sp.

The cyanobacterial cultures were grown under standard conditions. Figure 5.2A show the time course evolution of *Chroococcidiopsis* sp. The cultures showed a lag phase until day 5, followed by an exponential growth phase between day 5 and 13. During this phase, maximum turbidity of 3 at 750 nm was reached. After this growth period, the culture reached the stationary phase (day 14). Similar growth curve was obtained by representing dry weight time course evolution (Figure 5.2B).

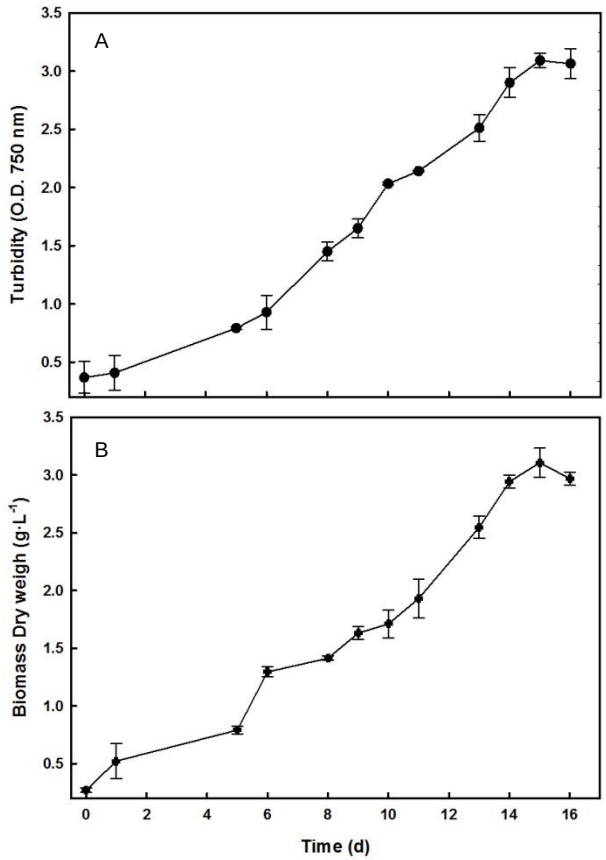

Figure 5.2. *Chroococcidiopsis* sp. growth evolution. O.D. at 750nm (A) or dry weight of culture broth (B) were determined at the indicated times. Error bars show standard deviation of replicates. More details in the Materials and Methods section.

The maximum photosynthetic efficiency of PSII gives relevant information about the cell viability of the cultures, being a useful parameter to evaluate the effect that the light causes on the photosynthetic activity. As it can be seen in Figure 5.3., cultures grown under 70 µmoles of photons·m^2·s^{-1} maintain a constant photosynthetic efficiency of 40%, proving to be in line with the values reported for non-stressed cyanobacteria (Baqué et al. 2013).

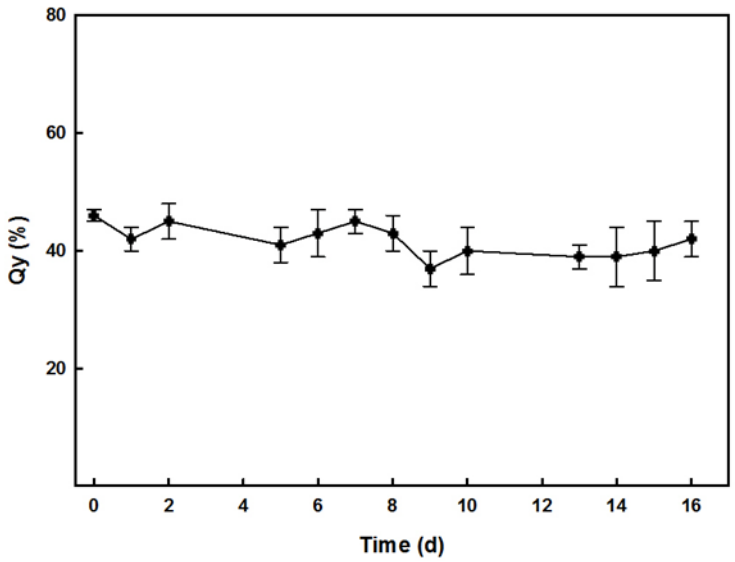

Figure 5.3. Maximum photosynthetic efficiency of *Chroococcidiopsis* sp. growing cultures. Time-course evolution of quantum yield (Qy) of *Chroococcidiopsis* sp. cultures under constant light intensity of 70 µmol of photons·m^{-2}·s^{-1}. Details of growth conditions are described in the Materials and Methods section.

As can be seen in Figure 5.4, the content of phycobiliproteins in *Chroococcidiopsis* sp. was affected by the age of the culture, the largest productivity being obtained during the lag phase. Specifically, a slight increase in PBP content was observed during the first days of growth. This may be caused by the lower cell density in the culture which therefore results in a higher amount of light per cell is higher which may induce a ROS-dependent photoinhibition of the photosynthesis apparatus. Phycobiliproteins

and especially allophycocyanin have been shown to display antioxidant and radical scavenging activity (Ge et al. 2006).

On the other hand, a slight increase in phycobiliprotein content is observed after 10 days of growth if compared with early exponential phase values (Figure 5.4). At the exponential phase, an increase of the cell density was produced and therefore decreases the amount of light available per cell. Consequently, the increased PBP content might be related to the need for light harvesting in dense cultures. Since nutrient starvation has a negative effect on the production of PBP, this pigment accumulation cannot be a consequence of nutrient limitation at this stage. It has been shown that phosphorus, nitrogen and sulfur starvation cause a decrease in phycobiliproteins content in cyanobacteria, because under starvation conditions cyanobacteria can use phycobiliproteins as a source of nutrients (Khatoon et al. 2018; Markou and Nerantzis 2013).

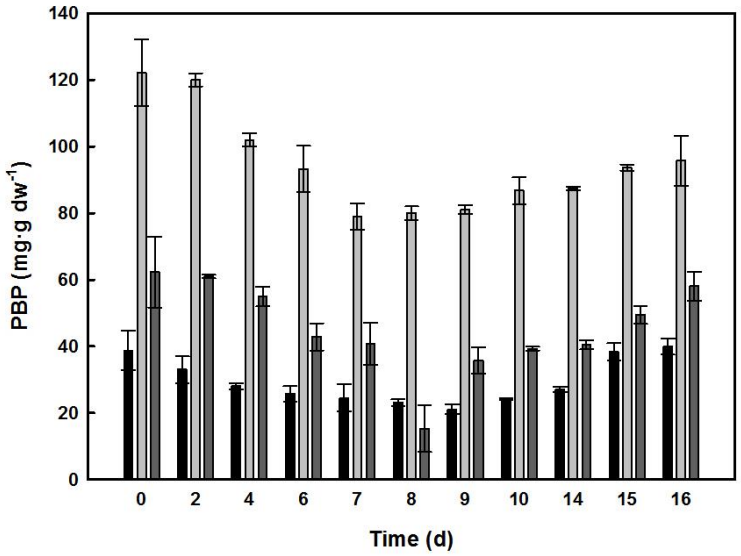

Figure 5.4. Time-course evolution of phycobiliproteins content in *Chroococcidiopsis* sp. cells. Dark bars represent the content in phycocianin (PC), light gray bars represent the content in allophycocyanin (APC); and dark gray bars represent the content in phycoerythrin (PE). Details of growth conditions and pigment extraction and determination are described in the Materials and Methods section.

Quantity and profile of phycobiliproteins has been analyzed according to the age of the culture, as the profile of phycobiliproteins is species-dependent and their abundance changes depending on cultivation conditions (Rizzo et al. 2015). The maximum APC content of 122.25 $mg \cdot g^{-1}$ was reached during the first day of growth. The maximum PC values were reached during the stationary phase (39.88 $mg \cdot g^{-1}$). Finally, the maximum PE content was reached on day 1 (62.35 mg·g-1) and followed same trend as PC and APC. Interestingly, all the intracellular pigments accumulation decreased during the exponential growth phase while the maximum amount is achieved during lag and stationary phase. An intracellular accumulation during the stationary phase could be related to the powerful antioxidant role reported for phycobiliproteins which could be expressed to attenuate the oxidative stress generates during senescence.

Total content of phycobiliproteins does not differ from other cyanobacteria (Khatoon et al. 2018; Khazi et al. 2018) which roughly accounts for about 20% of total dry weight. However, the content of APC in *Chroococcidiopsis* sp. is particularly high compared to other works (Khazi et al. 2018). APC content of *Chroococcidiopsis* sp. becomes more than 10% of the total dry weight which suggests high potential of the cyanobacterium for large scale production of this valuable pigment.

Effect of light intensity on the growth and phycobiliproteins production of *Chroococcidiopsis* sp.

Once light availability was suspected to influence PBP production in batch cultures of *Chroococcidiopsis* sp., an experiment to determine the effect of light intensity on PBP production was carried out. In batch mode, a given light intensity is set up but cell concentration varies throughout the cultivation period so the amount of light received per cell decreases continuously in the course of time, which impedes an accurate analysis of light-dependent PBP accumulation.

Thus, the experiment was run in repeated-batch mode so that biomass concentration, in each culture was kept within a narrow values range. This allowed studying the effect of a specific light intensity on growth and PBP production

In order to assess the phycobiliproteins production in *Chroococcidiopsis* sp. cultures under semicontinuous mode, independent cultures were incubated under an incident light of 10, 50, 70, 100 and 150 µmoles of photons · m^{-2}·s^{-1}. The cultures turbidity was kept within an optical density range between 0.7 and 0.9 (Figure 5.5A) which corresponds to a dry weight of 0.47± 0.016 g·L^{-1} (Figure 5.5B).

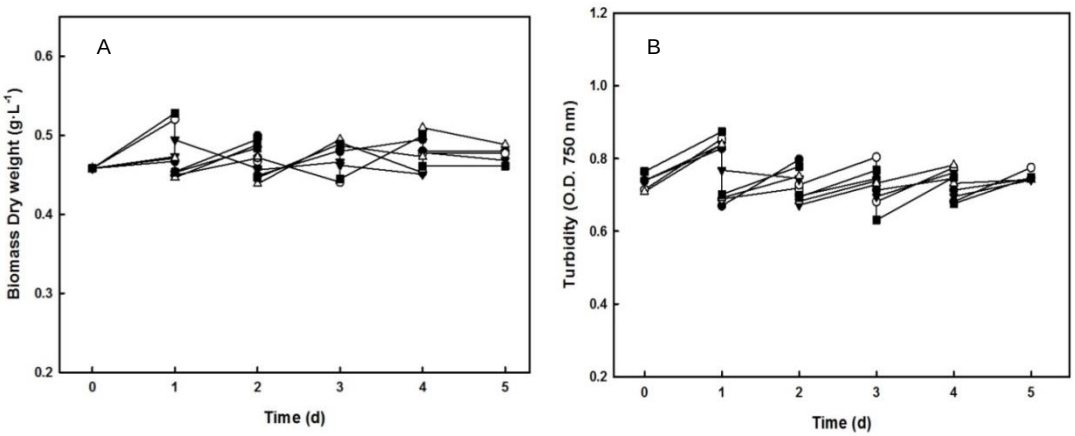

Figure 5.5. Semicontinuous cultivation of *Chroococcidiopsis* sp. grown under different light intensity. Cells from standard cultures at 70 µmoles of photons ·m^2·s^{-1} were harvested during the exponential phase of growth, washed and resuspended in fresh standard culture medium. Each culture was growth in semicontinuous mode, and continuously illuminated with continuous white fluorescent light of 10 (-●-); 50 (-○-); 70 (-▼-); 100 (-Δ-) and 150 µmol photons m^{-2} s^{-1} (-■-). When indicated, optical density at 700 nm (A) and dry weight (B) were determined as indicated in the Materials and Methods section.

Figure 5.5 shows the semicontinuous cultivation of *Chroococcidiopsis* sp. cultures irradiated with different light intensities and Figure 5.6 shows the maximum efficiency of PSII (Qy). During the first 24h of the experiment, a decrease in Qy of 76% and 84%, with respect to the value at zero time of culture under irradiances of 100 and 150 µmol photons m^{-2} s^{-1} is observed. This could be due to the cells adaptation to the higher light intensity as the inoculum was taken from a stock culture adapted to 70 µmol photons m^{-2} s^{-1}. On the contrary, no significant effects were observed in cells subjected to low light intensity (10 and 50 µmol photons m^{-2} s^{-1}) with respect to control culture. After 5 days of cultivation, all cultures showed Qy values that may be

compatible with healthy cyanobacterial cells, but cultures with high light intensity showed a significant inhibition of photosynthesis, with respect to the other cultures.

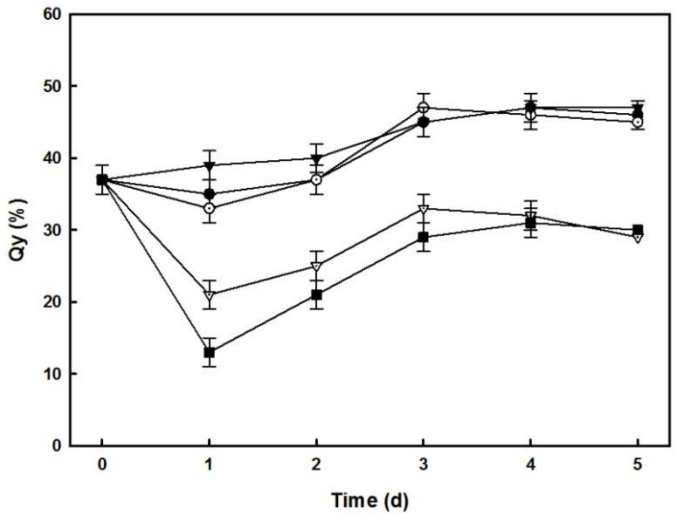

Figure 5.6. Effect of light intensity on the maximum photosynthetic efficiency of *Chroococcidiopsis* sp. The cyanobacterium was grown in semicontinuous mode, under continuous irradiance of 10 (-●-); 50 (-○-); 70 (-▼-); 100 (-Δ-) and 150 μmol photons m^{-2} s^{-1} (-■-). Details of growth conditions are described in the Materials and Methods section.

The intracellular PBP content is affected by the incident light intensity on the cultures. Table 5.2 shows an increase in the production of total PBP was produced at low irradiance in *Chroococcidiopsis* sp. The maximum values were reached in cultures subjected to 10 μmol photons m^{-2} s^{-1}, with 204.9 mg·g^{-1} of PBP, followed by cultures subjected to 50 μmol photons m^{-2} s^{-1} with values of 187 mg·g^{-1} of PBP. However, no differences were found between the productivity of PBP at irradiances of 70 μmol photons m^{-2} s^{-1} and higher. The results suggest that PBP accumulation in *Chroococcidiopsis* sp. could be enhanced at low light irradiance. This effect has been described in other cyanobacteria (Pandey et al. 2011).

Table 5.2. Total PBP in *Chroococcidiopsis* sp. under different light intensities (% dw).

Light intensity (µmol photons m^{-2} s^{-1})	Total PBP (% dry weight)
10	20.5 ± 0.3
50	18.7 ± 0.7
70	14.8 ± 0.8
100	15.0 ± 1.3
150	15.6 ± 0.7

Data represents the means of three independent experiments ± standard deviation.

Phycocyanin and phycoerythrin are maximal at the lower light intensity of 10 µmol photons m^{-2} $s^{-1,}$ while allophycocyanin is lower than control culture (70 µmol photons m^{-2} s^{-1}) (Fig. 5.7). Although Atacama Desert has the record in high light intensity in the world, *Chroococcidiopsis* sp. grows inside the gypsum rock and thus it will receive low light intensity (Viteck et al. 2017). Cultures under low irradiance (10 and 50 µmol photons m^{-2} s^{-1}) produced the highest PC values reaching 86.4 and 61.5 $mg \cdot g^{-1}$ on day 5, which represents an increase of 49.8% and 86.4%, respectively, if compared to the control culture illuminated with PAR light of 70 µmol photons m^{-2} s^{-1}. This delay might be related to the required adaptation period to light shift by adjusting the PBP content and maximizing photosynthetic efficiency (Grossman 2003).

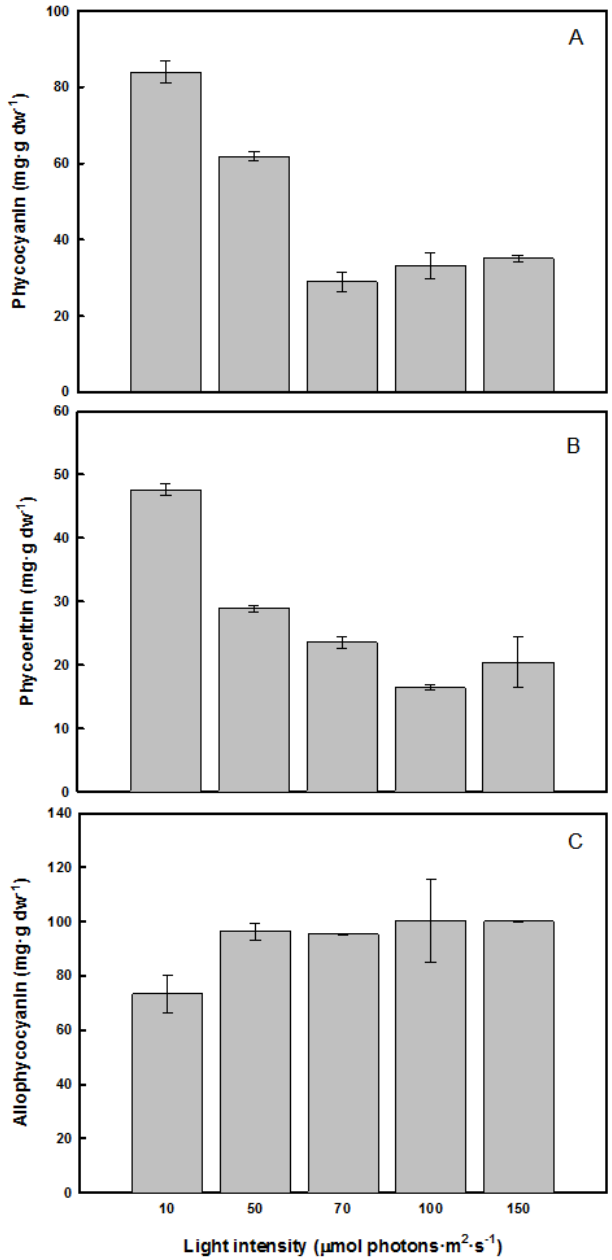

Figure 5.7. Effect of light intensity on phycobiliproteins content of *Chroococcidiopsis* sp. Cultures of the cyanobacterium were continuously illuminated, during 5days, with light intensity of 10 (-●-); 50 (-○-); 70 (-▼-); 100 (-Δ-) and 150 µmol photons m^{-2} s^{-1} (-■-).Details of growth conditions are described in the Materials and Methods section.

Figure 5.7B shows the maximum intracellular accumulation of PE. Cultures subjected to low irradiance (10 and 50 μmol photons m^{-2} s^{-1}) had the highest PE values, of 47.6 mg·g^{-1} and 28.9 mg·g^{-1} respectively after 5 days of semi-continuous cultivation at constant irradiance. For cultures under high irradiance (100 and 150 μmol photons m^{-2} s^{-1}) no differences were observed in the production of PE. The results showed that PE content decreased as light intensity increased, which is in good agreement with that observed in other species of cyanobacteria (Guihéneuf et al. 2015) since at low light intensity more PE is produced in order to harvest the maximum amount of photons (Sosa-Hernández et al. 2019).

The genus *Chroococcidiopsis* isolated from Qatar's environment (Das et al. 2018) produced PBP of 22.6 mg·g^{-1} that comprised of both PC (11.4 mg·g^{-1}) and PE (10.6 mg·g^{-1}), (APC was not tested) under 700 μmol photons m^{-2} s^{-1}. In our study we found a maximum production of 200 mg·g^{-1} of PBP in cultures of *Chroococcidiopsis* sp. from the Atacama Desert (Chile). This variability in PBP content between species might reflect the adaptation capacity of the *Chroococcidiopsis* genus to extreme variations in environmental conditions and, in addition, it point out the relevance to optimize conditions to trigger accumulation of these valuable molecules.

Regarding the accumulation of APC, according to Figure 5.7C, after 5 days of cell growth, no differences are found in APC production with the exception of cultures subjected to very low irradiance (10 μmol photons m^{-2} s^{-1}). APC had a different response to light intensity than PE and PC under very low irradiance (10 μmol photons m^{-2} s^{-1}), because the intracellular APC content was 23.1% lower than that of control cultures. The rest of the cultures subjected to high irradiance (100 and 150 μmol photons m^{-2} s^{-1}) showed no change with respect to the control at 70 μmol photons m^{-2} s^{-1}.

The ratio PC: PE: APC (normalized to APC) is species-dependent in cyanobacteria (Bodemer 2004). In addition, the PBP composition may vary depending on the intensity and type of incident light in the culture. It is well known that cyanobacteria species exhibit chromatic acclimation that leads to characteristic green or red/orange colors. In this study the PC: PE: APC ratio was calculated in *Chroococcidiopsis* sp. with the aim of comparing its profile with other cyanobacteria (Table 5.3). The results show APC it is the major

PBP in *Chroococcidiopsis* sp., while in some species is almost absent (Van Den Hoek et al. 1995). As an example of PC: PE: APC ratio variability, in *Anabaena variabilis* the ratio was 1.9: 0.8: 1, and in *Aphanizomenon issatschenkoi* it was 1.3: 0.5: 1.

Table 5.3. PC:PE:APC ratio in *Chroococcidiopsis* sp. grown under different light intensity.

Light intensity (µmol photons m^{-2} s^{-1})	**PC:PE:APC**
10	1.1:0.6:1
50	0.6:0.3:1
70	0.3:0.2:1
100	0.3:0.2:1
150	0.4:0.2:1

According to literature, phycobiliproteins are produced under low light irradiance in order to increase the efficiency of light collection. Kumar et al (2011) showed that *Spirulina platensis* grown under approximately 27 µmol photons m^{-2} s^{-1} produces 127.5 $mg \cdot g^{-1}$ of total PBP 76.5 $mg \cdot g^{-1}$ of PC, 34 $mg \cdot g^{-1}$ of APC and 17 $mg \cdot g^{-1}$ of PE. In addition, an increase in light irradiance produced a decrease in PBP production. In addition, other factors like carbon availability may influence the ratio; for instance, Sharma et al. (2014) showed that cultures with a CO_2 deficiency showed an increase in APC in *S. platensis*. This may be because the APC has a role in dissipating excess energy (Tamary et al. 2012).

In summary, light irradiance was found to regulate PBP accumulation in *Chroococcidiopsis* sp. Interestingly, each specific phycobiliprotein accumulated on a different quantitative pattern as a function of the light irradiance; this would allow to suggest different roles for specific PBP (PC, PE, APC) depending on the intensity of the incident light. Particularly, APC could play a leading role among PBP in light dissipation under excess photons, in good agreement with similar role described in literature for APC in other cyanobacteria. Besides, the high APC maximum intracellular levels

produced at relatively high light intensities suggest *Chroococcidiopsi*s sp. could have potential for production of this valuable PBP.

CHAPTER VI

EFFECTS OF FERTILIZER-BASED CULTURE MEDIA ON THE GROWTH AND PRODUCTION OF VALUABLE COMPOUNDS BY *CHROOCOCCIDIOPSIS* SP.

Abstract

Chroococcidiopsis sp. can produce exopolysaccharides (EPS) with the objective to provide cells with a highly hydrated matrix to protect them against dissecation. In addition, cyanobacterial EPS have been reported to display valuable bioactivities to human health, thus the biotechnological production of these compounds is broadly worthy. In this Chapter, biomass production and accumulation of carbohydrates, including EPS and phycobiliproteins, was analyzed in cultures of *Chroococcidiopsis* sp. growing on commercial fertilizers (NPK media) which usually lead to reduce biomass production costs. From the obtained results it can be concluded that the use of commercial fertilizers increased the productivity of the biomass and stimulated the biosynthesis and accumulation of carbohydrates, exopolysaccharides and phycobiliproteins.

Keywords: Cyanobacterium; NPK medium; Sulfated exopolysaccharides; Fertilizers

Chapter VI

Introduction

The cyanobacterium *Chroococcidiopsis* sp. has been found in the most extreme zones of the planet, both in the hottest (Atacama Desert) and coldest (Antarctica) extreme deserts (Bahl et al. 2011). This cyanobacterium produces compounds of biotechnological interest which enable it to survive under extreme environmental conditions. Some of these compounds were already described in the Introduction section of this Thesis.

Some cyanobacteria species produce exopolysaccharides (EPS) to survive in extreme arid environment where water is scarce and hardly bioavailable. A highly hydrated EPS-coated cell cover envelops the cyanobacterial cells and stabilizes enzymes and molecules in a dry environment (Billi et al. 2017). Cyanobacterial EPS have diverse structural complexity that sometimes includes up to 9-different monosaccharides linked by *O*-glucoside bonds. It is also known that the monosaccharides xylose, galactose and glucose are usually found in significant proportions in the EPS from microalgae and cyanobacteria (Rossi and De Philippis 2016).

EPS and sulfated have an array of applications related to their bioactive properties. In addition, sulfated EPS may have the capacity to reduce total cholesterol and are also promising substances in the reduction of coronary heart diseases, due to their hypocholesterolemic effects (Korcz et al. 2018). Moreover, adhesion of pathogenic microorganisms to cyanobacterial cells has been reported to be avoided by EPS, which has made it possible to suggest their use in anti-adhesive therapies (Delattre et al. 2016). A potentially promising bioactivity of sulfated EPS is the ability to prevent the growth of tumor cells (De Jesus Raposo et al. 2013). These bioactive properties are valuable to human health and have attracted attention from the scientific community towards the production of EPS from cyanobacteria. Moreover, these sulfated polysaccharides also have been reported to display antiviral effect and antioxidants properties; they also have anti-inflammatory properties and have a role in the immunomodulatory system (De Jesus Raposo et al. 2013; Wijesekara 2011). Its production depends on the growing conditions (Rinker and Kelly 1996).

The cultivation of cyanobacteria and microalgae for biotechnological purposes has a high production cost associated with their biomass and metabolites production at large scale. Culture media composition is an important factor to be taken into account in cyanobacterial cultivation due to the current high cost of associated chemicals. With the purpose of reducing costs and even reaching better productivity results, the replacement of chemical compounds by commercial fertilizers should be a way to make photosynthetic microorganism production easier and cheaper (Scardoelli-Truzzi and Sipaúba-Tavares 2017).

In this work, the influence of commercial fertilizers of different chemical composition on the growth of *Chroococcidiopsis* sp. was studied in order to save cost during biomass production. The influence of those commercial fertilizers on the accumulation of carbohydrates, exopolysaccharides and phycobiliproteins (PBP) by the aforementioned cyanobacterium was analyzed.

Chapter VI

Materials and Methods

Microorganism and growth conditions

The cyanobacterium *Chroococcidiopsis* sp. was obtained from gypsum samples provided by researchers from the CSIC (Museum of Natural Sciences, Madrid) within a research project in collaboration with the University of Huelva, as described in Chapter IV of this Thesis.

The cyanobacterium *Chroococcidiopsis* sp. was cultured at pH 7 in a sterile BBM (Bold's basal Medium) culture medium and the concentration of sodium nitrate was modified in order to have a nitrogen concentration of 9 mM, previously determined as optimal in Chapter IV of this Thesis.

CO_2 was used as inorganic carbon source and was supplied to the cultures by bubbling CO_2-enriched air (2.5% v/v) through a hollow glass rod immersed in the medium. These cultivation conditions are called "standard" in this Thesis. The light source used to provide light energy to the cultures in the culture room was generated by Philips lamps (Philips 30W/33), (Philips Ibérica SAU, Spain), which supplied 70 $\mu E\ m^{-2}\ s^{-1}$ of white light on the surface of the culture flasks.

The culture media, named as F1, F2, F3, F4 and F5, were elaborated using the following fertilizers: NPK 12-6-4, NPK 4-10-10, NPK 18-6-6, NPK 2.4-4.8-6 and NPK 8-6-6, respectively (Agroliq NPKs, Agralia Fertilizers, S.L, Spain). These numbers refer to the fertilizer composition in Nitrogen (Total), Phosphorus (P_2O_5) and Potassium (K_2O) expressed as a percentage (%, w/w). These fertilizers have a different N/P ratio, which influences the growth of photosynthetic microorganisms (Rasdi and Qin 2015).

The composition of the NPK fertilizer is shown in Figure 6.1 and its composition was calculated by using the density of the NPK fertilizer: NPK 12-6-4 (ρ= 1.19 $g \cdot cc^{-1}$), NPK 4-10-10 (ρ; 1.20 $g \cdot cc^{-1}$), NPK 18-6-6 (ρ=1.24 $g \cdot cc^{-1}$), NPK 2.4-4.8-6 (ρ=1.13 $g \cdot cc^{-1}$) and NPK 8-6-6 (ρ= 1.17 $g \cdot cc^{-1}$). The nitrogen contained in the fertilizers can be in the form of urea, ammonium or nitrate, depending on the NPK fertilizers.

Table 6.1. Composition of the NPK fertilizers.

Commercial fertilizers	N ($mol \cdot L^{-1}$)	P ($mol \cdot L^{-1}$)	K ($mol \cdot L^{-1}$)	N/P	Nitrogen source
NPK 12-6-4	5.544	0.503	1.011	11.0	9% Urea, 3% Ammonium
NPK 4-10-10	2.661	0.845	2.548	3.1	4% Ammonium
NPK 18-6-6	8.009	0.524	1. 580	15.3	15.9% Urea, 2.1% Ammonium
NPK 2.4-4.8-6	1.084	0.382	1.439	2.8	1.6% Nitrate, 0.8% Ammonium
NPK 8-6-6	3.635	0.495	1.490	7.3	6% Urea, 2% Ammonium

In order to prepare the fertilizer-based culture media by using NPK fertilizers, the volume of NPK added to each culture medium was calculated, to make a final nitrogen concentration of 9 mM (Table 6.2).

Table 6.2. Nitrogen and Phosphorous composition of the fertilized-based culture medium used in this Thesis.

Culture medium	NPK added ($mL \cdot L^{-1}$)	N (mM)	P (mM)	N/P
F1	1.6	9	0.81	11
F2	3.4	9	2.84	3.1
F3	1.1	9	0.58	15.3
F4	8.3	9	3.15	2.8
F5	2.5	9	1.22	7.3
BBM*	-	9	1.71	5.2

*The data corresponding to the standard medium are included as reference.

In addition, the culture media were completed with a commercial solution of micronutrients (Table 6.3). The amount of macronutrients added to the each culture medium was 0.415 $ml \cdot L^{-1}$, in order to make a final iron concentration of 0.018 mM.

Table 6.3. Composition of micronutrients solution used for the cultivation of *Chroococcidiopsis* sp. in media prepared with fertilizers.

Micronutrients	% p/v	mol·L^{-1}
B	0.54	0.370
Cu	0.41	0.031
Fe	1.20	0.913
Mn	0.63	0.473
Mo	0.04	0.010
Zn	0.34	0.092

Finally, $CaCl_2$ and $MgSO_4$ were added up to a final concentration of 0.018 g·L^{-1} and 0.153 g·L^{-1} respectively, with the objective of avoiding calcium and magnesium deficiencies. These solutions would be eventually replaced by other water sources in large-scale cultivation.

The pH of all the media was adjusted to 7, so in some cases it was necessary to increase or decrease pH by adding 10% NaOH (v/v) or 3.7% HCl (v/v) solutions. These media were autoclaved for 25 min at 120°C and 1 atm overpressure.

The resulting culture media were inoculated with cyanobacterial cells grown in BBM medium. These inoculated media were cultured in repeated-batch mode. Repeated-batch cultivation consisted of periodically collecting part of the biomass and diluting the culture with fresh culture medium plenty of nutrients, maintaining the initial volume.

Adapted and non-adapted cultures

Cells of *Chroococcidiopsis* sp. were grown in BBM culture medium until the exponential phase was reached. Then, cells were harvested and used to inoculate fertilizer-based culture media. The cells in the inoculated media could require a time period to get adapted to the new nutritional conditions (Picardo et al. 2013). The cultures of the cyanobacterium were considered

adapted to the fertilizers-based culture media when biomass productivity was maximal and remained constant.

In addition, the maximum photosynthetic efficiency of the cultures (Qy) should also be taken into account to judge the adaptation state (Zhao et al. 2017). The cyanobacterial cultures were considered adapted when the Qy reached at least 0.4 and remained constant.

Biomass productivity measurement

The cyanobacterial growth was daily assessed spectrophotometrically (Evolution 201, Thermo Fisher Scientific, USA) by following optical density (O.D.) at 750 nm. Dry weight measurements were carried out using 10 mL samples of each culture. The samples were filtered through glass microfiber filters of 47 mm diameter and 0.7 μm pore size (MFV-5, AnoiaFilterlab, Spain).

Biomass productivity was calculated as the increase in dry weight in a given culture volume and for a specific period of time in the linear phase of the cyanobacterial growth. Similarly, the specific productivity of each targeted metabolite produced by the cyanobacterium was calculated as the increase in the quantity of this product in a given culture volume or per dry weight.

Maximum photosynthetic efficiency

The photosynthetic performance was evaluated by measuring the chlorophyll fluorescence in dark-acclimated cells, considered as maximum photosynthetic efficiency of photosystem II (Fv/Fm). This parameter was determined using a PAM device (Pulse Amplitude Modulation, model AquaPEN AP100; Photon System Instruments, Czech Republic) in accordance with Cuaresma et al. (2012).

Sulphated polysaccharides (EPS-S)

The quantification of carbohydrates was carried out following the method reported by Dubois et al. (1956) as described in Chapter IV of this Thesis.

EPS-S content was determined as described by (Ramus 1977). Culture samples (1 mL) were centrifuged and the supernatants were used to determine

EPS-S. The supernatants were added 4 mL of 0.5 M acetic acid and 500 µL of alcian blue (1 mg·mL^{-1} in acetic acid 0.5 M, pH 2.5). Samples were incubated overnight at 22 ºC to precipitate all the extracellular EPS-S. Samples were centrifuged at 3000 x *g* for 30 min. The optical density of supernatant was read at 610 nm. The difference in absorbance between negative control (distilled water) and samples was proportional to the concentration of the polyanion (Guzmán-Murillo 2007).

Pigment extraction and PBP determination

The pigment extraction was carried out as described in Chapter IV. The phycobiliproteins content was analyzed by measuring the absorbance of the obtained cyanobacterial extracts at 565 nm, 620 nm and 650 nm. After that, the concentration of phycobiliproteins was calculated by the following equations, as described by Round et al. (1989):

$$Phycocianin[PC; mg/mL] = [A_{620} - (0.72\ x\ A_{650})]/6.29$$

$$Allophycocianin\ [AC; mg/mL] = \ [A_{650} - (0.191\ x\ A_{620})]/5.79$$

$$Phycoerythrin\ [mg/mL] = \ \left[A_{565} - \left((2.41\ x\ CPC) - (1.41\ x\ CAPC)\right)\right]/13.02$$

Statistics

Unless otherwise indicated, tables and figures show the average value of three replicates in each experiment and they were expressed as means with ± standard deviation (SD). Mean values are presented in the corresponding figures with error bars as standard deviation.

Results and Discussion

Effect of the commercial fertilizers on the growth and viability of *Chroococcidiopsis* sp. cultures

The experimentation of this section aims at identifying suitable chemical compositions of fertilizers to address the massive production of the extreme environment cyanobacterium, a necessary stage for the biotechnological exploitation of the microorganism.

Biomass samples analyzed in this Chapter were taken from repeated-batch cultures of *Chroococcidiopsis* sp. which were grown as described in the Materials and Methods section. The results shown in Figure 6.1 correspond to the time-course evolution of optical density through the repeated-batch cultivation process. The results demonstrate that *Chroococcidiopsis* sp. cultures adapt repeated-batch cultivation after a number of dilution cycles.

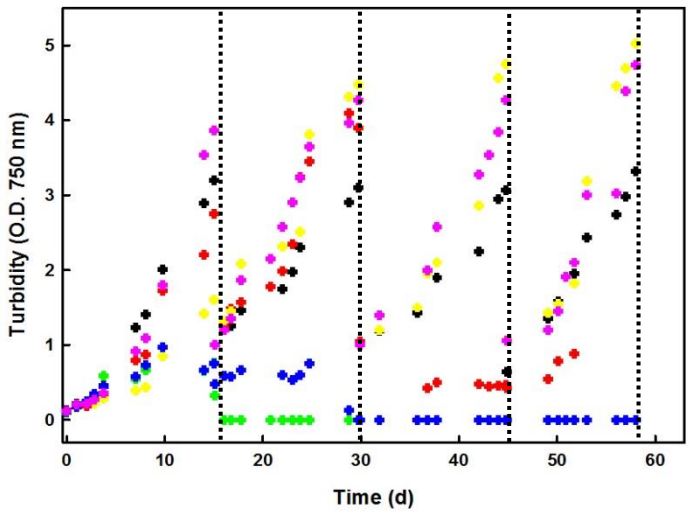

Figure 6.1. Repeated-batch cultivation of *Chroococcidiopsis* sp. Cells from standard cultivation, at the beginning of the logarithmic phase of growth, were harvested and resuspended in fresh standard culture medium (black circle, control) and in different fertilizers-based culture media. (●) F1; (●) F2; (●) F3; (●) F4 and (●) F5. More details in the Materials and Methods section.

Cultures were considered "adapted" or "non-adapted" according to the criteria defined in the Materials and Methods section. Accordingly, Figure

6.2. shows growth parameters (optical density and dry weight) and viability (photosynthetic) of both adapted and non-adapted cyanobacterial cultures grown on the different culture media.

Figure 6.2.(1) shows growth and Figure 6.2.(2) shows cell viability (data of the cyanobacterium *Chroococcidiopsis* sp. cultivated in liquid medium and using various commercial fertilizers.

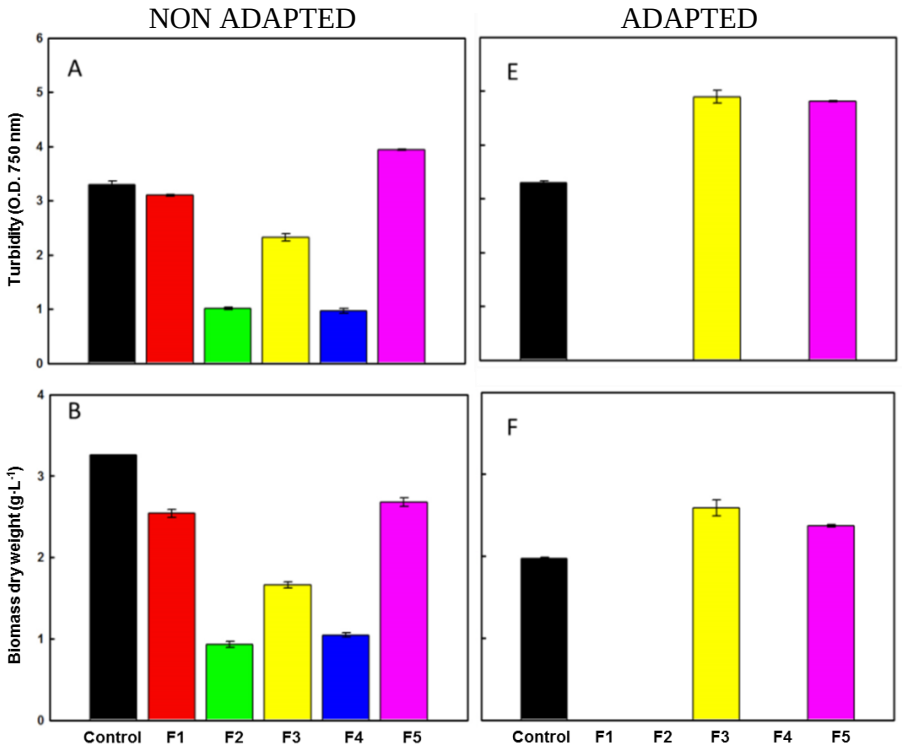

Figure 6.2.(1). Growth of the cyanobacterium *Chroococcidiopsis* sp. incubated in fertilizers-based culture media. The graphs of the left part of the Figure (A, B) correspond to non-adapted cultures to commercial fertilizers, and the graphs on the right (E and F) correspond to cultures that were previously adapted to commercial fertilizers following the procedures described in the Materials and Methods section. The graphics correspond to data obtained after 15 days of incubation (non adapted cultures) or four dilution cycles (adapted cultures). (Control black circle, control) and in different fertilizers-based culture media, (●) F1; (●) F2; (●) F3; (●) F4 and (●) F5.

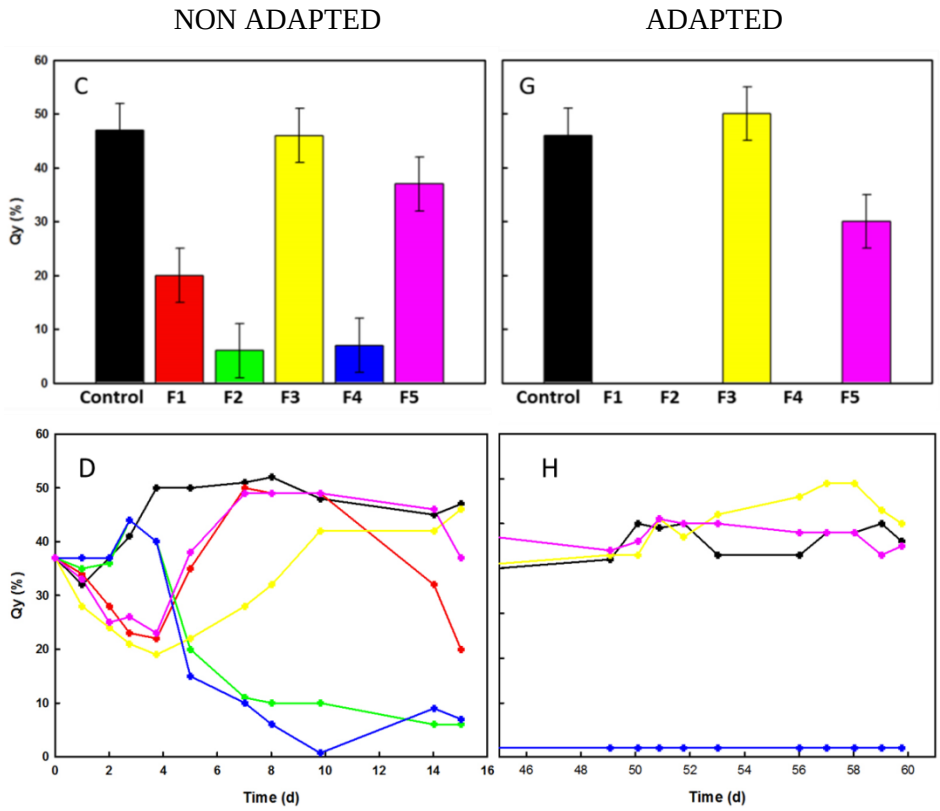

Figure 6.2.(2). Cell viability in cultures of the cyanobacterium *Chroococcidiopsis* sp. incubated in fertilizers-based culture media. The graphs of the left part of the Figure (C and D) correspond to non-adapted cultures to commercial fertilizers, and the graphs on the right (G and H) correspond to cultures that were previously adapted to commercial fertilizers. The graphics C and D correspond to data obtained after 15 days of incubation (non adapted cultures) and G and H correspond to four dilution cycles (adapted cultures). D and H show the time-course evolution of maximum photosynthetic efficiency (Qy). (Control black circle, control) and in different fertilizers-based culture media, (●) F1; (●) F2; (●) F3; (●) F4 and (●) F5.

Figures 6.2.(1)A and 6.2.(1)B show that the growth of control cultures and non-adapted F1 and F5 cultures, measured as turbidity at 750 nm and dry weight, was faster and more productive than non-adapted F2, F3 and F4 cultures. In addition, a detailed analysis of Figure 6.2.(1) reveals significantly large differences in the maximum concentration of biomass reached in the cultures depending on the type of commercial fertilizer used. Thus, again the

cultures F1 and F5 gave rise to maximum biomass concentrations. Both fertilizers, F1 and F5, allowed to obtain relatively high growth values close to 80% of that obtained for control culture.

Cell viability in the cultures of *Chroococcidiopsis* sp. is shown in Figure 6.2.(2)D and 6.2.(2)H, and reveal an expected correspondence between viability and growth data of the cyanobacterium (Figure 6.2.(1)). Figure 6.2.(2)D shows two trends in the evolution of the maximum photosynthetic efficiency: (i) In control cultures and F1, F3 and F5, *Chroococcidiopsis* sp. required increasing times of partial adaptation to the culture conditions until reaching the maximum average photosynthetic efficiency characteristic of that cyanobacterium, between 40% and 50%; (ii) In cultures F2 and F4, *Chroococcidiopsis* sp. showed a rapid fall in maximum photosynthetic efficiency to values below 10%.

Figures 6.2(1)E, 6.2.(1)F, 6.1.(2)G and 6.2.(2)H show growth and cell viability data, at the exponential phase, of *Chroococcidiopsis* sp. cultures grown in the same fertilizers-based culture media but with a previous adaptation period. Unlike non-adapted cultures, Figures E and F show that growth of the adapted cultures in F3 and F5 is roughly between 20% and 30% greater than that of the control cultures. The above data correlate well with the cell viability data measured as maximum photosynthetic efficiency (Figure 6.2.(2) H of adapted cultures).

The production of photosynthetic microorganisms enriched in valuable compounds for human health, food or energy sectors is an exponentially growing biotechnological activity (Wijffels and Barbosa 2010). The industrial production of microalgae and cyanobacteria uses photobioreactors that are filled with thousands liters of culture per hectar, which entails a huge consumption of water and specific nutrients to obtain fast, stable and productive growth of the chosen microorganism. For the production process to be viable, besides selecting the optimal conditions -physical, chemical, physiological and operational- for the production of the microorganism, the economic feasibility of the process becomes crucial the further applicability at large scale. In this sense, the cost of nutrients is one of the significant components contributing to the total production costs (Norsker et al. 2011) and it is necessary to keep it as low as possible. From the economic point of

view, and also practical, the use of conventional culture media used in the laboratory is not the best option for mass production due to the complexity of its preparation on an industrial scale and the cost of the different chemical components. Therefore, commercial fertilizers, which commonly contain N, P and K and which can be specifically formulated according to each microalgal species requirements, are an attractive alternative to conventional culture media (Forján et al. 2015).

The selection of a suitable fertilizer is made based on its effectiveness in the production of biomass, witch can vary depending on the type and concentration of the N source contained in the formulation, as well as the proportion between the N content and the other components of the fertilizer (at least P and K). In this Thesis notable differences in biomass concentration were found in cultures non-adapted to fertilizers with respect to the adapted ones, and also the each specific fertilizer used had a large impact on biomass production. In general, it is observed that the fertilizers that gave the highest concentrations of biomass share the highest N and P ratios in their composition and all of them contained urea as a major source of N. On the contrary, apparently, the less favorable results of biomass production are obtained by using fertilizers with the lowest N/P ratios. All cultures have the same initial amount of nitrogen, in the form of urea and/or ammonium and/or nitrate but different N/P ratios. Consequently, the differences observed in biomass concentration after two weeks of cultivation should be due to the different N to P ratios and the nature of the nitrogen source.

Table 6.4 shows the data of maximum biomass productivity in both adapted and non-adapted cultures to the different fertilizers and show the relative differences between them. The value of 100% corresponds to the productivity of control cultures. In short, only the culture media F3 and F5 permitted the adaptation of *Chroococcidiopsis* sp. to long term production in repeated batch mode, achieving productivities higher than those of control cultures.

Table 6.4. Effect of the different commercial fertilizers on the biomass productivity of *Chroococcidiopsis* sp. Cultures were grown in the indicated fertilizer-based culture media, and the accumulated biomass was determined in each culture after 15 days of incubation (non adapted cultures) or four dilution cycles (adapted cultures).

Culture médium	NON-ADAPTED		ADAPTED	
	Productivity ($g \cdot L^{-1} \cdot d^{-1}$)	(%)	Productivity ($g \cdot L^{-1} \cdot d^{-1}$)	(%)
Control	0.22	100	0.20	100
F1	0.18	83.50	0.00	0
F2	0.02	10.31	0	0
F3	0.11	51.28	0.29	142.59
F4	0.03	15.47	0	0
F5	0.18	84.17	0.23	113.19

The maximum productivity of 0.29 $g \cdot L^{-1} \cdot d^{-1}$ obtained for the adapted F3 culture is similar to values published for other cyanobacteria produced at large scale (Das et al. 2018). According to it, the optimized fertilizer-based culture medium for lab-scale cultivation of *Chroococcidiopsis* sp. could be useful for large scale production (Patel et al. 2018; Danquah et al. 2009). In this respect, it should be borne in mind that cell growth is limited in the climatized algal culture room, among other factors due to the low, non-optimized light intensity, which means that there is still room for improving the productivity of the cultures.

Therefore, it can be concluded that the use of commercial fertilizers could favor the increase in the productivity of the cyanobacterium in liquid medium, and that the chemical composition of the fertilizers determines their effectiveness. The results obtained suggest the potential of the fertilizers to develop the massive production, on a large scale, of the cyanobacterium of extreme arid environment *Chroococcidiopsis* sp., opening opportunities for its biotechnological applications.

Effect of the commercial fertilizers on the accumulation of carbohydrates and exopolysaccharides by *Chroococcidiopsis* sp.

This section shows the results corresponding to the evolution of carbohydrates and polysaccharides of cyanobacterial cultures grown in

different fertilizer-based culture media previously described in the Materials and Methods section. Part of the carbohydrates accumulate in the cyanobacterium in the form of exopolysaccharides (EPS), depending on species and cultivation conditions (Trabelsi et al. 2009; Nicolaus et al. 1999). According to literature, most of these polysaccharides would be sulfated and have some interesting bioactive properties, such as antioxidant and anti-inflammatory properties (De Jesus Raposo et al. 2015).

The experimentation shown in this section was aimed at identifing chemical compositions of fertilizers that enhance the accumulation of polysaccharides of the cyanobacterium.

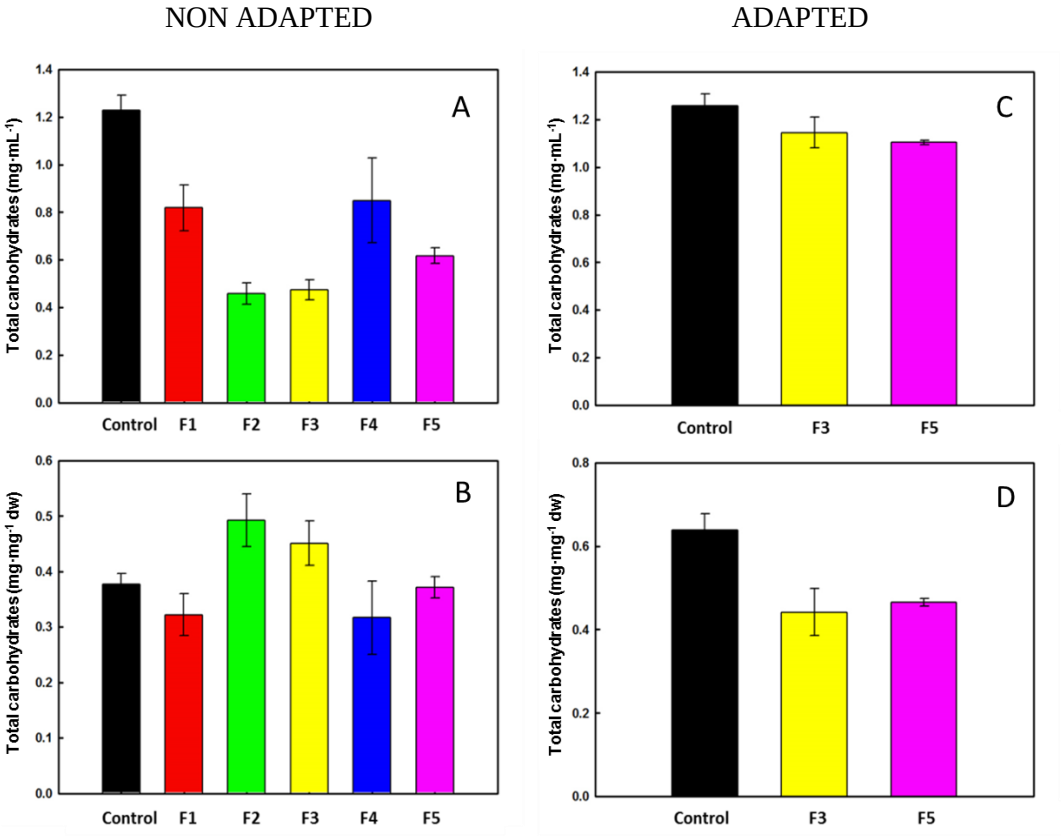

Figure 6.3. Intracellular production of carbohydrates content in different cultures of the cyanobacterium *Chroococcidiopsis* sp. Aliquots of cyanobacterium stock cultures were incubated in different NPK-commercial fertilizers-based culture media as source of nutrients. The culture media (F1 to F5) were prepared according to the compositions described in the Materials and Methods section. The cultures which were grown during 15 days of incubation (non adapted cultures) or four dilution cycles (adapted cultures) and then carbohydrates content in the cells were analyzed. More details as indicated in the Materials and Methods section.

Figure 6.3 shows notable differences in carbohydrates productivity depending on the culture medium used. Thus, in Figure 6.3A it can be seen that the highest production of carbohydrates occurs in the control culture. Among the cultures grown on fertilizers-based media, those corresponding F1 and F4 achieved the maximum carbohydrate production values, behind the data corresponding to the control cultures. The maximum concentration of carbohydrates per volume of culture was achieved in the culture grown on F4.

The low carbohydrate content in cultures F2, F3 and F5 could be related to those reasons already discussed for the low growth of these cultures, Figure 6.2(2).

As described previously, those cultures grown with F3 and F5 got adapted to the new media, then allowing production of stable cultures and continuous accumulation of carbohydrates. The volumetric productivities of these molecules were slightly lower than those obtained for control cultures.

To determine what fertilizers-based culture led to higher intracellular carbohydrate accumulation, the concentration of carbohydrates per dry weight unit of biomass was measured in both adapted and non-adapted cultures. In general, as shown in Figure 6.3 the cultures that yielded the highest concentrations of biomass accumulated less carbohydrates per unit of biomass, therefore they showed a less intense carbohydrates biosynthesis. On the contrary, cultures showing little growth accumulated higher carbohydrates content per unit of biomass, suggesting that carbohydrates accumulate in *Chroococcidiopsis* cultures under stress situations such as limited nutrient availability. Indeed, this would be the case for the highest intracellular carbohydrate accumulation observed in the F2 and F3 non-adapted cultures, those that produced the lowest concentrations of cyanobacteria. The maximum concentration of carbohydrates in the biomass of *Chroococcidiopsis* sp. was roughly 50% (0.5 mg mg dw^{1}), obtained in the cultures grown with the F2 medium. The fact that the lower accumulation of carbohydrates corresponds to the cultures that showed more productive growth is consistent with the growth under non-stressing conditions, which generally favors protein accumulation over that of carbohydrates and lipids (Markou et al. 2012).

In coherence with the described results, F2 cultures (containing the highest intracellular accumulation of carbohydrates), decreased the Qy to 25% of the maximum value after one week of incubation (Figure 6.2.(2)D). This fact indicates a loss of viability of the cells of the cyanobacterium that leads to continuous loss of biomass, consequence of the decrease in primary anabolic activity and cell lysis, as demonstrated in numerous studies with microalgae grown under stress (Wang and Lan 2018; Imase et al. 2013). In situations of stress that limit or inhibit cellular growth, especially in those related to the limitation or lack of any nutrient, the microalgal metabolism tends to

accumulate, preferably, energy reserve molecules, in particular carbohydrates and lipids (Kannan and Pattarkine 2014).

In short, from the results described and discussed, it is suggested that in the extreme environment cyanobacterium *Chroococcidiopsis* sp. the biosynthesis and accumulation of carbohydrates could be stimulated under stress conditions that limit or inhibit their growth. Those conditions could be related to the limitation or lack of any nutrient, particularly N and/or inorganic P, or even to the replacement of a given nutrient which produces an adaptation period. This reasoning is based on the fact that the intracellular accumulation of carbohydrates seems to be slightly stimulated in non-adapted cultures.

In this work the influence of fertilizers on the production of exopolysaccharides by the cyanobacterium was also studied.

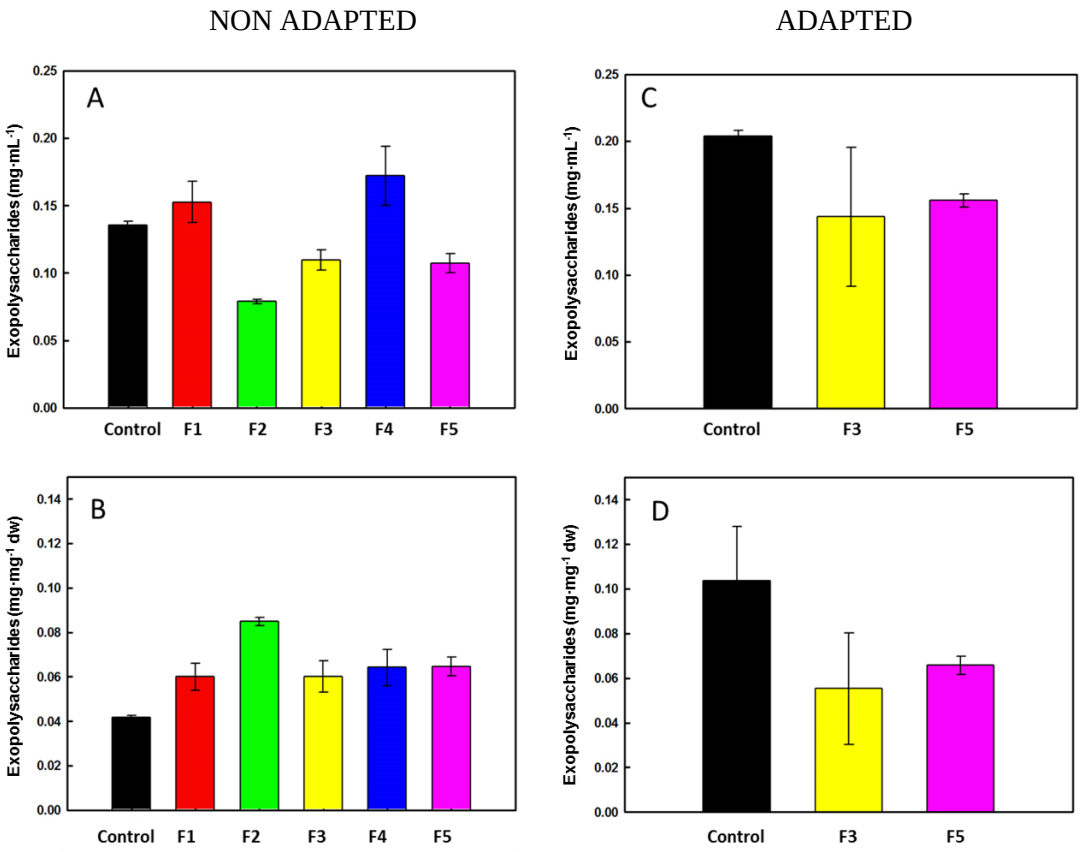

Figure 6.4. Production of exopolysaccharides in cultures of the cyanobacterium *Chroococcidiopsis* sp. Aliquots of cyanobacterium stock cultures were grown in different fertilizers-based culture media (F1 to F5). The graphics correspond to data obtained after 15 days of incubation (non adapted cultures) or four dilution cycles (adapted cultures).

Figure 6.4 shows production of exopolysaccharides (EPS) of *Chroococcidiopsis* sp. grown in control culture medium and in fertilizers-based culture media, in adapted and non-adapted cultures. Figure 6.4A shows, in non-adapted cultures, that the accumulation of EPS dependent on the fertilizer follows a similar pattern to the accumulation of total carbohydrates. The culture medium that produces the lowest EPS is that corresponding to F2, characterized by the rapid loss of cells viability, as shown in Figures 6.2.(2). The high volumetric productivity of EPS observed in cultures F2 and F4, which cells shows low viability after 2 weeks of growth (Figure 6.2.(1)),

support the idea that physico-chemical stress could favor the carbohydrate accumulation in *Chroococcidiopsis* sp.

To evaluate the hypothesis that stress could be among the causes that increase the production of EPS in cultures of *Chroococcidiopsis* sp. grown on fertilizers-based culture media, Figure 6.5 was made. The X axis of the graph represents the time, in days, that each culture remained with a maximum PSII efficiency below 0.35, considered this as a normal average value for a photosynthetically active culture of cyanobacterium, and the Y axis represents the maximum EPS content of each culture, based on biomass.

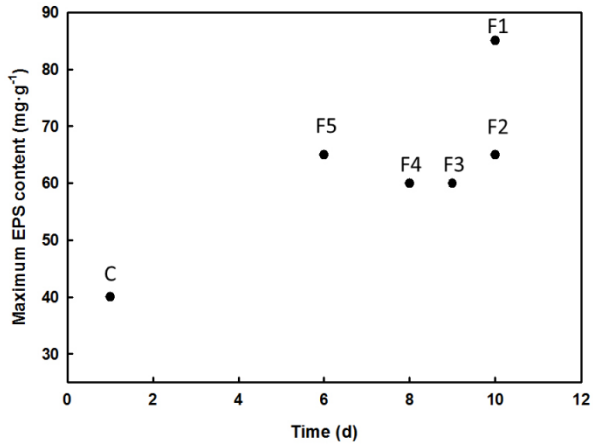

Figure 6.5. EPS accumulation in *Chroococcidiopsis* sp. under low maximum photosynthetic efficiency. The EPS content and the photosynthetic efficiency were measured as described in the Materials and Methods section of this work. The x-axis represents the number of days in which the culture had a Qy below 35.

The graph shows correlation between the amplitude of the time period that the cultures remained exhibiting at low photosynthetic efficiency (< 0.35, low viability) and the production of EPS. The Figure 6.5 also suggests that the influence of the composition of the NPK-fertilizer on EPS accumulation used could be significant.

These results suggest that situations of stress, either due to a change in the diet or the type of nutrients or derived from variations in other physical-chemical parameters, favor the production of EPS by the cyanobacterium. The

impact of the specific composition of each NPK-fertilizer may have on EPS production compared to the influence of other possible stress factors, requires to be studied.

The results suggest that the research should continue conducting studies of EPS production under conditions of nutrient limitation or deficiency, or by inducing stress through variation in salinity, temperature or irradiance conditions in the culture. The results obtained in this Chapter suggest EPS would accumulate in *Chroococcidiopsis* in response to those changes.

Effect of fertilizer on the production of sulfated exopolysaccharides by *Chroococcidiopsis* sp.

The presence of EPS-S in cyanobacteria has been previously reported (De Philippis and Vincenzini 1998). Figure 6.6 shows the data of EPS-S concentration in *Chroococcidiopsis* sp. grown in fertilizer-based cultures media. This Figure indicates that the maximum EPS-S productivity value was reached at the end of the cultivation period, only in adapted cultures, grown on F3 and F5.

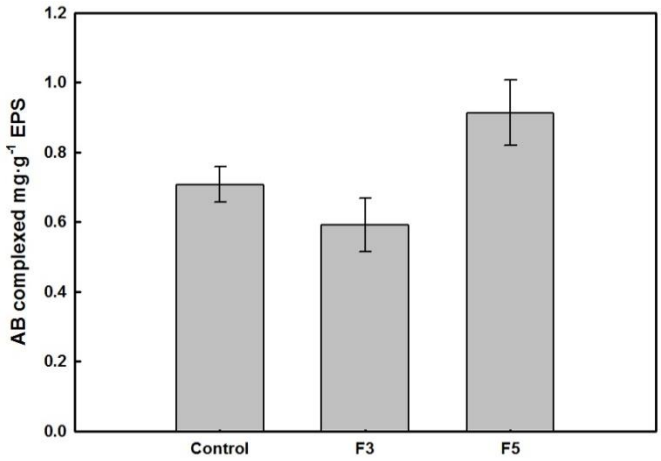

Figure 6.6. Production of EPS-S in cultures of *Chroococcidiopsis* sp. The graph shows the indirect measure of EPS-S in the form of AB complexed EPS, as is described in the Materials and Methods section. The graph corresponds to adapted cultures to fertilizer-based culture media. Adapted cells were grown in the indicated media and the intracellular EPS-S content was determined after four dilution cycles. More details in the Materials and Methods section.

Figure 6.6 shows that F5 culture cells contain the highest values of EPS-S being 30% higher with respect to the control, however, the cells in F3 culture resulted in a 16% decrease.

Effect of fertilizer on the phycobiliproteins production by *Chroococcidiopsis* sp.

In order to analyze the potential use of based-fertilizers cultures in the production of valuable interest compounds by *Chroococcidiopsis* sp., the PBP content was analyzed in adapted and non-adapted cultures. Figure 6.7 shows that, with the exception of F3, there are no significant differences in the PBP production in the different fertilizers-based cultures and the cultures control. Higher concentrations of PBP were obtained in cultures grown in F3 medium. That may be due to the higher N/P ratio in F3 medium. Therefore F3 could be used for the production of biomass enriches in PBP.

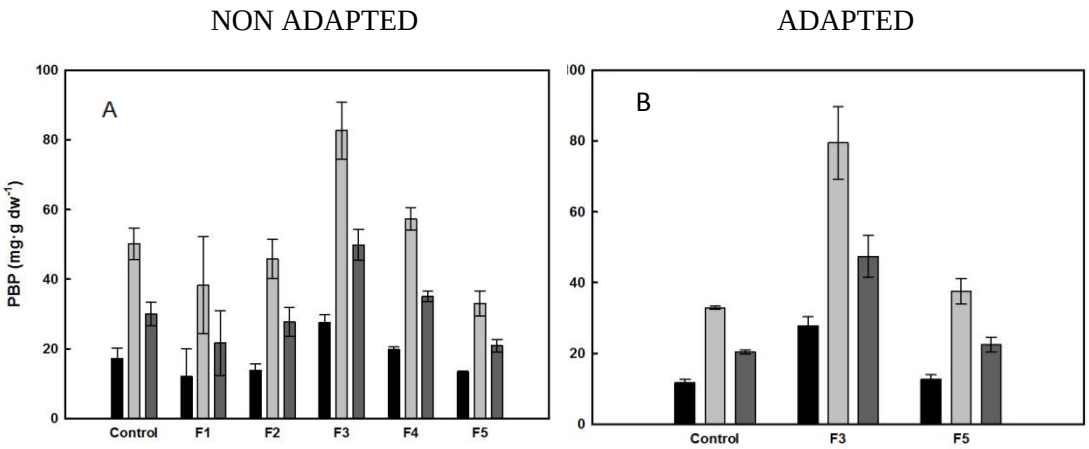

Figure 6.7. PBP production in fertilizer-based cultures of *Chroococcidiopsis* sp. Cells were grown in the indicated media and the PBP content was determined after 15days (non adapted) or four dilution cycles (adapted). Dark bars represent the content of phycocianin (PC), light gray bars represent the content of allophycocyanin (APC); and dark gray bars represent the content in phycoerythrin (PE). Details of growth conditions, pigment extraction and determination are described in the Materials and Methods section.

In short, *Chroococcidiopsis* sp. can be satisfactorily grown on nutrients contained in commercial fertilizers, whose composition determines their

effectiveness. In this respect, a suitable choice of fertilizer would be based on selecting those with adequate N to P ratios -to satisfy cell nutrient demand- and an N source (urea or nitrate). The use of NPK formula instead of standard culture medium is advisable as long as it results in reduced biomass production costs and should ease operation at large scale. From the obtained results, it was also inferred that the specific NPK composition of the used fertilizer definitely affects the carbohydrate and phycobiliprotein content of *Chroococcidiopsis* sp., which highlights the importance of selecting NPK formula according to the biomass quality required. Stress conditions might trigger exopolysaccharides accumulation in *Chroococcidiopsis* sp. which needs being further investigated.

CHAPTER VII

GENERAL DISCUSSION

Chapter VII

General discussion

The use of extremophiles for biotechnological purposes presents a wide range of possibilities. These microorganisms are source of unique compounds and, in addition, the cultivation of extremophiles has certain advantages over the cultivation of non-extremophiles (Forján et al. 2015). As we have described in this Thesis, extremophiles require special conditions to grow. These conditions are harmful to non-extremophiles, so large-scale cultures will present the advantage of lower contamination risk (Varshney et al. 2015; Forján et al. 2015). In addition, obtaining metabolites from extremophilic microorganisms could be more economically profitable, depending on each species specific features. For example, as described in Chapter III, the process to obtain carotenoids from halophilic cells can be simple because under low NaCl concentrations cell lysis is induced, and consequently carotenoids extraction could be conducted directly from the cells without any mechanical cell disruption operation (Rodrigo-Baños et al. 2015). In short, understanding the specific features of extremophiles physiology and biochemistry is of great relevance in order to exploit their biotechnological potential.

However, due to the high production cost, only a small number of applications are currently marketed (Coker 2016). In this context, the study of the parameters that influence the production of these microorganisms and their valuable molecules should contribute to make these processes economically feasible. This Thesis intends to be a valuable contribution to the aforementioned scientific aims. The results obtained in this Thesis allowed us to enhance biomass productivity and suggest valuable biotechnological potential of two extremophilic microorganisms: the halophilic archaea *H. mediterranei* and *Chroococcidiopsis* sp., a cyanobacterium from extreme arid environment.

H. mediterranei, transferred to our group by our colleague Prof. Rosa Martínez from the University of Alicante, stands out for its ability to synthesize bacterioruberin (BR), a carotenoid with high antioxidant capacity. It has been shown in previous studies that BR presents higher antioxidant capacity than others carotenoids produced by extremophilic or not extremophilic microorganisms (Rodrigo-Baños et al. 2015). BR is therefore a very powerful carotenoid that can have significant benefits to health, in the

food, feed, and pharmaceutical industries (Cardoso et al. 2017). This Thesis propose a wide range of conditions to effectively produce BR which should case further design and optimization of large-scale production processes.

Furthermore, a novel cyanobacterium *Chroococcidiopsis* sp. was isolated from extreme arid environment as described in Chapter IV of this Thesis and its biotechnological potential has also been studied in this work. Based on our findings, *Chroococcidiopsis* sp. is proposed as a potential source of phycobiliproteins (PBP) and exopolysaccharides (EPS). The results obtained from the experiments carried out with *Chroococcidiopsis* sp. have shown that biomass and targeted metabolites productivity can be largely improved based on physicochemical and nutritional key parameters optimization.

Improved growth and carotenoids production of *H. mediterranei*

Effect of environmental conditions for growth and carotenoid production of *H. mediterranei*

One of the limitations for the commercial exploitation of carotenoids is the high cost associated with the production and purification of pigments (Gong and Bassi 2016). In this Thesis, the potential of *H. mediterranei* to produce BR has been analyzed.

BR biosynthesis pathway in haloarchaea is shown in Figure 7.1, published by Yang et al. (2015). Essentially, most of the C_{50} carotenoids structures are based on C_{40} symmetric backbone phytoene, formed by condensation of two molecules of geranylgeranyl pyrophosphate (Yang et al. 2015). Phytoene further evolves to the biosynthesis of lycopene. Main enzyme-mediated steps of lycopene biosynthesis pathway in *H. mediterranei* are also present in the carotenoid biosynthesis pathway of microalgae (Yang et al. 2015). C_{50} carotenoid biosynthesis pathway in haloarchaea starts from the precursor lycopene, which is elongated by the catalytic action of the lycopene elongase (lye) reactions. Bacterioruberin is synthesized after a series of hydroxylation at the lateral chain ends of both sides of the skeleton (Yang et al. 2015).

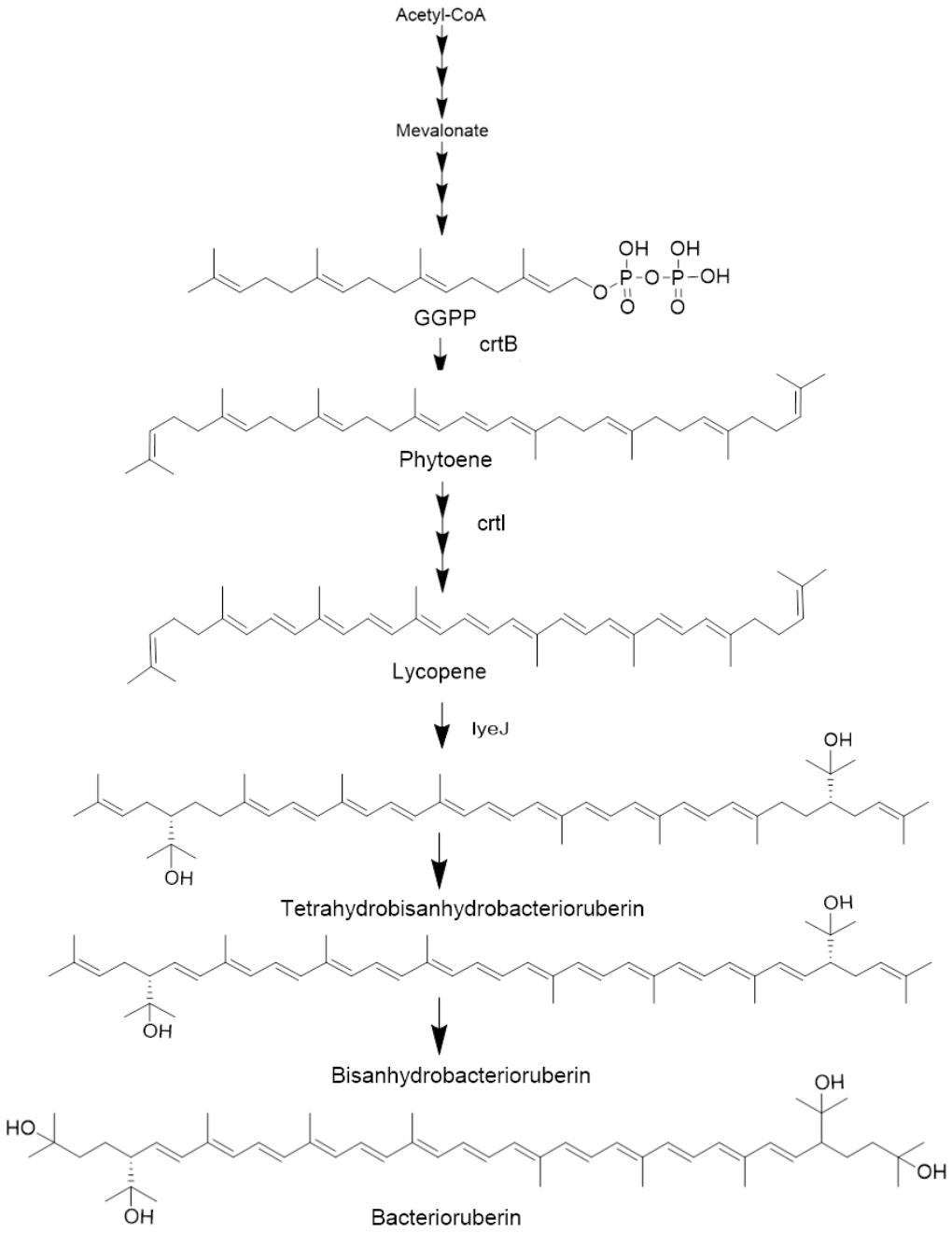

Figure 7.1. Biosynthesis pathway of bacterioruberin in haloarchaea. Geranylgeranyl pyrophosphate (GGPP); Phytoene synthase (CrtB); phytoene desaturase (Crtl); lycopene elongase / lycopene 1,2-hydratase (LyeJ).

The results of this Thesis unveiled the large impact of key cultivation conditions and nutrients on the productivity of BR by *H. mediterranei*. Therefore, specific culture conditions such as temperature, pH and salinity values should be set beforehand to maximize biomass yield and carotenoid production, thereby improving yields and reducing production costs (Rodrigo-Baños et al. 2015). However, the approach to standardize and optimize the conditions of growth and carotenoid production simultaneously, particularly at large-scale, is complex (Calegari-Santos et al. 2016; Fang et al. 2010). In this sense, a multivariate analysis can simplify the optimization process.

Statistical experimental methods such as Central composite design (CCD) and Response surface methodology (RSM) are tools that allow studying the effect of different parameters on a culture by analyzing wide ranges of those parameters. In the case of *H. mediterranei* we have studied the effect of 3 different parameters (temperature, salinity and pH) and our results provides highly valuable information on their effect on biomass and carotenoid accumulation in *H. mediterranei.* which is useful to design production processes at larger scale. Moreover, this tool provides information about the effect of the interaction of parameters. For instance, the effect of a given temperature value depends on other parameters such as salinity and pH. Using RSM, better decisions could be made for a profitable production of target substances (Latha et al. 2017; Hamidi et at. 2014; Ibrahim et al. 2011). In this Thesis, the optimal conditions to obtain maximum values of growth and carotenoid production are obtained by the haloarchaea are also determined (Chapter II).

Conditions addressing maximum carotenoid accumulation may lead to reduced growth. In this sense, RSM was also useful to find conditions to balance growth and carotenoid production, which is a key aspect regarding the economic feasibility of the process. Accordingly, the model showed to be a flexible tool for making decisions to set operational process parameters that favor either growth, carotenoid accumulation of both.

The predictive model also allowed to propose ranges of conditions under which haloarchaea growth rates still remain high, for instance above 90% of the maximum values. This can be of great importance in order to select conditions for production at large scale, keeping high productivity

whereas costs remain as low as possible. For instance, temperature control results in a strongly increased cost of the final product (Béchet et al. 2013). In order to save energy costs, lower operational temperature values can be adopted while keeping growth rates above 90%. Even though RSM helps to define a suitable balance between productivity and cost, it is highly advisable to analyze in detail the most economically convenient strategy: either finding operation conditions to balance growth and accumulation or developing a 2-stages cultivation strategy.

Effect of nutritional factors on growth and carotenoids production by *H. mediterranei*

In addition to studying the effect of parameters such as temperature, salinity and pH, the effect of nutritional parameters on the accumulation of carotenoids has also been studied in this Thesis.

As shown in Chapter III, *H. mediterranei* was grown until the stationary phase, in which an increase in carotenoid content was observed at the end of the exponential phase. This strategy turned out to be very convenient for carotenoids production. The high carotenoid production found in *H. mediterranei* cultures, and also per cell, at the end of the exponential phase (10-fold higher compared to early exponential phase) was hypothesized to be associated to nutrients deficiency. This fact led us to analyze the effect of glucose and/or yeast extract starvation in carotenoid production. These nutrients are two of the major components in the culture medium of *H. mediterranei*. As shown in Chapter III, glucose and yeast extract starvation had a large positive impact on carotenoids accumulation but negative on biomass production. The fact that under glucose depletion carotenoid accumulates rapidly and largely in *H. mediterranei* cells, possibly unveil a role for glucose as carotenoid biosynthesis inhibitor. In this sense, it has been published that carotenoid production is especially low in the presence of high glucose concentrations (Marcoleta et al. 2011).

The results led us to study two different strategies for BR production: (1) a culture grown up until nutrient depletion or (2) a 2-stages cultivation. The 2-stages cultivation strategy consists of a first phase in which biomass is produced under optimal growth conditions, and a second stage in which carotenogenesis is induced by nutrient starvation.

The results obtained and discussed in Chapter III indicated that, in terms of final productivity of carotenoids, similar results are obtained in both scenarios under the same period of time (7 days). It is necessary to take into account the operational costs that are attributed to a two-stage cultivation system in order to decide the most strategies for carotenoids production by *H. mediterranei.*

H. mediterranei produces mostly BR as shown in Chapter III. This could make downstream processes related to carotenoid isolation relatively quicker, easier, and cheaper (Giani et al. 2019). Low salt concentrations in the culture medium, roughly below 5%, cause cell lysis in *H. mediterranei*, which therefore avoids cost investments in terms of energy required to enable efficient cell breaking (Asker et al. 2002). It also makes carotenoids easily available by solvent-mediated extraction if compared to direct extraction from not broken cells. This means that haloarchaeal cells might be suitable for eventually maximizing pigment recovery at lower costs compared to other microorganisms (Giani et al. 2019).

Furthermore, the production of other carotenoids can be stimulated in *H. mediterranei*. This archaea might be explored as a potential cell factory for lycopene production. Firstly, blocking PHB synthesis could divert in more acetyl-CoA flux to lycopene synthesis; and secondly, disrupting the bacterioruberin synthesis significantly can increase the lycopene production as it was reported by Zuo et al. 2018 (Figure 7.2).

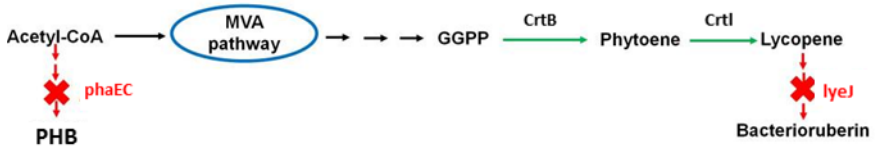

Figure 7.2. Schematic illustration of lycopene production by *H. mediterranei*. The figure shows the triggered lycopene synthesis by enhancing the lycopene biosynthetic pathway and blocking its use as BR precursor and the competitive pathway for poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHB) biosynthesis. Green arrows represent enhanced steps and red arrows and crosses represent blocked steps. Geranylgeranyl pyrophosphate (GGPP); Phytoene synthase (CrtB); phytoene desaturase (Crtl); lycopene elongase/ lycopene 1,2-hydratase (LyeJ).

In addition to the parameters assayed in this Thesis, other stress strategies (nutrient limitation, high light intensities, UV light, low oxygen tension) should be assessed as triggering factors to induce and improve the accumulation of BR. The relationship between these factors and increased levels of reactive oxygen species (ROS) has been previously described in haloarchaea (De Jager et al. 2017; Jones and Baxter 2017; Rodrigo-Baños et al. 2015). As BR protects the cells against oxidative damage, these parameters could improve the accumulation of carotenoids and new large-scale production strategies could be proposed.

In summary, the ability of *H. mediterranei* to accumulate bacterioruberin under stress conditions was demonstrated. Besides, this haloarchaea accumulates polyhydroxybutyrate (PHB) as reported in literature (Melanie et al. 2018). This polymer production process should be further analyzed in *H. mediterranei* grown under the optimal conditions proposed by the statistical model, in order to assess the production feasibility of this valuable product.

Exploring the biotechnological potential of an extremophilic cyanobacterium isolated from extreme arid environment

Isolation and identification

The use of microorganisms for biotechnological purposes usually requires stable, fast growth cultivation. In spite of their potentiality in biotechnology, extremophilic microorganisms may present difficulties to grow under laboratory conditions, most of which differ largely from those specific harsh conditions of the natural environments where extremophiles thrive (Stewart 2012; Alain and Querellou 2009). In this Thesis highlights, the convenience of adapting culture media and procedures to isolate and cultivate extremophilic specific microorganisms according to their specific physiological features.

The results of this Thesis show that was possible to isolate a cyanobacterium species from endolithic colonizations in gypsum rocks from the Atacama Desert. The isolation technique favored the selection of fast-growing photosynthetic microorganisms which is desirable considering their eventual use at industrial scale (Alain and Querellou 2009). However, previous studies have shown that the gypsum rock fragments contain a high

biodiversity of microorganisms that include cyanobacteria, archaea or even microalgae (Wierzchos et al. 2015; Wierzchos et al. 2012). The biotechnological potential of some of these microorganisms has been previously discussed by other authors. For instance, a previous work reported the ability of some endolithic microalgae to produce Orange Carotenoid Protein (OCP) (Wierzchos et al. 2015), which is used by the cell to dissipate excess energy (Sedoud et al. 2014) and it can provide novel tools for photoprotection engineering and for applications in optogenetics (Kirilovsky and Kerfeld 2013).

Nonetheless, the cultivation of these microalgae in liquid medium in the lab was reported to be difficult which probably has limited further trails at a larger scale. Several technical strategies may help improve cultivation of extremophilic microorganisms. For instance, using traditional cultivation techniques (petri plates, liquid cultures) combined with culture media of similar composition to that of the extreme environment where the microorganism thrives; or using cell encapsulation techniques in polymers-based solidified microdroplets which has shown positive results in the cultivation of bacteria (Stewart 2012; Alain and Querellou 2009) and microalgae (Sung et al. 2016).

As described in Chapter IV of this Thesis, a cyanobacterium from extreme arid environment was isolated and identified within the genus *Chroococcidiopsis*, a cyanobacterium that has the ability to survive under extreme water scarcity, but whose biotechnological potential has been so far scarcely studied as described in the Introduction section. This Thesis work is an attempt to screen part of the biotechnological potential of this cyanobacterium.

Biomass production

Optimizing the growth of the cyanobacterium in liquid cultures is one of the main steps in the research process towards its biotechnological application. This Thesis unveiled that vigorous culture agitation, and a suitable nitrogen source and concentration are key aspects to obtain productive cultures of *Chroococcidiopsis* sp.

Agitation turned out to be an important parameter since *Chroococcidiopsis* sp. settled spontaneously in a short period under our

experimental conditions. The spontaneous settling of *Chroococcidiopsis* sp. was described previously by Das et al. 2018, who reported a settling efficiency of 97% of the biomass in 1h. This cyanobacterial feature becomes an advantage since the harvesting, based on the settling, accounts for 20–30% of the total costs of the biomass production process (Ryan Georgianna and Mayfield 2012). Cost savings in the production of cyanobacterial biomass becomes particularly relevant due to the low growth rates of cyanobacteria when compared to microalgae (Mur et al. 1999). Furthermore, the fact that the cultures are more productive if urea is used as nitrogen source makes the use of commercial fertilizers-based artificial culture media more attractive as a common strategy to lower the production cost (Bermejo 2018). The latter is briefly discussed below in this discussion.

The production of *Chroococcidiopsis* sp. at large scale remains a challenge. The biomass productivity values achieved in the experiments of this Thesis in lab scale cultures of *Chroococcidiopsis* sp. were all below 0.22 $g \cdot L \cdot d^{-1}$. The productivity of *Chroococcidiopsis* sp. seems to be limited by low inorganic carbon bioavailability (Schipper et al. 2019), which possibly makes production in outdoor systems for dense cultures. Schipper et al. (2019) also studied the optimal CO_2 concentration for *Chroococcidiopsis* sp. and showed that an increase in CO_2 concentration, from 5% to 30% (v/v) in the gas supplied to the cultures, should not necessarily lead to increased biomass productivities. This fact may be related to the limited CO_2 diffusion through the EPS envelope of *Chroococcidiopsis* sp., typical feature of these cyanobacterium. Besides, the cells exhibit aggregation; this could also limit the diffusion rate of the dissolved inorganic carbon towards inner cells in the aggregates. Moreover, carbon uptake has been reported to be limited by low rate carbon concentration mechanisms (Schipper et al. 2019) which consequently impact the growth of the cyanobacterium negatively.

In short, although *Chroococcidiopsis* sp. might tolerate high CO_2 concentrations, this must be optimized to avoid CO_2 squandering. In this sense, the maximum productivities reported so far in outdoor cultivation of *Chroococcidiopsis* sp. with CO_2 as carbon source are roughly around 0.12 g $L\ d^{-1}$ (Schipper et al. 2019), still below those values obtained in laboratory cultures (this Thesis), which means there is still room for improvement of the *Chroococcidiopsis* sp. productivity at large scale. In addition, temperature

optimization could become relevant to produce *Chroococcidiopsis* sp. at large scale. In its natural habitat, this cyanobacterium is able to withstand a wide range of temperatures since the differences in temperatures between day and night, in the area where *Chroococcidiopsis* sp. was isolated in the Atacama Desert, range between -5 ºC and 50 ºC (Meslier et al. 2018). This could be an advantage for large-scale cultivation, where temperature control for most of the microalgae and cyanobacteria production processes increases the cost of the final product (Ruiz et al 2016).

Part of this Thesis was aimed at studying how to reduce the production costs of biomass production or the accumulation of interesting compounds at large-scale cultivation. Production of cyanobacterial biomass implies a high demand for optimum analytical grade reagents, in addition to a considerable amount of time required to prepare culture medium for massive production. All together may increase the final cost of the product noticeably (Kumari et al. 2015). The replacement of the culture medium for agricultural NPK fertilizers has been studied in this thesis for the extreme environment cyanobacterium *Chroococcidiopsis* sp. This strategy has been previously used for production of other cyanobacteria and microalgae which, result reduced of production costs and even higher productivities than those obtained by means of conventional culture media (Nayak et al. 2016; Silva-Benavides 2016). The approximate cost of 1L of BBM culture medium is approximately 0.13 €. The commercial fertilizer-based culture media cost was found to be approximately 16 times cheaper than the BBM medium, which becomes highly advantageous if applied in production processes at large scale.

Overall, taking in mind the economic advantages related to using NPK based culture media, it can be said that the use of fertilizers should be considered as a promising tool for the sustainable production of *Chroococcidiopsis* sp. Besides, it would also be interesting to study the effect of fertilizer-based media on the biomass composition and their impact on target metabolites production which is of great importance for the end commercial use of the products.

Biotechnological potential

As referred in the Introduction section, *Chroococcidiopsis* sp. can be a valuable source of novel compounds of outstanding properties to human health. These compounds are mostly produced as a result of metabolic

adaptations of the extremophilic cyanobacteria to cope with the harsh conditions of their habitat. A crucial structural mechanism in the adaptation of extreme environment cyanobacteria to anhydrobiosis is the production of abundant exopolysaccharide-rich envelopes (EPS) (Wierzchos et al. 2015). These cyanobacterial EPS have been reported to display outstanding anti-inflammatory and antibiotic properties (Moscovici 2015). The production of these EPS can be induced by variations in the physicochemical parameters of the cultures, such as nutrient limitation or starvation (Sengupta et al. 2018). Determining the chemical composition and structure of the EPS produced by the *Chroococcidiopsis* sp. isolated in this Thesis work would allow assessing its potential as bioactive compound. In this sense, the presence of sulphate groups in the EPS structure confers this molecule proven antioxidant and anti-inflammatory activities (Montero et al. 2018).

In the *Chroococcidiopsis* sp. strain isolated in this Thesis, the accumulation of carbohydrates and EPS was found to be stimulated under stress conditions by limiting or inhibiting the cyanobacterial growth (Chapter VI). These conditions could be related to the limitation or lack of an essential nutrient, particularly N and/or inorganic P, as described by Moreno et al. 1998, and even by changing the nitrogen source of the culture medium. The latter has also been described for other microorganisms (Khani et al. 2016) The inorganic N to C assimilation imbalance in N-lacking cultures of microalgae and cyanobacteria is known to address accumulation of carbohydrates and lipids whilst reducing the intracellular protein content even to less than 50% the initial content (Schipper et al. 2019). The consequence of this strategy in our *Chroococcidiopsis* sp. strain is the significant increase of EPS to total carbohydrate ratio. Thus, N to C imbalance should help establish better production strategies for enriching the cyanobacterium in EPS.

Cyanobacteria contain water-soluble proteins known as phycobiliproteins (PBP) which are interesting from the biotechnological point of view. In this Thesis, the effect of the light intensity was analyzed in cultures of *Chroococcidiopsis* sp. paying special attention to the production of PBP, given the high content of PBP of the isolated strain (Chapter VI). Likely other cyanobacteria, the PBP content of *Chroococcidiopsis* sp. increased under low light intensity (Guihéneuf et al. 2015; Pandey et al. 2011). In our study we found promising PBP production results in relation to other PBP

producers. Further studies are needed to analyze the effect of other influencing parameters that could improve the production of PBP in *Chroococcidiopsis* sp. cultures. Nevertheless, it is necessary to take into account that the abiotic stress induced by suboptimal pH and temperature values is known to produce a degradation effect in the PBP structure, which affects its biotechnological production (Stanic-Vucinic et al. 2018; Kannaujiya et al. 2017).

The PBP profile revealed interesting results since *Chroococcidiopsis* sp. produced a higher content of allophycocyanin (APC) compared to other cyanobacteria (Kumar et al 2011). This could possibly be due to the influence of the specific type of light supplied to the cultures (Ojit et al. 2014), an evolutionary adaptation that allows *Chroococcidiopsis* sp. to be more efficient by capturing light of certain wavelengths (Sharma et al. 2014). Although the APC can be used to develop specific applications such as quantum yield assays or flow cytometry techniques, it has received little attention so far. This is due to some challenges in APC processing, mainly the fact that APC is a minor component of the total phycobiliproteins content of a cyanobacterial cell (Tavanandi et al. 2019). The results obtained in this Thesis for *Chroococcidiopsis* sp. place this cyanobacterium as a promising source of APC.

Overall, extremophile-based products development still must face technological and economic challenges to become really competitive. Nevertheless, the knowledge generated by academies, new companies and multinational companies is growing rapidly, and this is due to the extraordinary biotechnological potential of these extremophilic microorganisms.

CONCLUSIONS

CONCLUSIONS

1. Temperature (36.51 °C or 36.81 °C), pH (8.20 or 8.96), and salinity (15.01 or 12.03%, w/v) are the optimal conditions for the *H. mediterranei* biomass and carotenoid production. RMS approach serves to accurately predict both the biomass and carotenoid production by the haloarchaea at any temperature, pH, and salinity of the media, which is valuable for performing C_{50} carotenoid production particularly bacterioruberin by *H. mediterranei* in a one-step process.

2. *H. mediterranei* can be an outstanding producer of the red antioxidant C_{50} pigment bacterioruberin. Bacterioruberin accounts roughly for about 90% of the total pigment content in *H. mediterranei*. Furthermore, most carotenoids are produced at the end stages of growth.

3. For first time, in *H. mediterranei*, the definite positive influence of nutrient starvation on the accumulation of pigments (mostly bacterioruberin) was shown by *H. mediterranei*. Glucose starvation was the most productive condition for pigment accumulation suggesting a role for glucose as BR biosynthesis inhibitor. Low levels of magnesium sulphate were found to be sufficient for sustaining fast growth. These observations allow suggest that large scale bacterioruberin production could be developed by two different strategies: (i) cultivating *H. mediterranei* in conditions which simultaneously allow growth and bacterioruberin accumulation, and (ii) running two phase processes, with a first phase for biomass production in nutrient-replete culture medium and a second phase under glucose and/or yeast extract starvation.

4. The cyanobacterium *Chroococcidiopsis* sp. from extreme arid environment was isolated and systematically grown on a suitable, selected culture medium. Improved growth was achieved by vigorous agitation of the liquid cultures and using specifically urea or nitrate as the most suitable nitrogen sources at relatively high concentrations. Moderate productivity values of roughly 0.20 $g \cdot L^{-1} \cdot d^{-1}$ and the cell aggregates tendency to produce sediment, point out *Chroococcidiopsis* sp. as a promising strain for large scale production.

5. Compared to commercial cyanobacteria such as *Spirulina platensis*, *Chroococcidiopsis* sp. biomass is relatively rich in carbohydrates, and could

be suitable as source of linoleic and linolenic fatty acids which account for more than 50% of the total unsaturated fatty acids content of the cyanobacterium. Together with phycocyanin, the aforementioned molecules point out potential for *Chroococcidiopsis* sp. as a food valuable ingredient.

6. Light irradiance regulates PBP accumulation in *Chroococcidiopsis* sp. Each specific phycobiliprotein accumulated on a different quantitative pattern as a function of the light irradiance, thus each of them probably having a different role depending on the intensity of the incident light. Particularly, APC could play a leading role among PBP in light dissipation under excess photons. The high APC maximum intracellular levels produced at relatively high light intensities suggest *Chroococcidiopsis* sp. could have potential for production of this valuable PBP.

7. The use of commercial fertilizers could favor the increase of the productivity of the hyper-arid cyanobacterium *Chroococcidiopsis* sp. in cultures in liquid medium, and the chemical composition of fertilizers determines their effectiveness, being especially productive those that contain urea in its composition. The specific fertilizer composition in N and P also influences carbohydrate and phycobiliprotein accumulation in *Chroococcidiopsis* sp., thus producing biomass of shifted quality.

8. In *Chroococcidiopsis* sp. the biosynthesis and accumulation of carbohydrates could be stimulated under stress conditions that limit or inhibit their growth. These stress conditions could be related to the limitation or lack of an essential nutrient, particularly N, which would allow determining and optimizing production strategies for production of carbohydrates-enriched biomass.

9. The referred stress conditions might favor the production of exopolysaccharides of *Chroococcidiopsis sp*. The specific composition of each NPK medium might in addition influence EPS production differently, this requires being further researched.

CONCLUSIONES

1. La temperatura (36.51 °C y 36.81 °C), pH (8.20 y 8.96) y la salinidad (15.01 y 12.03%, p/v) son las condiciones óptimas para la producción de carotenoides y biomasa, respectivamente, de *H. mediterranei*. El enfoque RMS nos permite predecir con precisión tanto la producción de biomasa como de carotenoides por las haloarchaea a cualquier temperatura, pH y salinidad dentro del rango estudiado, lo cual es valioso para llevar a cabo la producción de carotenoides C_{50}, particularmente bacterioruberina por *H. mediterranei*, en un proceso de una sola fase.

2. *H. mediterranei* puede ser un excelente productor del pigmento rojo antioxidante $C_{50,}$ la bacterioruberina. La bacterioruberina representa aproximadamente el 90% del contenido total de pigmento en *H. mediterranei*. Además, la mayoría de los carotenoides se producen en las etapas finales de crecimiento.

3. Por primera vez, en *H. mediterranei*, se demostró la influencia positiva de la deficiencia de nutrientes en la acumulación de pigmentos (principalmente bacterioruberina). La deficiencia de glucosa fue la condición más productiva para la acumulación de pigmento, lo que podría demostrar un papel inhibidor de la glucosa en cuanto a la biosíntesis de BR. Se demostró también que niveles bajos de sulfato de magnesio son suficientes para mantener un crecimiento rápido. Estas observaciones permiten sugerir que la producción de bacterioruberina a gran escala podría desarrollarse mediante dos estrategias diferentes: (i) cultivar *H. mediterranei* en condiciones que permitan simultáneamente el crecimiento y la acumulación de bacterioruberina, y (ii) ejecutar procesos de dos fases, con una primera fase para la producción de biomasa en medio de cultivo pleno en nutrientes y una segunda fase con deficiencia de glucosa y/o extracto de levadura.

4. La cianobacteria *Chroococcidiopsis* sp., de ambiente árido extremo, se aisló y se cultivó sistemáticamente en un medio de cultivo adecuado para su crecimiento. Se logró un crecimiento mejorado mediante la agitación vigorosa de los cultivos líquidos y se determinó específicamente urea o nitrato como las fuentes de nitrógeno más adecuadas para el crecimiento de la cianobacteria a relativamente altas concentraciones de dichas fuentes de nitrogeno. Valores moderados de productividad de aproximadamente 0,20 g

$\cdot L^{-1} \cdot d^{-1}$ y la tendencia de los agregados celulares sedimentar, señalan a *Chroococcidiopsis* sp. como una cepa prometedora para su producción a gran escala.

5. En comparación con cianobacterias comerciales como *Spirulina platensis*, la biomasa de *Chroococcidiopsis* sp. es relativamente rica en hidratos de carbono, y podría ser adecuada como fuente de ácidos grasos linoleico y linolénico, los cuales representan más del 50% del contenido total de ácidos grasos insaturados de la cianobacteria. Junto con la ficocianina, las moléculas antes mencionadas señalan el potencial de *Chroococcidiopsis* sp. como valioso ingrediente en la elaboración de productos en alimentación.

6. La irradiancia de luz regula la acumulación de PBP en *Chroococcidiopsis* sp. Cada ficobiliproteína específica se acumula en un patrón cuantitativo diferente en función de la irradiancia de la luz, por lo que cada uno de ellos probablemente tenga un papel diferente dependiendo de la intensidad de la luz incidente. En particular, APC podría desempeñar un papel específico en la disipación de luz bajo exceso de fotones. Los altos niveles intracelulares de APC producidos a intensidades de luz relativamente altas sugieren que *Chroococcidiopsis* sp. podría tener potencial para la producción de esta valiosa PBP.

7. El uso de fertilizantes comerciales podría mejorar la productividad de la cianobacteria de ambiente árido extremo *Chroococcidiopsis* sp. en cultivos en medio líquido, y la composición química de los fertilizantes determina su efectividad, siendo especialmente productivos aquellos que contienen urea en su composición. La proporción de N y P también influye en la acumulación de carbohidratos y ficobiliproteínas en C*hroococcidiopsis* sp., produciendo así biomasa de distinta calidad.

8. En *Chroococcidiopsis* sp. la biosíntesis y la acumulación de carbohidratos podrían estimularse en condiciones de estrés que limitan o inhiben su crecimiento. Estas condiciones de estrés podrían estar relacionadas con la limitación o falta de un nutriente esencial, particularmente N, que permitiría determinar y optimizar las estrategias de producción para la producción de biomasa enriquecida con carbohidratos.

9. Las condiciones de estrés referidas podrían favorecer la producción de exopolisacáridos de *Chroococcidiopsis* sp. La composición específica de cada medio NPK podría además influir en la producción de EPS de manera diferente, lo cual requiere una mayor investigación.

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About the author

Zaida Montero Lobato was born in Paradas (Sevilla, Spain), on the 2dn of June 1991. In 2010 she started her studies on "Environmental Science" at the University of Huelva, Spain. In 2014 she graduated with Special Mention for the best academic record. During her studies she started to collaborate as student research trainee inside of the department of "Environmental Biology and Health" at the University of Huelva. There, she developed her first research on extremophilic microorganisms which was awarded with the 1st Prize "Sapere Aude", organized by the University of Huelva and Catedra CEPSA. In 2014, she started a one year MSc in Biohazard Assessment-Industrial hygiene-Prevention of Occupational Hazards. She carried out her practices for the Master's degree at the Juan Ramón Jimenez Hospital in Huelva where she worked carrying out the biohazard assessment to which hospital workers are exposed. During this work, she studied the antimicrobial effect of an extremophilic microalgae on pathogenic bacteria commonly associated to hospital environments and which showed resistance to common antibiotics.

In 2015, she started to collaborate inside the Biotechnology of Algae Group (Bital) within the "Department of Chemistry" at the University of Huelva. In December of this year she obtained a contract in the European Project "SPLASH" (Sustainable PoLymers from Algae Sugars and Hydrocarbons) from the Seventh Framework Programme, in which she was working up to September 2016.

In 2017, she started her PhD student contract as part of the Bital Group. She focused on the isolation, screening of the potential application, optimization of the culture media and conditions to obtain added-value products from extremophilic microorganisms. During her three years of PhD she attended different congresses and conferences, and she had the opportunity to move to Bergen (Norway) for a research internship in UniResearch. There, she worked on the screening and cultivation of various cold environment microalgae and she also worked on medium re-use and she evaluated the effects of media recycling on microalgae cultivation in order to lower the costs for large-scale microalgae production.