# Exploring the biotechnological potential of *Cyanobium* sp. pigments

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# Abstract

Cyanobacteria are a group of prokaryote photosynthetic organisms able to synthesize multiple high-value compounds, such as pigments, which are known for its multiple bioactivities and several uses in industries.

This dissertation aims to exploit the biotechnological potential of a poorly studied cyanobacterium, *Cyanobium* sp. LEGE 06113, particularly in what concerns the pigments bioactive potential. Therefore, the work was divided into two major parts: (i) Evaluation and characterization of bioactive potential of *Cyanobium* sp. pigments-rich extracts, and (ii) Optimization of biomass and bioactive pigments production by *Cyanobium* sp..

In the first part, organic and aqueous pigment-rich extracts were obtained by a classic extraction methodology using several solvents – organic solvents: acetone (**A**), ethyl acetate (**EA**) and ethanol (**E**); and an inorganic solvent: water (**W**) – in order to extract different groups of pigments. Also, attempting to increase the efficiency of extraction from *Cyanobium* sp. biomass, successive extractions were performed using **W** if the first extraction was performed with an organic solvent (**A**, **EA** or **E**), and with **A** if the first extraction was with **W**. A total of eight extracts was obtained and the extracts named according to the solvents used for its obtention - four organic extracts: **A**, **EA**, **E**, W-**A**; and four aqueous extracts: **W**, A-**W**, E-**W**, EA-**W**. The bioactive potential of *Cyanobium* sp. extracts was assessed in terms of antioxidant capacity by performing $ABTS^{\bullet+}$, $^{\bullet}NO$, $O_2^{\bullet-}$ scavenging assays. The pigment profile and content of carotenoids and phycobiliproteins, and the content in phenolic compounds of each extract were determined, since this group of compounds is usually related to antioxidant capacity. Moreover, in order to evaluate the possible cytotoxic features of the extracts, a cell viability assay (MTT) was also performed for the most promising pigment-rich extracts (W-**A** and E-**W**).

In the second part, the optimization of biomass and pigments production was attained using a factorial Box-Behnken modelling for a range of three abiotic factors – temperature (20 - 30 ºC), pH (6.0 - 9.0) and salinity (10 - 30 $g.L^{-1}$). The interaction of these abiotic factors was set according to a factorial plan, resulting in 13 runs of combinations tested during 18 days, each one. Biomass, photosynthetic activity, carotenoid and phycobiliproteins production and antioxidant capacity of acetonic and aqueous extracts were determined over time and plotted into quadratic models. Using the quadratic models, the optimal T, pH and [NaCl], for simultaneous production of biomass and pigments (carotenoids and phycobiliproteins) were calculated and, in order to validate the model, the optimal condition set was tested.

Regarding the first part, generally, the most promising extracts were the successive extracts of acetone after water (W-**A**) and water after ethanol (E-**W**). W-**A** showed the higher antioxidant capacity results in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ scavenging assays and was one of the organic extracts with higher content in carotenoids. E-**W** showed the highest content in phycobiliproteins and was one of the best aqueous extracts in terms of antioxidant capacity. In what concerns the cytotoxic evaluation, all the tested concentrations of W-**A** revealed to have a high cytotoxic effects, being more suitable for pharmaceutical industries, on the other hand, E-**W** seems more promising for food and feed industries since it revealed to be non-cytotoxic for all the range of concentrations tested.

In terms of the optimization of bioactive pigments production by *Cyanobium* sp., results revealed that temperature and pH had a more significant impact than salinity, on its metabolism, and it was possible to determine a significant quadratic model for all tested parameters. According to the factorial modelling, the optimal condition for biomass, carotenoids and phycobiliproteins productivity would be obtained at 20 ºC, pH 9.0 and 10 $g.L^{-1}$ of NaCl, as was subsequently confirmed.

The work developed along this dissertation may constitute a valid contribution for the valorisation of *Cyanobium* sp. as an interesting strain for biotechnological applications and to help solving the high-cost constrains related to cyanobacteria-based bioprocesses, especially on the biomass production step.

Keywords: Cyanobacteria, antioxidant capacity, cytotoxicity, carotenoids, phycobiliproteins, Box-Behnken factorial

# Resumo

As cianobactérias são um grupo de organismos procariotas capazes de sintetizar múltiplos compostos de elevado valor económico, tais os como pigmentos que são conhecidos pelas suas propriedades bioativas e pelos seus possíveis usos em diversas indústrias.

Esta dissertação tem como objetivo a exploração do potencial biotecnológico de uma cianobactéria pouco estudada, o *Cyanobium* sp. LEGE 06113, focando-se particularmente no potencial bioativo dos seus pigmentos. Com este objetivo, o trabalho foi dividido em duas partes: (i) Avaliação e caracterização do potencial bioativo de extratos de *Cyanobium* sp. ricos em pigmentos, e (ii) Otimização da produção de pigmentos bioativos pelo *Cyanobium* sp..

Na primeira parte, foram obtidos extratos, orgânicos e aquosos, ricos em pigmentos, usando uma metodologia clássica de extração assistida com solventes – orgânicos, acetona (**A**), acetato de etilo (**EA**) e etanol (**E**) e um solvente inorgânico, água (**W**) – de forma a extrair diferentes grupos de pigmentos. Para aumentar a eficiência de extração, foram realizadas extrações sucessivas usando **W**, quando a primeira extração tinha sido realizada com um solvente orgânico (**A**, **EA** ou **E**), e usando **A**, quando a primeira extração tinha sido realizada com **W**. Foi obtido um total de oito extratos denominados de acordo com os solventes usados para a sua obtenção – quatro extratos orgânicos: **A**, **EA**, **E**, W-**A;** e quatro extratos aquosos: **W**, A-**W**, E-**W**, EA-**W**. O potencial bioativo dos extratos de *Cyanobium* sp. foi avaliado em relação à sua capacidade antioxidante utilizando ensaios de sequestro dos radicais $ABTS^{\bullet+}$, $^{\bullet}NO$, $O_2^{\bullet-}$. O perfil e quantificação de pigmentos (carotenóides e ficobiliproteinas) e o conteúdo em compostos fenólicos de cada extrato foi também determinado, pois estes compostos estão normalmente relacionados com capacidade antioxidante. Para os dois extratos mais promissores (W-**A** e E-**W**) foi também realizado o ensaio de viabilidade celular (MTT) de modo a avaliar a sua citotoxicidade.

Na segunda parte, foi realizada a otimização de produção de pigmentos usando um modelo fatorial do tipo Box-Behnken para uma gama de três fatores abióticos – temperatura (20 - 30 ºC), pH (6.0 - 9.0) e salinidade (10 - 30 $g.L^{-1}$). A interação destes três fatores abióticos foi estabelecida de acordo com o plano fatorial, resultando em 13 combinações, testadas durante 18 dias cada uma. A produção de biomassa, carotenóides e ficobiliproteinas, assim como a atividade fotossintética e a capacidade antioxidante de extratos acetónicos e aquosos, foi determinada ao longo do tempo e os dados obtidos inseridos num modelo quadrático. Usando o modelo quadrático, foram calculados os valores ótimos de T, pH e [NaCl] para produção simultânea de biomassa

e pigmentos (carotenoides e ficobiliproteinas). De modo a validar o modelo quadrático obtido, a condição ótima calculada foi testada.

Relativamente à primeira parte, os extratos mais promissores foram os sucessivos de acetona a seguir a água (W-**A**) e de água a seguir a etanol (E-**W**). W-**A** mostrou ser o extrato com mais capacidade antioxidante para os ensaios $ABTS^{\bullet+}$ e $^{\bullet}NO$ além de ser um dos extratos com maior conteúdo e carotenoides. Por outro lado, E-**W**, revelou ser o extrato com mais conteúdo em ficobiliproteinas e, de entre os extratos aquosos, apresentou um dos melhores resultados no que toca a capacidade antioxidante. A avaliação da citotoxicidade dos extratos revelou que todas as concentrações testadas de W-**A** apresentaram elevados efeitos citotóxicos, sendo então a sua utilização mais viável em indústrias farmacêuticas. E-**A**, pelo contrário, não demonstrou efeitos citotóxicos para nenhuma das concentrações testadas demonstrando ser exequível a sua utilização na indústria alimentar.

No que toca à otimização de pigmentos bioativos, os resultados revelaram que a temperatura e o pH tiveram um impacto mais significativo no metabolismo do *Cyanobium* sp. que a salinidade, e foi possível obter um modelo quadrático para todos os parâmetros testados. De acordo com o modelo obtido, a condição ótima para produção de biomassa, carotenóides e ficobiliproteinas seria 20 ºC, pH 9.0 e 10 $g.L^{-1}$ de NaCl, como foi subsequentemente confirmado.

O trabalho desenvolvido ao longo desta dissertação poderá constituir uma contribuição para a valorização do *Cyanobium* sp. como uma estirpe interessante do ponto de vista biotecnológico, e para ultrapassar alguns problemas relacionados com os custos elevados de produção de biomassa de cianobactérias para fins industriais.

Palavras chave: Cianobactéria, capacidade antioxidante, citotoxicidade, carotenóides, ficobiliproteinas, fatorial Box-Behnken

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# List of Abbreviations

| | |
|---|---|
| **A** | Acetone |
| **ABTS** | 2,2'-azino-bis(3-thylbenzothiazoline-6-sulphonic acid) |
| **AOX-A** | Antioxidant compounds for acetonic extract |
| **AOX-W** | Antioxidant compounds for aqueous extract |
| **APC** | Allophycocyanin |
| **Carot** | Carotenoids |
| **Chl** | Chlorophyll |
| **DMSO** | Dimethyl sulfoxide |
| **DMH** | Dimethylhydrazine |
| **DW** | Dry weight |
| **E** | Ethanol |
| **EA** | Ethyl Acetate |
| **Fv/Fm** | Maximal quantum yield |
| **GAE** | Gallic Acid Equivalent |
| **HepG2** | Human hepatoma cell line |
| **HPLC** | High performance liquid chromatography |
| **IC** | Inhibitory concentration |
| **LEGE-CC** | LEGE Culture Collection |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantification |
| **MTT** | 3-(4,5-dimethylthiazole-2- yl)-2,5-diphenyltetrazolium bromide |
| **NADH** | β-nicotinamide adenine dinucleotide |
| **NBT** | Nitrotetrazolium blue chloride |
| **W** | Water |
| **PAM** | Pulse-Amplitude Modulation |
| **PB** | Phosphate buffer |
| **PC** | Phycocyanin |
| **PDA** | Photodiode array |
| **Phyco** | Phycobiliproteins |
| **PMS** | Phenazine methosulphate |
| **$P_X$** | Biomass Productivity |
| **PS** | Photosystem |
| **OD** | Optical Density |
| **ROS** | Reactive oxygen species |

| | |
|---|---|
| **rpm** | Revolutions per minute |
| **SNP** | Sodium nitroprusside |
| **T** | Temperature |
| **TC** | Total carotenoids |
| **TE** | Trolox equivalent |
| **TPB** | Total phycobiliproteins |
| **$X_0$** | Initial biomass concentration |

# 1. Introduction

## 1.1. Cyanobacteria

Cyanobacteria represent a unique group of organisms. They are the only known prokaryote organisms capable of oxygenic photosynthesis, having a major role in the carbon dioxide fixation and oxygen release – being thus a fundamental source of primary production in many ecosystems (Hess, 2011; Beck et al., 2012).

As ubiquitous organisms, with a large diversity of species, they cover a vast amount of habitats, including: deserts, lakes, oceans, soil, glaciers, streams and hot springs – due to their wide range of physiological properties and tolerance to environmental stress (Sánchez-Baracaldo et al., 2005; Uzair et al., 2012). In fact, cyanobacteria are considered one of the most successful groups of organisms on earth having fossil records from 3.3 to 3.5 billion years ago (Singh et al., 2011).

Their morphology is equally diverse, the simplest one is unicellular, free-living (Fig. 1a) or surrounded by a mucilaginous envelope (glycocalyx, sheath, capsule or slime) (Fig. 1b) (Geada et al., 2017), but evolution lead to a new morphology: cells organized in a row, designated trichomes. The trichomes can be surrounded by a sheath being then called filaments (Fig. 1c), and the most complex structure that these organisms can present is branched filaments (Fig. 1d) (Abed et al., 2009; Lee, 2018). Some filamentous strains also present climate-resistant cells called alkinetes (Singh et al., 2011). In terms of cell composition, cyanobacteria cell wall is similar to Gram-negative bacteria, formed by a peptidoglycan layer right after a periplasmatic space that is then surrounded by an outer membrane (Geada et al., 2017; Lee, 2018).

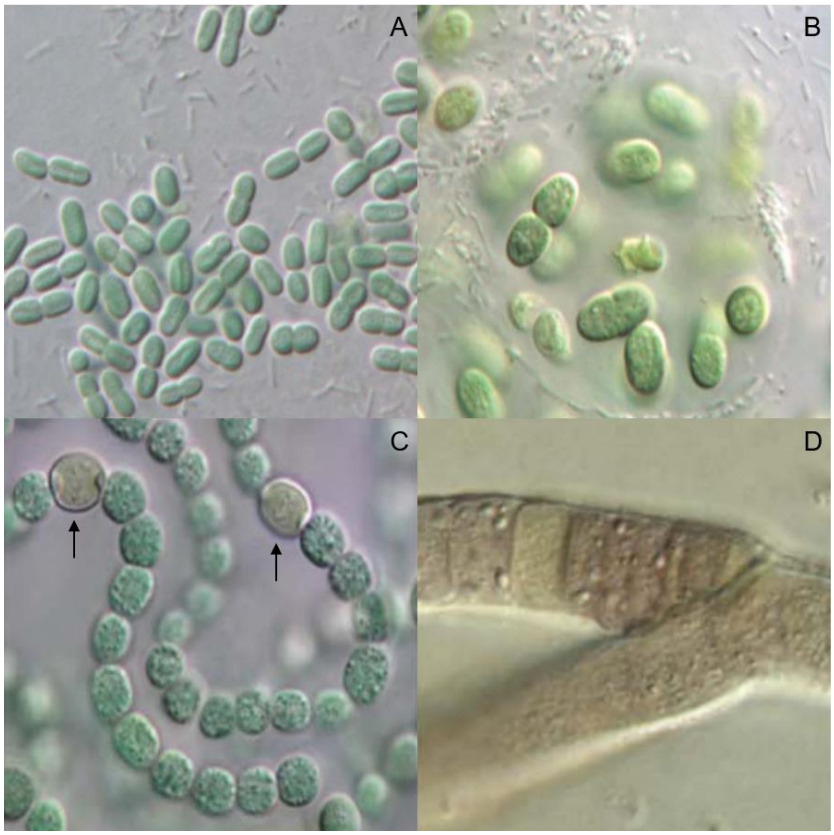


Figure 1. Morphological cyanobacteria diversity. A) *Synechococcus* sp.; B) *Aphanothece* sp.; C) *Nostoc* sp. (heterocysts pointed out with arrows); D) *Scytonema ocellatum*. Adapted from Neustupa and Škaloud, 2008 and Brinkmann et al., 2015.

Cyanobacteria are also capable of multiple metabolic processes - some strains are capable of anoxygenic photosynthesis (Cohen et al., 1986; Stal, 1995), various types of fermentation (homolatic, heterolatic, mix acid and homoacetate) (Stal and Moezelaar, 2006), and atmospheric nitrogen fixation. For this last one, cyanobacteria can be grouped in heterocystous and non-heterocystous forms, where the first have specific vegetative cells designed to fix $N_2$, called heterocysts (Fig. 1c) (Bergman et al., 1997). Nevertheless, the main metabolic process performed by this group of organisms is oxygenic photosynthesis. In the cyanobacteria cells, the machinery for the photosynthetic light-reactions is located in the thylakoids. Thylakoids are an intracellular membrane system that can show several intracellular organizations (Mullineaux, 1999; Geada et al., 2017). Associated with the thylakoid membrane there are pigments that take part in light-harvesting and energy transfer to the reaction centres – processes essential for photosynthesis. Pigments in the thylakoids are structured in form of complexes called antennae, and are usually grouped in three major classes: chlorophylls (Chl), carotenoids and phycobiliproteins (Richmond 2004).

Although the pigment composition present in the photosynthetic machinery differs between different cyanobacteria groups, photosynthetic organisms have Chl *a* as a part of their core reaction centre. Chl *b*, *c* or *d* can also be present as accessory pigments in the antenna widening the light absorption range (Masojdek et al. 2007). Furthermore, Chl show two absorption bands - 450-475 nm and 630-675 nm (Fig. 2).

In cyanobacteria, the main class of light harvesting pigments-protein complexes are phycobiliproteins. These pigments are organized to form a complexes called phycobilisomes which are attached to the cytoplasmatic face of the thylakoid membranes (Gantt 1980; Bogorad 2003; Pagels et al. 2019). In cyanobacteria, the photosystem (PS) II is mainly composed of phycobilisomes while the PS I is exclusively constituted by Chl *a* (Masojdek et al. 2007). Phycobiliproteins absorb light in the visible spectrum between 500-650 nm (Fig. 2) and work together with Chl *a* to maximize light harvesting (Gantt, 1980). These pigments are divided in four main classes: phycocyanin, phycoerythrin, phycoerythrocyanin and allophycocyanin, being phycocyanin the most common one in cyanobacteria. Nevertheless, some cyanobacteria can synthesise phycoerythrin or can have phycoerythrocyanin as a substitute. Allophycocyanin, works as a mediator in the energy transfer between other phycobiliproteins and the photosynthetic reaction centres (Dumay and Morançais, 2016; Pagels et al., 2019).

Carotenoids are a vast group of terpenoid pigments considered essential for the survival of photosynthetic organisms (Cogdell and Frank, 1987; Guedes et al., 2011b). Within this large group of more than 600 pigments there are two major classes, the carotenes and the xanthophylls. Carotenes, e.g. α and β-carotene, are composed only by carbon and hydrogen while xanthophylls, which include pigments like violaxanthin and astaxanthin, have also oxygen-containing functional groups in their composition (Higuera-Ciapara et al., 2006; Del Campo et al., 2007; Masojdek et al., 2007; Guedes et al., 2011b).

Carotenoids can also be classified according to their role in the cell – primary carotenoids (e.g. α -carotene, β -carotene and lutein) that are crucial for cell survivance and are directly involved in the photosynthesis; and secondary carotenoids (e.g. astaxanthin and canthaxanthin) that are only produced and accumulated when cells are exposed to certain stimuli by a process denominated carotenogenesis (Guedes et al., 2011; Zhang et al.,2014).

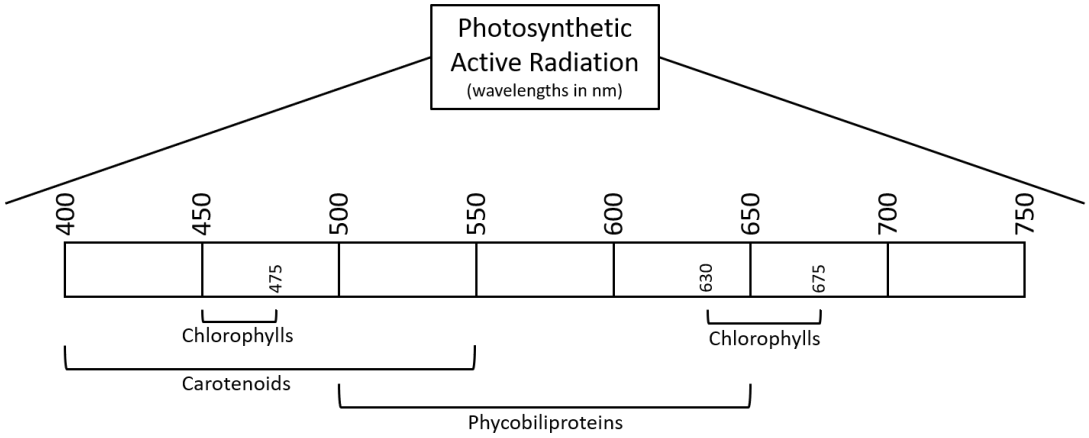


Figure 2. Squematic representation of the absobtion range of the three main classes of pigments (chlorophylls, carotenoids and phycobiliproteins) in the visible light spectrum.

Carotenoids play two major roles in photosynthesis. They work as accessory light-harvesting pigments, with a light absorption range between 400 and 550 nm (Fig. 2), by transferring energy to Chl *a* and expanding the wavelength range in which light is able to be used for photosynthesis (Cogdell and Frank, 1987; Masojdek et al., 2007). These compounds are also important agents for the protection of the photosynthetic machinery (membranes, photosystems and antenna). Simultaneously, carotenoids can quench reactive oxygen species (ROS) and Chl triplets resulting in triple state carotenoids, which release the excess of energy safely as heat (Varela et al., 2015). These pigments are also involved in a process called non-photosynthetic quenching. This process lowers the levels of Chl singlets by thermal dissipation, meaning that the probability of chlorophyll triplets formation decreases and consequently the ROS production, specifically singlet oxygen, is prevented (Müller et al., 2001).

Also involved in the protection of the cells is another group of compounds – polyphenols. They are thought to be part of the antioxidant response to UV-light since their content increased in microalgal biomass after an exposure (Goiris et al., 2012; Safafar et al., 2015). Although the high content in phenolic compounds is usually associated with terrestrial plants, several studies have reported that microalgae and cyanobacteria also produce several classes of flavonoids. Polyphenols compounds like, gallate, chlorogenate, cinnamate, pinostrobate, and p-OH-benzoates were found in *Arthorspira* sp. (Klejdus et al., 2009). These compounds also show antioxidant properties since they scavenge free-radicals directly through single electron or hydrogen atom transfer, and due to their ability of metal chelation, which inhibits the formation of free-radicals catalysed by transition metals (Rice-Evans et al., 1997).

## 1.2. Biotechnological uses for cyanobacteria pigments

Among all the compounds that can be obtained from cyanobacteria, pigments emerged as the components with higher market prices, being the main source of revenues for industries when compared to carbohydrates, proteins and lipids also obtained from these organisms (Ruiz et al., 2016). Pigments are already used in pharmaceutical, nutraceutical, cosmetic, food and feed industries due to their vast properties. These several applications raised the interest of many companies, leading to a cyanobacteria mass production, as well as phycobiliproteins extraction and purification at an industrial scale. *Arthorspira platensis* is the main cyanobacterium used to produce phycobiliproteins, although the existence of other promising species (Pandey et al., 2013).

In what concern carotenoids the most used organisms are the microalgae, *Dunaliella salina* to produce β-carotene and *Haematococcus pluvialis* to produce astaxanthin. Pilot scale projects for the production of lutein using *Muriellopsis* sp. and *Scenedesmus almeriensis* are also being carried (Del Campo et al., 2007).

Regarding extraction and purification processes, phycocyanin is sometimes sold in form of raw biomass product (*Spirulina* containing phycocyanin), however, the biological benefit of the pigment can be increased within the purification process (Yu et al., 2017; Pagels et al., 2019). The extraction process requires a mixture of mechanical and chemical extraction due to a high resistance of the cyanobacteria cells, and thus the methodology needs to be chosen according to the organism (Pan-utai and Iamtham, 2018). The chemical extraction is based on the high solubility of phycocyanin in aqueous solvents, using one (classical) or more (two-phase or successive) solvents, according to the desired purity (Stewart and Farmer, 1984; Moreno et al., 1997).

In the case of carotenoids, the extraction is usually made from dried biomass and consist in several methodologies, including the conventional extraction using organic solvents (Ruane, 1974; Nonomura, 1987; Gamlieli-Bonshtein et al., 2002). Nevertheless, cell disruption processes are also used together with solvents. Carotenoids are usually sold as carotenoids rich oils, called oleoresin, which contains a great amount of a specific carotenoid (e.g. 25% of lutein) (Cerón et al., 2008; Panis and Carreon, 2016).

### 1.2.1. Pharmaceutical applications

Nowadays, society faces many issues related to several diseases that are induced or intensified by oxidative stress, like cancer, atherosclerosis or Alzheimer's, among others (Lobo et al., 2010; Guedes et al., 2011c).

Oxidative stress is caused by several molecular reactive species, among them, and most commonly, ROS. ROS are naturally produced by the cellular metabolism, but can also have an exogenous origin, triggered by physiological stresses caused, for example, by air pollution, cigarette smoke or excessive radiation (Guedes et al., 2011c; Assunção et al., 2017).

Some ROS fit into the class of free radicals, meaning that they have one or more unpaired electrons that may become unstable and consequently, highly reactive. Hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet and nitric oxide radical are some of the main ROS also produced in many disease states (Lobo et al., 2010). Humans have natural antioxidant defences, i.e. molecules that have the ability to remove, prevent or delay oxidative damages, such as glutathione, uric acid, and an enzyme system that includes superoxide dismutase and catalase (Halliwell, 2007; Lobo et al., 2010). However, if the balance between those natural defences and free radical production is destabilized, oxidative damages can occur, damaging lipids, nucleic acids and proteins (Lobo et al., 2010; Assunção et al., 2017). When this occurs, antioxidants from exogenous origin should be included in the diet in order to aid in homeostasis restore (Lobo et al., 2010; Assunção et al., 2017).

Oxidative stress is typically associated with the development of several human conditions like inflammatory diseases (Romay et al., 1998). Inflammation can be defined as a physiological defence of the tissues in order to respond to endogenous or exogenous harmful stimulus (Zamora et al., 2000; Khansari et al., 2009). The inflammatory response can be divided in two stages: acute and chronic inflammation. The first corresponds to an initial stage of inflammation, triggered by the activation of the immune system (innate immunity), usually bringing benefits to the host and persists for short times. However, if the inflammation persist for a longer period of time it can lead to a chronic inflammation stage which can affect the human health, once this stage has been correlated with several chronic diseases (Reuter et al., 2010). Free radicals, ROS and reactive nitrogen species (RNS), are also generated in the inflammatory process. When the innate immune system is activated in response to a certain stimulus, pro-inflammatory molecules, cytokines and chemokines, are secreted by the immune system cells. These molecules induce the production of ROS and RNS which helps to eliminate pathogens for instance, however, an overproduction of these free radicals may occur if the inflammatory response persists which can lead to DNA damages and diseases like cancer (Khansari et al., 2009).

In order to restore the oxidative homeostasis, synthetic antioxidants have been produced, however these have been associated with toxic side effects like liver damages

and carcinogenesis and consequently raised safety issues (Guedes et al., 2013b). In this perspective, cyanobacteria emerged as a solution since they can be a source for natural antioxidants, like pigments (Goiris et al., 2012; Guedes et al., 2013b; Pandey et al., 2013).

Phycobiliproteins are a group of pigments that, holds a lot of potential to be used in pharmaceutical formulations. These pigments in addition to its proved antioxidant and anti-inflammatory properties also shows neuroprotective and hepatoprotective properties (Sekar and Chandramohan, 2008).

Phycocyanin derived from *Aphanizomenon flos-aquae* showed to be a strong antioxidant when applied *in vitro* against oxidative damage (Benedetti et al., 2004). Also, phycocyanin radical scavenging properties showed to inhibit microsomal lipid peroxidation (Sekar and Chandramohan, 2008), and phycocyanin derived from *Arthorspira platensis* exhibited hypocholesterolemic activity by modelling serum cholesterol concentrations (Nagaoka et al., 2005). As an anti-inflammatory, phycocyanin also decreases oedema, histamine release, myeloperoxidase activity, and levels of prostaglandin and leukotrienes in inflammation tissues (Sekar and Chandramohan, 2008) and is known to have the capacity to suppress inflammatory cofactors like NF-kB which is linked to cancer and autoimmune diseases (Reddy et al., 2000; Bhat and Madyastha, 2001). Phycocyanin antitumor effect have been also proven by decreasing the tumour necrosis factor in mice blood serum treated with endotoxin, and particularly one isolated from *Arthrospira platensis* was found to inhibit the growth of human leukaemia K562 cells (Liu et al., 2000). In terms of hepatoprotective properties, phycocyanin also revealed to play a role in a human hepatitis animal model. This pigment reduced the serum alanine amino transferase, aspartate amino transferase, and malondialdehyde (González et al., 2003).

Among carotenoids, β-carotene also exerts several benefits for the human health. Its antioxidant capacity aid in: immune system stimulation; prevention of free radicals life-threatening diseases such as arthritis, coronary heart diseases, premature aging; some types of cancer and specifically Alzheimer's disease, caused by persistent oxidative stress in the brain (Mattson, 2004; Törnwall et al., 2004). Nakashima et al. (2009) also observed that cognitive impairment was prevented in transgenic mice fed with extracts from *Chlorella* sp. containing β-carotene and lutein. As specific examples, it was found that β-carotene from *Dunaliella* sp. was able to lower the incidence of several types of cancer and degenerative diseases (Ben-Amotz and Fishler, 1998); β-carotene from *Chlorella ellipsoidea* and *Chlorella vulgaris* was able to inhibited the development of colon cancer (Plaza et al., 2009). Moreover, in the human body β-carotene can be

converted to vitamin A which have a wide range of diverse biological functions related to human health, for example helping in the immunity cataracts prevention, night blindness and skin diseases (Pisal and Lele, 2005).

Among xanthophylls, lutein and zeaxanthin also revealed to hold specific biological roles in decreasing cancer development, enhancing immune function and age-related macular degeneration (Lakshminarayana et al., 2010). Moreover, in terms of antioxidant capacity, lutein revealed to be more effective than β-carotene in inhibiting auto-oxidation of cellular lipids, and in protecting against oxidant-induced cell damage (Lakshminarayana et al., 2010). Indeed, lutein has been recommended for prevention of cancer and diseases related to retinal degeneration. This xanthophyll can also significantly inhibit growth of androgen-dependent and androgen-independent prostate cancer cell lines *in vitro*, as well as, prevent colon carcinogenesis *in vivo* (Narisawa et al., 1996); they also play a role upon inhibition of proliferation of human mouth epithelial cancer line KB (Sun et al., 2006). Additionally, Reynoso-Camacho et al. (2011) demonstrated an additional chemoprotective effect of lutein against colon cancer induced by DMH. In mice, lutein fed at 0.002% of the diet, showed to decreased the number of tumors by 55% and 32%, respectively (Reynoso-Camacho et al., 2011).

### 1.2.2. Food and nutraceutical applications

Pigments can also have application in the food and nutraceutical industries. One of the main uses for microalgae pigments in these industries is to substitute synthetic dyes and antioxidants since some have adverse effects to human health and consumers associate synthetic with unhealthy leading their preferences towards more “natural” products (Carocho et al., 2014; Caporgno and Mathys, 2018). Phycocyanin obtained from *A. platensis* is already used as a colorant in food items such as candies, jellies, chewing gum, ice sherbets, popsicles, soft drinks, dairy products and wasabi (Santiago-Santos et al., 2004; Spolaore et al., 2006). Moreover, phycocyanin extracts and whole *A. platensis* were incorporated to produce cookies to enhance protein and fibre content, with potential health benefits (El Baky et al., 2015).

Carotenes, like β-carotene from *Dunaliella,* are commonly used as food colorants to improve the appearance of margarine, fruit juices, cheese, baked goods, canned foods, dairy products and confectionary, to attract consumers. It is also being approved by United States of Food and Drug Administration as food colour, being recognized as safe natural colour. Moreover, as reported previously, β-carotene is also used as pro-vitamin A (retinol) in the formulation of healthy foods (Spolaore et al., 2006).

## 1.3. Influence of production conditions on pigments content

The synthesis of compounds, such as pigments, is influenced by biotic and abiotic factors. Changes in the pH, light, temperature, salinity, nutrient availability and other conditions modulate the productivity and the composition of biomass (Juneja et al., 2013; Guedes et al., 2014). Cyanobacteria quickly response to changes in the environment will induce their adjustment to nutritional needs which will consequently change their chemical composition (Guedes et al., 2011c). For that reason, the optimization of the growth conditions of specific strains has been studied in order to increase biomass production and bioactive compounds synthesis (Guedes et al., 2011c).

### 1.3.1. Temperature

One of the most important factor for all living organisms is temperature, it influences metabolic processes and the composition of the cells (Hemlata and Fatma, 2009), and for cyanobacteria it may affect the growth rate, cell size and nutrient requirements (Juneja et al., 2013). The optimal growth temperature and temperature tolerance range varies from strain to strain (Hemlata and Fatma, 2009). Temperature may cause photoinhibition, which has a great impact on growth rate, and stress by oxygen deficiency, since at high temperatures oxygen is much less soluble (Hemlata and Fatma, 2009; Juneja et al., 2013).

In the production of antioxidant compounds, temperature also plays an important role, particularly in pigments. In some species the production of phycocyanin has been described to be enhanced by higher temperatures – equal or higher than 30 ºC (Chaneva et al., 2007; Hemlata and Fatma, 2009; Hifney et al., 2013; Johnson et al., 2014; Maurya et al., 2014). For β-carotene it has been described that low temperatures (ca. 10 °C) can led to an increase of 4.0-fold in the carotenes production rather than higher temperatures (> 26 °C) (Ben-Amotz and Fishler, 1998; Gómez and González, 2004; Raja et al., 2007). On the other hand, Guedes et al. (2011c) reported that high temperatures induced the production of lutein and β-carotene in the microalgae *Scenedesmus obliquus*. In what concerns lutein production, high temperatures can favour the accumulation of lutein, although some strains are less tolerant and extra temperature increases can be damaging for the culture (Fernández-Sevilla et al., 2010).

### 1.3.2. pH

pH is also a key parameter in algal production once it defines the solubility and availability of $CO_2$ and others essential nutrients (Juneja et al., 2013). Cyanobacteria in general

mainly grows in neutral to alkaline pH (7.0 – 9.0), however, although changes in pH can affect their metabolism and inhibit growth, cyanobacteria have a great ability to adapt and thus to grown in other pH ranges (Juneja et al., 2013; Guedes et al., 2014). However, cells composition varies depending on pH values, e.g. it has been described that cyanobacteria tend to prefer more alkaline environment for both growth and phycocyanin accumulation, in a range from 8.0 to 10.0 (Poza-Carrión et al., 2001; Maurya et al., 2014; Keithellakpam et al., 2015; Kaushal et al., 2017). Hemlata and Fatma, (2009) also reported that the phycobiliprotein content of an *Anabaena* strain decreased 19% when the external pH was altered from 8.0 to 6.0. For lutein production, pH around 8.0 - 9.5 seem to be the best, although the effect and range varies depending on the strain. On the other hand, for β-carotene production at an industrial scale with *D. salina*, pH is kept around 7.5 for a higher β-carotene accumulation (Hosseini Tafreshi and Shariati, 2009).

### 1.3.3. Salinity

Another important factor to have in consideration in marine cyanobacteria production is salinity, and in the case of artificial media, NaCl is usually used to ascertain the salinity. Similarly to the former parameters, salinity affects the growth rate and biochemical composition of the cells (Juneja et al., 2013). Cyanobacteria acclimate better to salinity than higher plants who have a more restrict salt tolerance range (Sudhir and Murthy, 2004). High NaCl concentrations (ca. 0.5 M) have increased the phycocyanin production in some species, such as *Phormidium* sp., *Oscillatoria* sp. and *Arthrospira* sp. (Fuenmayor et al., 2009; Jonte Gómez et al., 2013). However, in other species, the high amounts of NaCl led to a decrease in the phycocyanin accumulation, as the case of *Anabaena* sp. and *Limnothrix* sp. (Hemlata and Fatma, 2009; Lemus et al., 2013). For carotenoids, Schubert et al. (1993) reported that higher salt concentrations lead to an increase in the production of carotenoids in *Synechocystis* sp. Similar results were found for the microalgae *Dunaliella tertiolecta* by Fazeli et al. (2006), although, the higher concentration of salt favoured total carotenoids production but reduced the growth rate of the culture and thus the total carotenoid productivity decreased.

## 1.4. Aims and framework

From a biotechnological point of view, cyanobacteria are a group of organisms with far potential but yet not well explored. More than a hundred thousand species are expected to exist (Guiry, 2012), but only a few thousands strains are maintained in culture collections, and from those not more than some hundreds have been screened and even less are produced in industrial scale. Moreover, taking in account the benefits and

industrial potential of cyanobacterial pigments, screening the bioactive potential of poorly studied strains might constitute a strategy to discover new marine resources since it is believed that this organisms can be a real source of bioactive compounds and that can be exploited for several applications (Guedes et al., 2011a).

*Cyanobium* sp. LEGE 06113, is a cyanobacteria strain isolated by LEGE Culture Collection (LEGE-CC) that has already shown to have potential biotechnological interest. Leão et al. (2013) reported the isolation of hierridin B from an extract of this *Cyanobium* strain which showed selective antitumoral activity against the colon adenocarcinomona HT-29 cell line. Later on, hierridin C was isolated from the same extract and showed antimalarial capacity (Leão et al., 2016). However, there is a lack of knowledge concerning other bioactive potential compounds, such as pigments.

This strain belongs to the *Cyanobium* genus, created by Rippka & Cohen-Bazire in 1983 when a more precise classification of cyanobacteria strains was attempted. Before this genera were created, these cyanobacteria belonged to the genus *Synechoeoccus* which included organisms with very different sizes, chemical and physiological properties and showed a wide range of mean DNA-base composition (Rippka and Cohen-Bazire, 1983). The new genus *Cyanobium* is now used as a taxonomic rank that comprises 14 different species, being the *Cyanobium gracile* Rippka & Cohen-Bazire the type specie of the genus (Guiry, 2018). The complete genomes of a few *Cyanobium* strains have already been sequenced, for example, ones isolated in Brazil and in Japan (Lima et al., 2014; Yamaguchi et al., 2016). The taxonomic classification of the *Cyanobium* genus is explicit on Table 1.

Table 1. Taxonomic framework of the genus *Cyanobium.*

| Taxonomic Rank | Taxon |
|---|---|
| Empire | Prokaryota |
| Kingdom | Eubacteria |
| Phylum | Cyanobacteria |
| Class | Cyanophyceae |
| Subclass | Synechococcophycidae |
| Order | Synechococcales |
| Family | Synechococcaceae |
| Genus | *Cyanobium* |

The cyanobacteria of this genus are usually oval to ellipsoid single cells, with 1.2 ± 0.2 μm of diameter, but might show up in pairs, right after division, and rarely appear in small

pseudofilaments. In what concerns to division process, it is symmetrical and occurs by binary fission in only one plane, parallel to the shorter cell axis. *Cyanobium* is distributed in a wide range of habitats like brackish water, freshwater and marine environments and when in culture they have the particularity of forming a homogenous suspension without a strong production of mucilage (Rippka and Cohen-Bazire, 1983; Komárek et al., 1999; G. Cronberg, E. J. Carpenter, 2004; Ramos et al., 2018). In terms of intracellular organization, the thylakoid pattern is parietal along the cell walls and the nucleoids show an elongated band-like form (Komárek et al., 1999; Hoffmann et al., 2005).

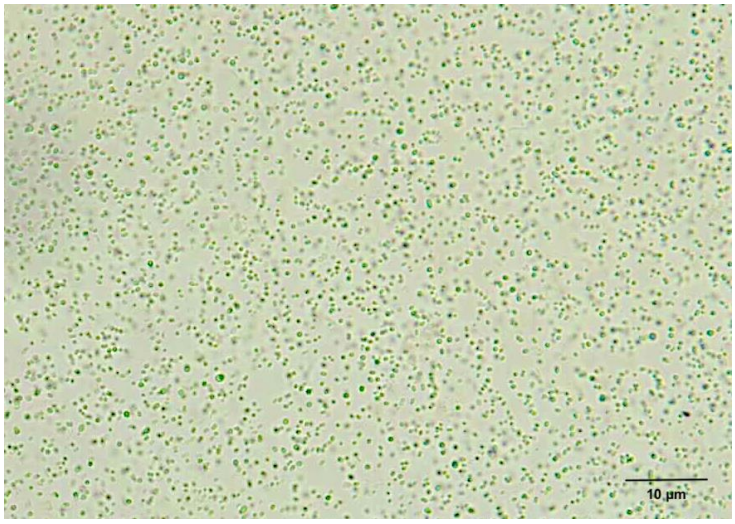


Figure 3. *Cyanobium* LEGE 06113 in optical microscopy 40X (Leica DM500).

Taking this into account, the main goal of this dissertation was to exploit the poorly studied biotechnological potential of *Cyanobium* sp. LEGE 06113 pigments.
To achieve the main goal of this dissertation, the work was divided in two major parts with specific aims:

1. Evaluation and characterization of the bioactive potential of *Cyanobium* sp. pigments-rich extracts:
   (a) Determination of the biochemical profile of the extracts, in terms of carotenoids, phycobiliproteins and phenolic compounds.
   (a) Evaluation of bioactive potential of the obtained pigment-rich extracts, in terms of their antioxidant capacity.
   (c) Determination of the cytotoxic effects of the most promising *Cyanobium* sp. pigments-rich extracts.

2. Optimization of biomass and bioactive pigments production by *Cyanobium* sp.:

(a) Evaluation of the effects of the interaction of three abiotic factors (temperature, pH and salinity) on pigments and biomass production, photosynthetic efficiency and antioxidant capacity of *Cyanobium* sp. pigment-rich extracts.

(b) Selection of the best temperature, pH and salinity conditions to increase the productivity of the culture in terms of biomass and bioactive pigments.

# 2. Material and Methods

## 2.1. *Cyanobium* sp. pigments extraction and bioactive potential assessment

### 2.1.1. Cyanobacterium source and biomass production

Biomass production of *Cyanobium* sp. LEGE 06113, obtained from LEGE Culture Collection (LEGE-CC), was performed in batch mode in 5 L glass flat bottom round flasks, at 25 °C, using as culture medium a modified Blue Green medium (BG11+) (Allen, 1968 adapted with twice phosphate and nitrates – data not yet published), with an initial pH of 7.5. Light was provided by fluorescent lamps (BIOLUX, OSRAM) with an intensity of 200 $\mu mol_{photons}.m^{-2}.s^{-1}$ and a light:dark cycle of 16:8 h. Constant air flow was also assured at 0.75 $L_{air}.L_{culture}^{-1}.min^{-1}$. For each batch, a pre-inoculum with an initial optical density of 0.1 (OD = $A_{680nm} - A_{750nm}$) was grown in 2 L glass flat bottom round flasks in the same conditions for 10 days, to ensure that the culture was in the exponential growth phase. The biomass was harvested by centrifugation, after 25 days, when the culture reached the stationary growth phase. The biomass was then freeze-dried and stored in low-humidity (exicator) and darkness conditions, for further analyses for prior to analysis.

### 2.1.2. Pigments-targeted extraction

In order to obtain pigment-rich extracts, single and successive extractions of *Cyanobium* sp. were performed using four solvents - acetone (**A**), ethyl acetate (**EA**), ethanol (**E**) and water (**W**). The methodology is explained in the following sections.

#### 2.1.2.1. Single extractions

The extractions were performed, in triplicate, using 250 mg of freeze-dried biomass and with final solvent volume of 15 mL. To improve the extraction rate, it was performed using a bead beater Precellys Homogenizer (Bertin, France) in three cycles with 5 mL of solvent each (6 series of 8000 rpm for 30 s with 45 s of pause); 670 mg of 0.1 mm beads were also added to the bead beater flasks together with the biomass to maximize cell disruption. Extracts were centrifuged at 2000 g for 10 min and the supernatant was dried – **A**, **EA** and **E** by a rotavapor system and **W** by a freeze-drying. All extracts were stored in low-humidity (exicator) and darkness conditions, for further analyses.

### 2.1.2.2. Successive extractions

The remaining biomass from the single extractions (see 2.1.2.1) was used for the attainment of four successive extracts. An aqueous extract was obtained from the biomass previously used in the extractions with **A**, **EA** and **E**, and an acetonic extract was obtained from the remaining biomass after W extraction, as described before (section 2.1.2.1). Extracts were centrifuged, and the supernatants were dried - acetonic extract by a rotavapor system and the aqueous extracts by a freeze-drying. All extracts were stored in low-humidity (exicator) and darkness conditions, for further analyses.

## 2.1.3. Antioxidant capacity assessment

The antioxidant capacity of each extract was accessed by $ABTS^{\bullet+}$, $O_2^{\bullet-}$ and $^{\bullet}NO$ scavenging assays. For all the assays the extracts were resuspended in phosphate buffer (100 µM) with 20 % of dimethyl sulfoxide (DMSO) (v/v) in an extract concentration of 10 $mg.mL^{-1}$. A series of seven successive dilutions of each extract was tested in order to determine the concentration able to scavenge 50 % and 25 % of the radicals (IC50 and IC25). For the three assays the IC values were calculated with Graphpad Prism® software (version 7.0) through curve spline interpolation. All the assays were performed in chemical and biological triplicates.

### 2.1.3.1. Determination of antioxidant capacity by $ABTS^{\bullet+}$ assay

The $ABTS^{\bullet+}$ [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] method is based on the generation of the blue/green ABTS radical cation due to the reaction between ABTS and potassium persulfate. This chromophore compound has a peak of absorbance at 734 nm and in the presence of antioxidant molecules, its generation is inhibited. The method was performed as described by Guedes et al. (2013a, 2013b) for each extract with concentrations ranging from 32 and 389 $µg.mL^{-1}$.

In detail, 63 µL of each dilution were transferred to a 96-well plate and mixed, with 180 µL of $ABTS^{\bullet+}$. After the addition of $ABTS^{\bullet+}$, the reaction was incubated for 6 min in darkness and the absorbance read at 734 nm. The extract absorbance interference was reduced by a blank using distilled water instead of $ABTS^{\bullet+}$.

### 2.1.3.2. Determination of antioxidant capacity by $O_2^{\bullet-}$ assay

The superoxide method consists in an indirect spectrophotometric assay to evaluate the capacity of the extracts to scavenge the superoxide radical. The superoxide radical is formed when the reduced form of phenazine methosulphate (PMS) reacts with oxygen.

The PMS is reduced by β-nicotinamide adenine dinucleotide (NADH) added to the reaction. The formed superoxide radical reacts with nitrotetrazolium blue chloride (NBT) reducing it to formazan, a blue compound that can be measured by reading the absorbance at 560 nm. Any molecule capable of scavenging the superoxide radical will decrease the reaction speed of NBT reduction. This assay was performed according Pinho et al. (2011) for a series of dilutions of each extract with concentrations between 42 and 333 $\mu g.mL^{-1}$.

In detail, 50 µL of each dilution were transferred to a 96-well plate and mixed, with 50 µL of 166 µM NADH, 150 µL of 43 µM NBT and 50 µL of 2.7µM PMS. After the addition of PMS, the reaction was incubated for 10 min in darkness. After the incubation time the superoxide radical scavenging capacity was determined by reading the absorbance at 560 nm. The extract interference was reduced by a blank using 19 µM phosphate buffer (PB) instead of PMS.

### 2.1.3.3. Determination of antioxidant capacity by $^{\bullet}NO$ assay

The $^{\bullet}NO$ scavenging capacity of the extracts was performed as described by Pinho et al. (2011). Nitric oxide, which is spontaneously formed when sodium nitroprusside (SNP), reacts with oxygen forming nitrite. The nitrite formation can be quantified by Griess reaction which forms a chromophore measured by optical density at 562 nm. This assay was performed for seven final extracts concentrations between 83 and 667 $\mu g.mL^{-1}$.

In detail, 100 µL of each dilution were transferred to a 96-well plate and mixed, with 100 µL of 20 mM SNP. The solution was incubated for 60 min exposed to ca. 20 $\mu mol_{photons}.m^{-2}.s^{-1}$ of fluorescent light. After the incubation time 100 µL of Griess reagent (1.0 % sulphanilamide and 0.1 % N-(1)-naphthylethylenediamine in 2 % phosphoric acid) were added to the solution and incubated for 10 min in darkness. After the incubation time the scavenging capacity was determined by reading the absorbance at 562 nm. To dismiss extracts interference, a blank was performed using 2 % phosphoric acid instead of Griess reagent.

## 2.1.4. Cytotoxicity evaluation

The cytotoxicity of the extracts was evaluated by the measurement of the viability of a human hepatoma cell line (HepG2) obtained from American Type Culture Collection (ATCC). The cellular viability was assessed by the mitochondria dependent reduction of the 3-(4,5-dimethylthiazole-2- yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan which can be quantified by optical density measurement at 510 nm as described by

Lopes et al. (2012). A series of dilutions of the extracts were tested, between 46.9 and 750.0 µg.mL$^{-1}$.

In detail, HepG2 cell line was seeded in 96 well plates at a density of 3.3 x 10$^4$ cells.mL$^{-1}$. After 24 h the cell medium was removed and replaced with new medium containing *Cyanobium* sp. extract, in the range of concentration mentioned before. Cell medium with 1 % and 20 % DMSO were also added as negative and positive control respectively. Cells were exposed to the range of extract concentrations and to the controls for 24 h. After the exposition time, 20 µL of a MTT solution (1 mg.mL$^{-1}$) dissolved in medium, were added to each well and incubated for 4 h at 37 ºC. After this period the medium was removed and 100 µL of DMSO were added to the wells in order to dissolve the formazan salts. Absorbance was read at 550 nm.

The assay was independently repeated four times, each one in duplicates for all the extracts. Cytotoxicity was expressed as a percentage of cell viability considering the values of the negative control as 100 % viability.

## 2.1.4. Total phenolic content quantification

Total soluble phenolic content of the extracts was determined by Folin-Ciocalteu method, as described by Maadane et al. (2015), using gallic acid as a standard. This method is based on the transfer of electrons from phenolic compounds to phomolybdic/phosphotungstic acid complexes, present in the Folin-Ciocalteu reagent, to form blue complexes that are determined spectroscopically at 760 nm (Ainsworth and Gillespie, 2007).

Extracts were resuspended at 1 mg.mL$^{-1}$, in PB (100 µM) with 20 % of DMSO (v/v). In a 96 well microplate, 25 µL of the resuspend extracts, 125 µL of distilled water and 65 µL of Folin-Ciocalteu reagent were added in triplicate and incubated for 5 minutes in the dark. After, 75 µL of sodium carbonate 6.6% were added to the wells and incubated for 1 h in the dark. After the incubation time, the absorbance was read at 760 nm. For the quantification of the total soluble phenolic content, a calibration curve of gallic acid was established and the results expressed as gallic acid equivalents (GAE) per mg of extract ($mg_{GAE}.g_{extract}^{-1}$).

## 2.1.5. Pigments characterization and quantification

### 2.1.5.1. Carotenoids

The profile and quantification of carotenoids was determined by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection (Waters Alliance 2695, USA), following the method described by Guedes et al. (2011). The extracts were

resuspended in acetone:acetonitrile (9:1) in a concentration of 10 $mg.mL^{-1}$, and an internal standard β-carotenol (170 $mg.L^{-1}$; Sigma) was added. A column heater (Waters, USA) was used during the analyses in order to maintain a constant temperature of 25 ºC.

The stationary-phase was constituted by a 4 x 250 mm Purospher Star RP-18e (5 µm) column (Merck), and mobile-phase by ethyl acetate (solvent A), and acetonitrile/water at 9:1 (v/v) (solvent B). An overall flow rate of 1 $mL.min^{-1}$ was set with various volumetric ratios of the solvents A and B along the elution time: 0–31 min (0–60 % A); 31–36 min (60 % A); 36–38 min (60–100 % A); 38–43 min (100 % A); 43–50 min (100-0 % A); and 50–55 min (0 % A). Spectra data from all peaks were collected in the range 250 to 750 nm. The compounds were identified by comparing the retention times and the UV-visible spectrum with the authentic standards. The elution times of the chromatographic authentic standards were: lutein 12.4 min, zeaxanthin 12.8 min, chl *a* 24.2 min, echinenone 24.6 min and β-carotene 32.5 min. Standards (HPLC grade) were purchased at Extrasynthese (lutein, zeaxanthin and β-carotene) and at Sigma (Chl *a* and echinenone). The calibration curves, correlation coefficient ($R^2$), limit of detection (LOD = 3*So/b) and limit of quantification (LOQ = 10*So/b; where So is the standard deviation of signal-to-noise ratio and b is the calibration plot slope) for the analysed carotenoids are shown in the Table 2. The results were expressed in mg of the respective carotenoid by g of extract ($mg_{carot}.g_{extract}^{-1}$).

Table 2. Calibration curves of authentic standards used for carotenoids profiling and its respective correlation coefficient ($R^2$), limit of detection (LOD) and limit of quantification (LOQ).

| Standards | Calibration curve ($mg.mL^{-1}$) | $R^2$ | LOD[b] | LOQ[c] |
|---|---|---|---|---|
| Lutein | y = 133772885*x - 22683 | 0.999 | 0.882 | 2.675 |
| Zeaxanthin | y = 1399289886*x - 119637 | 0.998 | 0.315 | 0.954 |
| β-carotene | y = 290230023*x + 172819 | 0.999 | 1.935 | 5.864 |
| Echinenone | y = 61438587*x + 12034 | 0.999 | 0.507 | 1.538 |

## 2.1.5.2. Phycobiliproteins quantification

For phycobiliproteins quantification, aqueous extracts were resuspended at 2 $mg.mL^{-1}$, in triplicate, and the absorbance read at 615 and 654 nm. The phycobiliproteins quantification was calculated as described by Bennett and Bogorad (1973) using the formulas: Phycocyanin (PC) = ($A_{615}$-0.474*$A_{652}$)/5.34; Allophycocyanin (APC) = ($A_{652}$-

$0.208*A_{615})/5.09$. The results were expressed in mg of the respective phycobiliprotein by g of extract ($mg_{phyco}.g_{extract}^{-1}$).

### 2.1.6. Statistical analysis

The experimental data were analysed using GraphPad Prism V. 7.0. A Shapiro-Wilk test of normality was performed before the analysis of variance (ANOVA) to confirm the normal distribution of the residuals. A two-way ANOVA with Tukey's multi-comparison test was used to found differences between extraction yields, antioxidant capacity, carotenoids, phycobiliproteins and phenolic content and cytotoxicity evaluation. Since each datum point had been replicated, a representative measure of variability was available in all cases to support said statistical analyses.

## 2.2. Optimization of bioactive pigments production by *Cyanobium* sp.

### 2.2.1. Experimental design and growth conditions

Aiming an optimization of pigments production by *Cyanobium* sp., a factorial Box-Behnken design was performed with three abiotic factors: temperature (T; 20 – 30 °C), pH (6.0 – 9.0) and NaCl concentration ([NaCl] 10 – 30 $g.L^{-1}$). The design and model were created and analysed in the Design-Expert 9.0 software (Stat-Ease, Inc., USA) (Hinkelmann, 2012). In total, 13 design points were performed in triplicate (Table 3) and a quadratic model was determined for each measured parameter: biomass, carotenoids, phycobiliproteins, antioxidant compounds productivity and photosynthetic efficiency.

Table 3. Box-Behnken matrix for the three variables (temperature, pH and [NaCl]) and respective levels for *Cyanobium* sp. batch experiments.

| Run | T (°C) | pH | [NaCl] ($g.L^{-1}$) |
|---|---|---|---|
| **1** | 20 | 6.0 | 20 |
| **2** | 20 | 7.5 | 10 |
| **3** | 20 | 7.5 | 30 |
| **4** | 20 | 9.0 | 20 |
| **5** | 25 | 6.0 | 10 |
| **6** | 25 | 6.0 | 30 |
| **7** | 25 | 7.5 | 20 |
| **8** | 25 | 9.0 | 10 |
| **9** | 25 | 9.0 | 30 |
| **10** | 30 | 6.0 | 20 |
| **11** | 30 | 7.5 | 10 |
| **12** | 30 | 7.5 | 30 |
| **13** | 30 | 9.0 | 20 |

A pre-inoculum was grown (for 7 days) in the central point condition (T: 25 °C; pH: 7.5; [NaCl]: 20 $g.L^{-1}$) in order standardize culture adaptation. Each design condition was tested in batch triplicate cultures, for 18 days, in glass flat bottom round flasks with 1800 mL of marine BG11 culture medium (Allen, 1968 adapted with twice phosphate and nitrates – data not yet published) with an initial optical density of 0.1 (OD = $A_{680nm}$ – $A_{750nm}$). Light intensity of 200 $\mu mol_{photons}.m^{-2}.s^{-1}$, light:dark cycle of 16:8 h and continuous air flow with a rate of 0.75 $L_{air}.L_{culture}.min^{-1}$ were set constant in all conditions. The pH was kept stable by the addition of buffers – MES (15 mM) for pH 6.0, HEPES (20 mM) for pH 7.5 and CHES (10 mM) for pH 9.0. Nine sampling days were set (0, 1, 4, 6, 8, 11, 13, 15, 18) for culture monitoring along time and all the referred parameters were evaluated in each one. Pigments characterization and quantification were only monitored in the days 1, 6, 11, 15 and 18.

## 2.2.2. Biomass quantification

### 2.2.2.1. OD determination

OD was determined, in duplicate, and the absorbance was read at 680 and 750 nm. The optical density of the culture was determined by the formula $A_{680nm}$-$A_{750nm}$ (Guedes et al., 2011c).

### 2.2.2.2. Chlorophyll *a* content

Chlorophyll *a* quantification was performed in triplicate as it follows: 1 mL of the culture was centrifuged at 4000 rpm for 5 min and the pellet washed twice with ultra-pure water and then centrifuged again. The supernatant was removed, and the pellet was dissolved in 1mL of methanol 90 %. The resuspended pellet was incubated for 15 min in the dark and absorbance was determined at 663 nm. The total chlorophyll *a* content was determined according to the following formula: $A_{664nm}$*12.7 ($mg_{Chl\ a}.L_{culture}^{-1}$), and results in terms of productivity of Chl *a* were expressed in mg of Chl *a* per litre of culture per day ($mg_{Chl\ a}.L_{culture}^{-1}.d^{-1}$) (Caroppo et al., 2012).

### 2.2.2.3. Dry weight determination

The dry weight (DW) was determined (in duplicate) by filtering a known volume of culture through preconditioned GF/C glass fibre filters (Whatman, UK), and further dried at 100 ºC for 24 h. In the conditions grown at 10 $g.L^{-1}$ of NaCl, 3 mL of culture were filtrated, and in the conditions grown at 20 $g.L^{-1}$ and 30 $g.L^{-1}$ of NaCl, to dismiss the NaCl interference, 3 mL of culture were previously diluted in distilled water to 10 $g.L^{-1}$ of NaCl and the total volume was filtrated.

### 2.2.2.4. Biomass productivity calculation

The biomass productivity ($P_X$) during the exponential phase were determined using the DW experimental data. $P_X$ ($g.L^{-1}.d^{-1}$) was calculated according to $P_X(t)=(X_t-X_0)/t$ where t is the sampling time, and $X_0$ the initial biomass concentration at the beginning of the experimental period (Guedes et al., 2011c).

## 2.2.3. *In vivo* chlorophyll *a* fluorescence monitoring

Photosynthetic activity was evaluated by *in vivo* chlorophyll fluorescence monitoring with Pulse-Amplitude Modulation (PAM) using a Junior-PAM/White fluorimeter (Walz, Germany). For that, 2 mL of the culture were centrifuged, and the medium removed in order to biomass concentration. Then, the biomass was incubated in darkness for 15 minutes. Before the measurement, the sample was exposed to a pulse of far-red light (0.8 s). For the maximal quantum yield (Fv/Fm), the culture was exposed to a basal measurement to estimate the minimum fluorescence (Fo), followed by a saturating pulse of 0.8 s (> 4000 $\mu mol_{photons}.m^{-2}.s^{-1}$) to obtain maximal fluorescence from the PSII (Fm) and the Fv/Fm (Figueroa et al., 2013).

### 2.2.4. Antioxidant capacity assessment

For monitorization of antioxidant capacity by $ABTS^{\bullet+}$ assay, an acetonic and a successive aqueous extraction were performed as it follows: 1 mL of each batch was centrifuged, at 2000 g for 5 min and the pellet was resuspended and homogenised in 1 mL of acetone 100 %. Cells were then broken in a bead beater (8000 rpm, 6 series of 30 s with 45 s pause) and the supernatant collected for further analysis. To extract water soluble compounds, the remaining pellet was resuspended in 1 mL of distilled water and the extract performed using the same beat beater settings mentioned before. The extract was centrifuged, and the supernatant collected for further analysis. Before performing the assay, the acetonic extracts were dried in a rotavapor and resuspended in the same volume of distilled water with 20 % DMSO. The antioxidant capacity assessment was evaluated in triplicate via the $ABTS^{\bullet+}$ assay as described by Guedes et al. (2011b; 2013b). For the quantification of the antioxidant capacity, a calibration curve with a known antioxidant, Trolox, was established and the antioxidant capacity expressed as trolox equivalents (TE) per DW of biomass ($mg_{TE}.g_{DW}^{-1}$). The productivity of antioxidant compounds was expressed as TE per litre of culture per day ($mg_{TE}.L_{culture}^{-1}.d^{-1}$).

### 2.2.5. Pigments characterization and quantification

#### 2.2.5.1. Sample preparation

For pigments characterization and quantification, acetonic and successive aqueous extractions were performed for, carotenoids and phycobiliproteins characterization and quantification, respectively. For that, 15 mL of each batch cultures were sampled and centrifuged at 2000 g for 15 min. The supernatant was discarded, and the pellet resuspended in 5 mL of acetone 100%. For control of the acetonic extraction process and quantification of carotenoids, 100 µL of β-carotenol internal standard (170 $mg.L^{-1}$; Sigma) were added. Extraction was performed in a bead beater (6 series of 8000 rpm for 30 s with 45 s of pause). After, the extract was centrifuged at 2000 g for 15 minutes, the supernatant was stored at -20 °C for carotenoid characterization and quantification.
For phycobiliproteins extraction, a successive aqueous extraction was then performed by resuspending the remaining pellet with 5 mL of distilled water and using the bead beater settled as referred before. After, the extract was centrifuged at 2000 g for 15 minutes and the supernatant was used for phycobiliproteins quantification.

### 2.2.5.2 Carotenoids

The characterization and quantification of carotenoids was performed using HPLC with PDA detection (Waters Alliance 2695, USA), following the method described by Guedes et al. (2011) as described in section 2.1.5.1. The results were expressed in terms of productivity of total carotenoids in mg per litre of culture per day ($mg_{carot}.L_{culture}^{-1}.d^{-1}$).

### 2.2.5.3. Phycobiliproteins

Phycobiliproteins quantification was performed in triplicated by reading the absorbance of the aqueous extracts at 615 and 654 nm. The phycobiliproteins quantification was calculated as described in section 2.1.5.2. The results were expressed in terms of productivity of total phycobiliproteins in mg per litre of culture per day ($mg_{phyco}.L_{culture}^{-1}.d^{-1}$).

## 2.2.6. Model Validation

In order to validate experimentally the quadratic model, a batch experiment was set with the optimal conditions of T, pH and [NaCl] obtained from the Box-Behnken design. All the assays for biomass, carotenoids, phycobiliproteins, antioxidant compounds productivity and photosynthetic efficiency were performed like described before (sections 2.2.2. – 2.5.3.).

## 2.2.7. Statistical analysis

Significance levels for each evaluated parameter were obtained by the analysis of variance (ANOVA). Also, a lack-of-fit test was applied to compare the residues of the model and the observed results. The model is validated whenever the statistical significance was higher than 0.05 in the lack-of-fit test. A Pearson correlation was also done for the biomass quantification parameters.

# 3. Results and discussion

## 3.1. Evaluation and characterization of *Cyanobium* sp. pigments bioactive potential

### 3.1.1. Extraction yields

For the attainment of the extracts, a solvent assisted extraction methodology was used. As referred before, cyanobacteria are able to synthesise different groups of pigments, from carotenoids to phycobiliproteins, that have different polarities and therefore for its extraction it is required to select the most proper solvent. In that way, three organic solvents with different polarities were chosen targeting the extraction of carotenoids. From the least polar to the most in terms of relative polarity, the organic solvents chosen were ethyl acetate, 0.23, (**EA**), acetone, 0.35, (**A**) and ethanol, 0.65, (**E**). For the extraction of phycobiliproteins, which are hydrophilic pigments, the chosen solvent was water, 1.00, (**W**). In order to maximize the extraction rate from *Cyanobium* sp. biomass, successive extractions were also performed using **W** after the extraction with organic solvents and using **A** after an extraction with **W**. A total of eight extracts was obtained and the extracts named after the solvents used to obtain them: four organic extracts – **A**, **AE**, **E** and W-**A**; and four aqueous extracts – **W**, A-**W**, AE-**W** and E-**W**. The yield of each extract is depicted on Table 4.

Table 4. Yield of the solvent assisted extractions for the *Cyanobium* sp.. Different lowercase letters show statistically significant differences ($p < 0.05$) among the organic extracts. Different capital letters show statistically significant differences ($p < 0.05$) among the aqueous extracts. Different symbols show statistically significant differences ($p < 0,05$) among the combined extracts yield.

| Organic | | Aqueous | | Total |
|---|---|---|---|---|
| **Solvent** | **yield (%)** | **Solvent** | **yield (%)** | **Combined yield (%)** |
| **A** | 9.27 ± 1,27 $^{a}$ | A-**W** | 36.03 ± 2.81 $^{B}$ | 45.31 ± 1.84 $^{*}$ |
| **EA** | 7,65 ± 0.10 $^{a}$ | EA-**W** | 45.74 ± 1.66 $^{A}$ | 54.40 ± 1.07 $^{+}$ |
| **E** | 26.98 ± 2.49 $^{b}$ | E-**W** | 23.59 ± 0.65 $^{C}$ | 50.57 ± 2.5 $^{*,+}$ |
| W-**A** | 10.32 ± 0.35 $^{a}$ | **W** | 47.04 ± 5.12 $^{A}$ | 55.94 ± 5.29 $^{+}$ |

Overall, the yield of aqueous extracts was higher than the organic ones. This happens probably due to the high content in aqueous soluble compounds in cyanobacteria cells, like phycobiliproteins – which is the main class of pigments in these organisms. Nevertheless, the use of different solvents led to different yields. Among the organic solvents, the higher extraction yield was 26.98 ± 2.49 % obtained with **E**, 3.5-fold higher

than the **EA** extraction, which showed the lowest extraction yield. In what concerns the aqueous extractions the higher yields were obtained with **W** and EA-**W** extractions attaining 47.04 ± 5.12 and 45.74 ± 1.66 %, respectively, with no significant differences. The **W** extraction showed an yield 2-fold higher than the E-**W** successive extraction, which revealed to be the lowest yield among the aqueous extracts. Concerning the organic solvent extractions similar results were reported by Giorgis et al. (2017), where the lowest yields were obtained using **A** as a solvent and the highest using **E**. The yield of extraction is dependent of the solvent used and evidences that polar solvents lead to higher yield percentages have already been reported before (Jerez-Martel et al., 2017). Moreover, successive extractions seem to represent a good strategy to maximize the use of biomass, these results demonstrate that the combined yield of the single extraction with the respective successive extraction can reach ca. 56 % being **EA**, EA-**W** and **W**, W-**A** the combinations that lead to higher yield percentages. These extraction yields calculations might be valuable as an economic viability indicator for industrial production.

## 3.1.2. Extract´s biochemical profile

In order to evaluate the extract's biochemical composition, targeting mainly carotenoids and phycobiliproteins, the quantification of these groups of compounds was performed. As phenolic compounds also exhibit antioxidant capacity, its content was determined in each extract. Results on the biochemical profile are presented in Fig. 4.

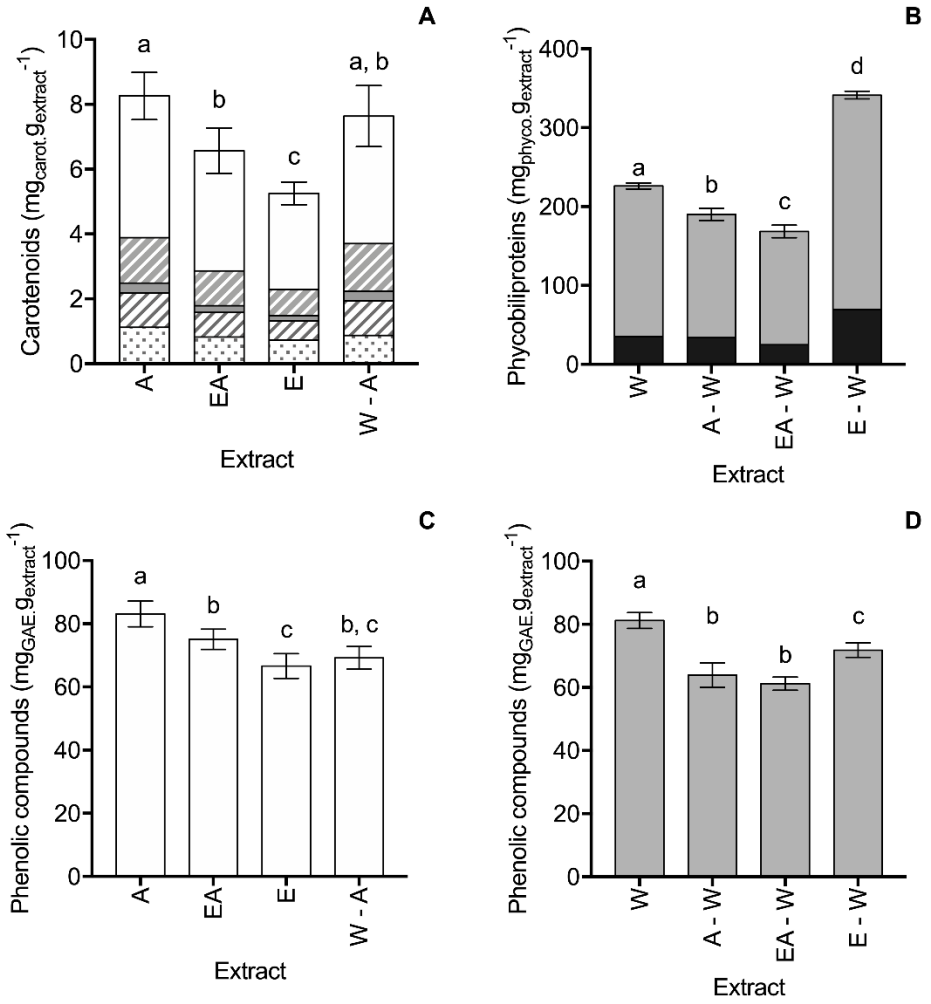


Figure 4. Biochemical composition (mean ± standard deviation, n =3) of *Cyanobium* sp. extracts, in terms of: (A) Total carotenoids (mg$_{carot}$.g$_{extract}^{-1}$) – bar colours represent specific identified carotenoids: Echinenone; Zeaxanthin; Lutein; β-carotene and Other carotenoids. (B) Total phycobiliproteins (mg$_{phyco}$.g$_{extract}^{-1}$) – bar colours represent specific identified phycobiliproteins: Phycocyanin and Allophyocyanin. (C) Total phenolic compounds in organic extracts (mg$_{GAE}$.g$_{extract}^{-1}$). (D) Total phenolic compounds (mg$_{GAE}$.g$_{extract}^{-1}$) in aquaous extracts. Different lowercase letters in the same graphic mean significant difference between extracts ($p < 0.05$).

The carotenoid profile and quantification were assed for all the extracts obtained with organic solvents – **A**, **E**, **AE** and W-**A**, results are depicted in Fig. 4A. The carotenoid profile of these extracts was similar but with different content, containing echinenone, zeaxanthin, lutein and β-carotene as the main carotenoids, and also unidentified derivates of those. Among these extracts, **A** and W-**A** showed the highest content in total

carotenoids, 4.37 ± 0,73 and 3.97 ± 0.94 $mg_{carot}.g_{extract}^{-1}$, respectively, with no significant differences ($p > 0.05$). These two extracts contained c.a. 1.4-fold more carotenoids than the extract with the least carotenoid content, **E**. The concentration of the identified carotenoids was also higher in the **A** and W-**A** extracts with equal amounts of lutein (0.31 ± 0.03 and 0.30 ± 0.03 $mg_{lutein}.g_{extract}^{-1}$), zeaxanthin (1.06 ± 0.10 and 1.07 ± 0.09 $mg_{zeaxanthin}.g_{extract}^{-1}$) and β-carotene (1.40 ± 0.16 and 1.46 ± 0.25 $mg_{\beta\text{-carotene}}.g_{extract}^{-1}$). An exception for echinenone which was highly extracted with **A**, 1.12 ± 0.11 mg $_{echinenone}.g_{extract}^{-1}$, 1.3-fold greater than the W-**A**. Similar results concerning the extraction of carotenoids were reported by Amaro et al., (2015) where the acetonic extracts showed to be the richest in total carotenoids. This study also reported that **E** could extract carotenoids but in a lower ratio when compared to the extraction with **A**.

The phycobiliprotein profile and quantification were assessed for the aqueous extracts – **W**, A-**W**, AE-**W** and E-**W**, which results are summarized in Fig. 4B. All extracts showed to have phycocyanin and allophycocyanin in its composition although in different concentrations, and phycoerythrin was not found in any extract. The E-**W** was the extract with higher content in total phycobiliproteins, 341.29 ± 4.72 $mg_{phyco}.g_{extract}^{-1}$, 2.0-fold more content than the one with lower concentration, EA-**W**. The content in specific phycobiliproteins was also higher in E-**W**, having 2.8-fold more phycocyanin and 1.9-fold more allophycocyanin than EA-**W**. Being phycocyanin the main pigment in cyanobacteria cells, it was expected that it would represent the main fraction of the aqueous extracts instead of allophycocyanin. Therefore the low content in phycocyanin may be due to degradation of the pigment since it is not stable in the long term and its degradation is easily induced by: light, temperature, microorganisms, drying methods and extraction cells disruption methods (Sarada et al., 1999; Cuellar-Bermudez et al., 2015). Nonetheless, the high content in allophycocyanin adds biotechnological potential to the extracts since this pigment, isolated from *Arthorspira platensis*, demonstrated a relevant antiviral activity (Shih et al., 2003).

The total content in phenolic compounds was determined for all extracts, results are depicted in Fig. 4C and 4D. For the extracts performed with organic solvents (Fig. 4C), **A** showed the highest content, 83.1 ± 4.1 ($mg_{GAE}.g_{extract}^{-1}$), being 1.2-fold higher than **E** and W-**A**, which were the ones with lower content in phenols, with no statistical differences between them ($p > 0.05$). For the aqueous extracts (Fig. 4D), **W** showed the highest content in phenolic compounds with 81.2 ± 2.5 ($mg_{GAE}.g_{extract}^{-1}$), showing a phenolic content 1.3-fold higher than the other extracts with lower phenolic content, A-**W** and EA-**W**, with no statistical differences between them ($p > 0.05$).

### 3.1.3. Antioxidant capacity of pigments-rich extracts

The antioxidant capacity of all extracts was assessed by three different assays - $ABTS^{\bullet+}$, $O_2^{\bullet-}$ and $^{\bullet}NO$ assay. The selection for these methodologies was based in their sensitivity to different molecules. The first one evaluates the general antioxidant capacity of the extracts to scavenge the synthetic $ABTS^{\bullet+}$ radical. The $O_2^{\bullet-}$ and $^{\bullet}NO$ assays evaluate the capacity of the extracts to scavenge specific biological radicals which have impacts on human health – helping in the assessment of biotechnological potential of *Cyanobium* sp. extracts for pharmaceutical and cosmetic industries. In order to compare the antioxidant capacity of the extracts, the IC50 and IC25 were calculated for each assay; these values represent the concentrations ($\mu g_{extract}.mL^{-1}$) of the extracts which are able to scavenge 50 % and 25 % of the radicals, respectively. The IC values calculated for all extracts in the three assays are depicted in Table 5.

Table 5. IC50 and IC25 of all *Cyanobium* sp. extracts for $ABTS^{\bullet+}$, $^{\bullet}NO$ and $O_2^{\bullet-}$ assays. Different lowercase letters show statistically significant differences ($p < 0.05$) among the organic extracts for each method. Different capital letters show statistically significant differences ($p < 0.05$) among the aqueous extracts for each method.

| Solvent | Inhibitory Concentration ($\mu g_{extract}.mL^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | $ABTS^{\bullet+}$ | | $^{\bullet}NO$ | | $O_2^{\bullet-}$ | |
| *Organic* | IC50 | IC25 | IC50 | IC25 | IC50 | IC25 |
| **A** | 297.0 ± 33.0 $^{a}$ | 119.0 ± 9.9 $^{a}$ | - | 180.7 ± 19.5 $^{a}$ | - | **199.6 ± 20.4** |
| **EA** | - | 108.4 ± 4.2 $^{a}$ | 336.7 ± 7.2 $^{a}$ | 116.7 ± 15.9 $^{b}$ | - | - |
| **E** | 139.2 ± 6.6 $^{b}$ | 55.7 ± 1.5 $^{b}$ | - | **94.0 ± 10.4** $^{c}$ | - | - |
| W-**A** | **69.7 ± 4.1** $^{c}$ | **34.1 ± 1.0** $^{c}$ | **162.0 ± 25.9** $^{b}$ | - | - | - |
| *Aqueous* | | | | | | |
| **W** | **172.1 ± 22.8** $^{A}$ | 59.9 ± 6.7 $^{A, B}$ | 298.7 ± 4.9 $^{A}$ | **121.3 ± 16.9** | - | - |
| A-**W** | **176.6 ± 22.8** $^{A}$ | 70.1 ± 1.4 $^{A, B}$ | **164.5 ± 27.6** $^{B}$ | - | - | **68.8 ± 7.3** $^{A}$ |
| EA-**W** | **177.5 ± 29.8** $^{A}$ | 71.9 ± 7.6 $^{A}$ | **130.7 ± 13.7** $^{B}$ | - | - | 174.0 ± 4.8 $^{B}$ |
| E-**W** | **136.3 ± 16.0** $^{A}$ | **48.5 ± 8.2** $^{B}$ | **183.5 ± 16.3** $^{B}$ | - | - | 106.1 ± 30.4 $^{C}$ |

The assays revealed that all extracts have antioxidant capacity, although it varies between extracts and assays. In the $ABTS^{\bullet+}$ assay, among the organic extracts, W-**A** revealed to be the one capable of inhibiting 50 % of the radical at a lower extract concentration, 69.7 ± 4.1 $\mu g_{extract}.mL^{-1}$. The **EA** extract was the only one that was not possible to determine the IC50 in the range of concentrations tested, being only possible to calculate an IC25 at 108.4 ± 4.2 $\mu g_{extract}.mL^{-1}$. Among the aqueous extracts, the IC50 was possible to be determined for all of them. The IC50 of the four aqueous extracts did

not have significant differences between them ($p > 0.05$), showing an overall IC50 average of 165.6 ± 19.7 $\mu g_{extract}.mL^{-1}$. However, when comparing IC25, E-**W** and EA-**W** showed significant differences between them, such difference is related to the non-linearity of IC curves.

In the $^{\bullet}NO$ scavenging assay, it was only possible to calculate the IC50 of two organic extracts, **EA** and W-**A**. Between these two, W-**A** had a significant lower value of IC 50, 160.0 ± 25.9 $\mu g_{extract}.mL^{-1}$, being 1.6-fold better than **EA**. Regarding the other two extracts, **E** and **A**, although it was not possible to calculate an IC50, an IC25 was obtained; among them, **E** showed a statistically lower IC25 value from **A**, 94.0 ± 10.4 $\mu g_{extract}.mL^{-1}$. In what concerns the aqueous extracts, all attained IC50 values, and particularly, A-**W**, EA-**W** and E-**W** extracts did not show any significative difference ($p > 0.05$), having an overall average of 159.7 ± 26.7 $\mu g_{extract}.mL^{-1}$, 1.9-fold better than **W**, which showed the highest IC50 value being significantly different from the others ($p < 0.05$).

In $O_2^{\bullet-}$ scavenging assay it was not possible to calculate an IC50 for any extract, revealing that extracts do not hold a strong scavenging capacity against this radical. Among the organic extracts, it was only possible to calculate the IC25 for **A** at 199.6 ± 20.4 $\mu g_{extract}.mL^{-1}$. For aqueous extracts, the lowest IC25 calculated was 68.8 ± 7.3 $\mu g_{extract}.mL^{-1}$ for the A-**W** extract being significantly lower than the EA-**W** and E-**W** values, the **W** extract did not show an IC25.

Among organic extracts, it is outstanding that W-**A** shows a stronger antioxidant capacity than **A** in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays even though both extracts showed similar (and the highest) content in carotenoids and that **A** had more phenolic content than W-**A**. Such detail of these extracts suggests that the **W** extraction prior to the acetonic one increased the antioxidant capacity of the extract, i.e. the first **W** extraction might have concentrated lipophilic compounds such as carotenoids into the biomass after the first extraction, increasing the efficiency of the **A** extraction and thereafter, leading to a higher antioxidant capacity of the extract. Results obtained by Trivedi et al., (2016) showed significant increases on the extraction efficiency when using successive extractions compared to singular ones. Furthermore, *Cyanobium* sp. extracts are a complex mixture of compounds and its antioxidant activity cannot be correlated to the content of a singular groups of compounds such as carotenoids. Synergic or antagonist interactions between different molecules should be take into account. Therefore, the high content of known antioxidant compounds, like phenols or carotenoids, in a crude extract like **A** do not necessarily imply a great antioxidant activity compared to other extracts (Amaro et al., 2015; Babić et al., 2016).

In the aqueous extracts, although the differences in the antioxidant capacity of the extracts be less accentuated than in the organic extracts, some similar trends can be found. Again, the increase on the antioxidant capacity seems to be more related to the use of successive extraction and consequently to possible concentration of hydrophilic compounds such as phycobiliproteins into the remain biomass. This can be observed in the **W** extract, although having a high content in phycobiliproteins and phenolic compounds, it was the extract with less $^{\bullet}NO$ scavenging capacity and it did not show $O_2^{\bullet-}$ scavenging capacity. Also, the $^{\bullet}NO$ and $O_2^{\bullet-}$ scavenging capacity increased in all aqueous successive extractions. Moreover, despite the phycobiliprotein content and the phenolic content be the highest and second highest, respectively, in E-**W**, this extract did not show a higher antioxidant capacity when compared to the other successive aqueous extracts.

In addition, the IC50 values obtained in the $ABTS^{\bullet+}$ assay revealed to be more promising than the ones reported by Padma Priya C (2014) for *Chroococcus minutus* and *Chlorella saccharophila*. Moreover, the IC50 values in the $ABTS^{\bullet+}$ assay were in the same range of the ones reported before, by Amaro et al. (2015), for *Gloeothece* sp. and *Scenedesmus obliquus* (M2-1). In the same way, the IC25 values obtained in the $O_2^{\bullet-}$ assay with *Cyanobium* sp. extracts showed to be more promising than the reported ones obtained with *Scenedesmus obliquus* (M2-1) extracts. However, the $^{\bullet}NO$ assay results of *Cyanobium* sp. extracts were less promising than the reported by Amaro et al. (2015).

### 3.1.4. Cytotoxicity of pigments-rich extracts

Based on the previous antioxidant capacity and biochemical profile results, the most promising extracts were chosen to undertake cytotoxicity analysis - one carotenoid rich extract, W-**A**, and one phycobiliprotein rich extract, E-**W**. The first one was chosen since it revealed to attain the best antioxidant capacity results in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays (Table 4) and higher content in total carotenoids (Fig. 4A). The E-**W** extract was chosen due its high content in total phycobiliproteins (Fig. 4B) when compared to the other aqueous extracts, and high antioxidant capacity in the $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays too (Table 4).

The cytotoxicity of W-**A** and E-**W** extracts was assessed by MTT assay, in a range of concentration from 46.9 to 750.0 $\mu g.mL^{-1}$ against HepG2 cells, results are shown on Fig. 5. The cytotoxicity assays were carried with a hepatic cell line since one of the possible biotechnological uses of the extracts is its incorporation on food, nutraceutical and pharmaceutical formulations. W-**A** revealed to have cytotoxic effects in all concentration, since the cell viability percentage was significantly different from the control ($p < 0.05$)

(Fig. 5A). E-**W** did not have a cytotoxic effect on the HepG2 cell lines in none of the concentrations tested reaching a cell viability with no significative differences from the control ($p > 0.05$) (Fig. 5B). None of the extracts showed a dose dependent effect on cell viability for the range of concentration tested.

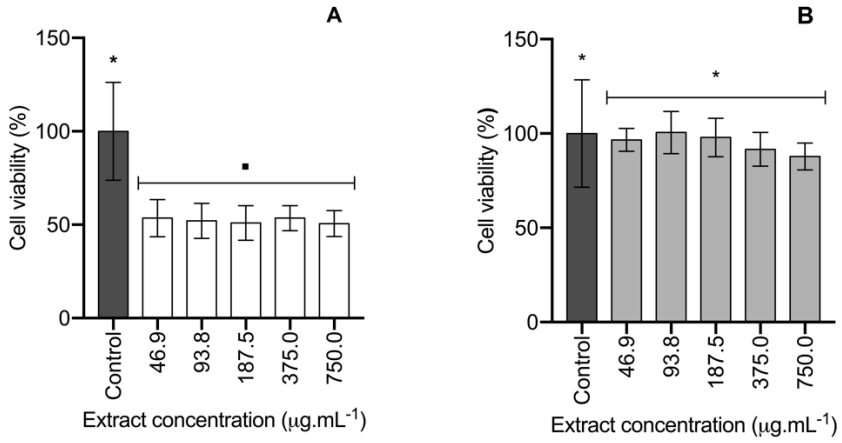


Figure 5. Cytotoxicity evaluation of *Cyanobium* sp. extracts (A) W–**A** and (B) E–**W** in a range of concentrations from 46.9 to 750.0 μg.mL$^{-1}$ against HepG2 cell line. Different symbols in the same graphic means significant difference between the extract and the control (cytotoxic) ($p < 0.05$).

These cytotoxicity results are important to indicate a possible biotechnological use of the extracts. Some cyanobacteria strains are capable to produce toxins with cytotoxic effects, harmful for human health (Codd et al., 2005; Jaiswal et al., 2008). Although no reports about toxin production by *Cyanobium* sp. LEGE 06113 were found, evidences of toxic blooms of another *Cyanobium* species have been described (G. Cronberg, E. J. Carpenter, 2004), suggesting that this specific strain may produce toxins which can be responsible for the low cell viability showed for W-**A**. Probably, W-**A** extracts would need to undertake purification processes in order to reduce its cytotoxicity, which, in turn, would lead to an increase in the production costs but would turn it more viable for pharmaceutical industries. On the other hand, the non-cytotoxic nature of the E-**W** extract makes it feasible to be used in feed and food industries.

## 3.2. Optimization of pigments production

The industrial applicability of cyanobacteria depends directly on finding a suitable organism with the desired features. *Cyanobium* sp. seems to be a promising organism for the production of bioactive compounds. Furthermore, the right combination of culture conditions is essential, since biomass productivity is a key factor for the potential use of a cyanobacterium in an industrial perspective, however the desire product may also need optimization. Temperature, pH and salinity (here, as [NaCl]), on an optimal condition, can induce to a higher production of desired compounds and, at the same time, produce enough biomass for an industrial use. Aiming the optimization of biomass and pigments production by *Cyanobium* sp., a factorial design was performed with three abiotic factors; temperature (T), pH and [NaCl], in the following range of levels: T (20 – 30 ºC), pH (6.0 – 9.0), [NaCl] (10 – 30 $g.L^{-1}$). In this work, the optimization of antioxidant compounds, and more specifically cyanobacterial pigments, was performed considering the productivity values, instead of cell content. The reason is that in a biotechnological perspective, the amount of the final product has a greater impact on the decision making than the biomass composition.

### 3.2.1. Factorial Model

The Box-Behnken design of thirteen runs was able to provide a significant quadratic model ($p < 0.01$) for all the studied parameters (Table 6). The three factors studied influenced the parameters in different ways, specific results will be discussed in the following sections. The impact of the three studied abiotic factors have been highlighted in several studies regarding cyanobacteria and other photosynthetic organisms' production. Overall, cyanobacteria grow in a great range of temperatures and have acclimation mechanisms to support such changes. In nature, variation on temperature happens daily and seasonally, with different thermic amplitudes depending on the zone of the globe. Temperature plays a crucial role in membrane stability and lipidic composition of cyanobacterial cell. In lower temperature, the fatty acid profile becomes more composed by polyunsaturated fatty acids, while in higher temperatures, the membrane fluidity is kept by saturated fatty acids (Paliwal et al., 2017). Furthermore, pH plays a fundamental role on cyanobacteria metabolism and physiology. pH is responsible for nutrient uptake, carbon availability and photosynthetic activity rate (Shruthi and Rajashekhar, 2014). Likewise, salinity – in the case of artificial media, NaCl concentration – is fundamental for ion regulation, osmotic balance and metabolic activity (Kannaujiya et al., 2017). Salt stress combine ionic and osmotic stresses, leading also

to an oxidative stress, thereupon many of the salt tolerance mechanisms are yet not clarified (Cepoi, 2019).

Table 6. Model analyses for all measured parameters**:** productivity of biomass ($P_X$), total phycobiliproteins (TBP), total carotenoids (TC)**,** antioxidant compounds (AOX-W for aqueous extract and AOX-A for acetonic extract) and photosynthetic activity (Fv/Fm). The quadratic model is set for temperature (T), pH (P) and NaCl concentration (N). Statistical parameters: probability value (p value) and correlation coefficient ($R^2$).

| **Parameter** | **p value** | **$R^2$** | **Quadratic model** |
|---|---|---|---|
| $P_X$ | <0.01 | 0.79 | - 0.5471 + 0.0104 T - 0.0046 [NaCl] + 0.1302 pH + 0.00007 T*[NaCl] - 0.00093 T*pH - 0.00013 [NaCl] *pH - 0.00015 $T^2$ + 0.00008 $[NaCl]^2$ - 0.0055 $pH^2$ |
| Fv/Fm | <0.01 | 0.96 | - 0.8564 + 0.0149 T - 0.0015 [NaCl] + 0.2026 pH + 0.00002 T*[NaCl] - 0.0010 T*pH - 0.0001 N*pH - 0.00003 $T^2$ + 0.00005 $[NaCl]^2$ - 0.0098 $pH^2$ |
| AOX-W | <0.01 | 0.81 | - 5.1639 + 0.0643 T - 0.030313 [NaCl] + 1.3217 pH + 0.0014 T*[NaCl] - 0.0083 T*pH - 0.0010 [NaCl]*pH - 0.0010 $T^2$ + 0.00006 $[NaCl]^2$ - 0.0636 $pH^2$ |
| AOX-A | <0.01 | 0.85 | - 4.6600 - 0.3428 T + 0.0293 pH - 0.0004 T*[NaCl] - 0.0196 T*pH - 0.0030 [NaCl] *pH + 0.0094 $T^2$ + 0.0002 $[NaCl]^2$ - 0.1020 $pH^2$ |
| TPB | <0.01 | 0.81 | - 31.1664 + 1.2292 T - 0.3185 [NaCl] + 5.4474 pH + 0.0062 T*[NaCl] - 0.0075 T*pH - 0.0045 N*pH - 0.0287 $T^2$ + 0.0043 $[NaCl]^2$ - 0.2906 $pH^2$ |
| TC | <0.01 | 0.79 | + 4.5485 - 0.9113 T - 0.2746 [NaCl] + 2.6901 pH + 0.0069 T*[NaCl] + 0.0214 T*pH + 0.00003 [NaCl] *pH + 0.1089 $T^2$ + 0.0020 $[NaCl]^2$ - 0.1922 $pH^2$ |

## 3.2.2. Biomass productivity

Biomass production and cyanobacterial growth were measured for eighteen days by dry weight (DW), optical density (OD) and chlorophyll *a* content. The three measurements were significantly correlated ($R^2 > 0.98$) (Table 7), but only DW values were used in the optimization process, since it is the most reliable for future industrial applications. However, OD and chlorophyll *a* content were used to monitor the culture growth on time, since it was possible to determine the value in the same day of sampling while the experiments were ongoing. Nevertheless, the correlation between the three methods validates the biomass quantification.

Table 7. Correlation analysis of *Cyanobium* sp. growth measurements: optical density (OD), dry weight (DW; $mg.mL_{culture}$) and chlorophyll *a* ($\mu g.mL_{culture}$), and its correlation coefficient ($R^2$).

| **Linear regression** | **$R^2$** |
|---|---|
| OD = 0.456*DW - 0.051 | 0.992 |
| OD = 0.118*Chl *a* - 0.050 | 0.986 |
| DW = 2.330*OD + 0.099 | 0.992 |
| DW = 0.251*Chl *a* + 0.026 | 0.981 |
| Chl *a* = 3.776*DW + 0.032 | 0.981 |
| Chl *a* = 8.185*OD + 0.488 | 0.986 |

The maximum productivity for each condition was calculated and modelled on the factorial design. The obtained equation (Table 5) was plotted and biomass productivity surface response is shown in Fig. 6.

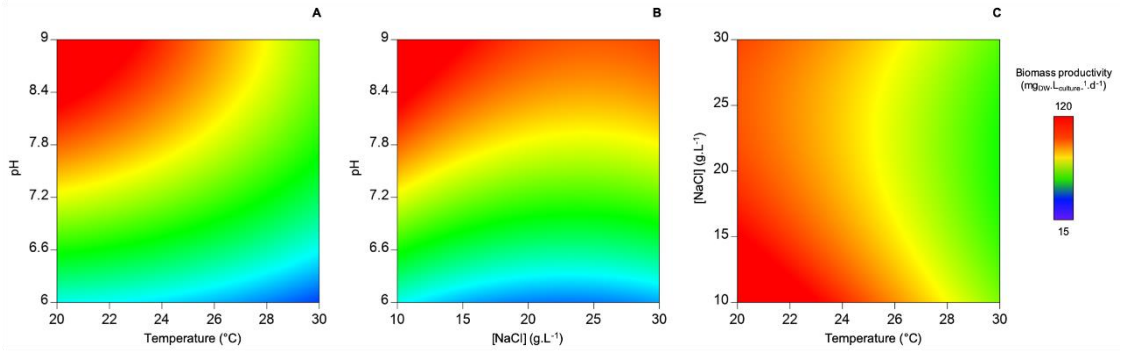


Figure 6. Response surface plots for maximum biomass productivity ($mg_{DW}.Lculture^{-1}.d^{-1}$) of *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 $g.L^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (20 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

The values for biomass productivity under tested conditions ranged from 16.33 ± 1.53 to 110.33 ± 0.58 $mg_{DW}.L_{culture}^{-1}.d^{-1}$. By the quadratic model, the optimal condition was found

at T = 20 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$, with a predicted maximum productivity of 122.66 ± 1.77 $mg_{DW}.L_{culture}^{-1}.d^{-1}$.

Regarding the influence of the three factors individually, some trends appears on the biomass productivity of *Cyanobium* sp. In terms of temperature, it can be set that as lower the temperature, higher is the biomass productivity. Cyanobacteria usually have a wide range of temperature that they can grow on, but in the range of tested temperatures *Cyanobium* sp. growth was not inhibited. The called thermal niche, is the temperature range in which the organism is able to survive. By reaching the upper critical temperature, the organism becomes exposed to heat stress, inducing inhibition on the photosynthetic apparatus and thereafter inducing to cell death (Nalley et al., 2018). The thermal niche and optimal temperature are species dependent. An example of this is *Anabaena doliolum,* that was grown from 30 to 40 °C with optimal growth at 30 °C (Reddy et al., 2019). On the case of *Leptolyngbya* sp. HS-16 and HS-36, isolated from a hot spring (c.a. 40 °C) could grow from 20 to 50 °C, being the optimal temperature 35 °C (Prihantini et al., 2019).

When it comes to pH, in the range studied, a alkaline pH seems to increase the growth and biomass production in *Cyanobium* sp. In general, cyanobacteria tend to grow better in neutral to alkaline environment, although they can survive in a great range of pH (Yang et al., 2018). Meng et al. (2018) have shown that *Cyanobacterium aponinum* could grow in a pH range from 3.0 to 8.0, but with higher productivity was obtained in more alkaline condition. Ismaiel et al. (2016) also reported that in the case of *A. platensis*, the best biomass productivity was found to be 9.0, when tested in a range from 7.5 to 11.0. Additionally, the tolerance to high pH can be used to overcome contaminations, such as other microalgal species, in cyanobacterial productions. Yang et al. (2018) observed that *Microcystis aeruginosa* could outcompete *Scenedesmus obliquus* in a pH of 9.0. On the other hand, the opposite effect happened when the pH was 6.0 and the cyanobacterium could not grow.

Additionally, in terms of [NaCl], a less evident trend was found in the present study, what means that the [NaCl] influenced in a less effective way the biomass productivity. Nevertheless, lower salinities seem to induce a slightly increase on biomass production of *Cyanobium* sp., and that's why the optimal condition was set as 10 $g_{NaCl}.L^{-1}$. The amount of salt needed in the media is very species dependent, due to the variability of these organism's habitat distribution and ability to adapt to extreme environments. Some studies have found similar response to NaCl concentration as was found here. Rai and Rajashekhar (2016) showed that *Oscillatoria fremyii*, *Oscillatoria geminata*, *Oscillatoria sancta*, *Phormidium corium*, *Phormidium tenue* and *Arthorspira major* could grow in

[NaCl] from 9 to 40 g.L$^{-1}$, with an optimal condition of 16 and 25 g.L$^{-1}$, depending on the strain. In the case of *Synechocystis* sp. CCNM 2501, this strain was able to survive in a NaCl range from 0 to 60 g.L$^{-1}$, with a decrease on the productivity with an increase on the salt concentration, being the optimal concentration 0 g.L$^{-1}$ (Paliwal et al., 2015).

Only a few studies have made optimizations with the interaction of the tested factors. An example was a study with *Nodularia spumigena* where temperature and salinity were optimized, and the interaction between the factors was determinant. In a lower salinity, temperature did not change growth, while in a high salinity, a higher temperature induced to a higher growth (Silveira and Odebrecht, 2019).

## 3.2.3. Photosynthetic activity

The measurement of the photosynthetic activity permits to monitor the status of the culture and also to see how efficient the growth and the photosynthetic metabolism are. The photosynthetic activity was measured every sampling day and the maximum rate (Fv/Fm) was plotted to the factorial model. The quadratic equation was significative (Table 5) and the response surface data is shown in Fig. 7.

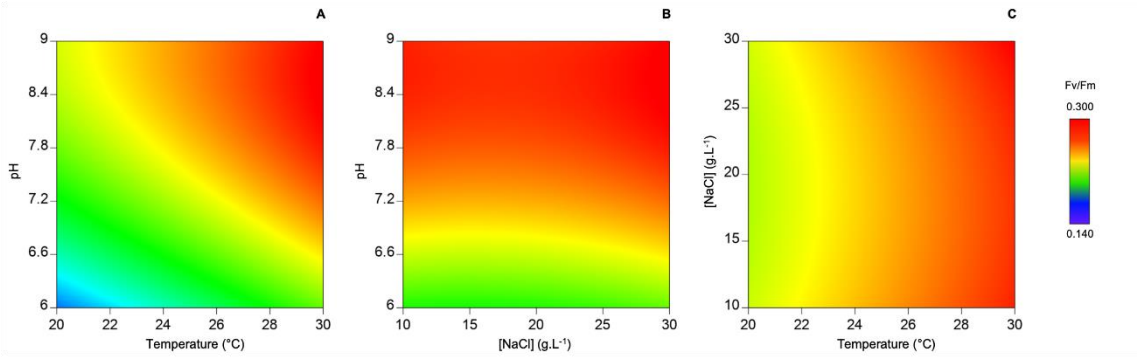


Figure 7. Response surface plots for maximum Fv/Fm in *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 g.L$^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (30 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

Photosynthetic rate values range between 0.143 ± 0.003 and 0.303 ± 0.003. From the quadratic model the optimal condition was found at T = 30 °C; pH = 9.0; [NaCl] = 10 g.L$^{-1}$, with a predicted maximum photosynthetic activity of 0.299 ± 0.009. On the contrary of the biomass, the highest photosynthetic activity was found in a high temperature. The photosynthetic metabolism is extremely heat-sensitive, the photosystem II is considered to be very sensitive, while PSI tolerate increases on temperature and hence in some cases, heat can stimulate PSI energy processing and a higher photosynthetic activity (Zhang and Liu, 2016). On the case of *Arthrospira* sp., temperatures over 30 °C increased the photosynthetic capacity, upregulating PSI. Only under 40 °C the photosynthetic capacity decreases (Zhang and Liu, 2016). Also, the correlation between

the increase of temperature and photosynthesis was also observed in a few *Synechococcus* sp. strains, where photosynthetic activity is higher during summer, when compared to other seasons (Kim et al., 2018).

Notwithstanding, optimal pH and [NaCl] were the same as biomass productivity. Thus, [NaCl] has shown to have less impact than the other variables, although the interaction of them is significative. In fact, growth and biomass accumulation is not dependent only on the photosynthetic metabolism, since cyanobacteria have other mechanisms that allow growth in non-optimal photosynthetic conditions. On the optimal biomass accumulation conditions, the photosynthetic activity would have a value of 0.257 ± 0.009, 1.2-fold lower than in the optimal condition for photosynthetic activity.

On the case of pH, the main influence is on the light-harvesting complex composition and the production of antennae pigments. In the present study, higher pH leads to a higher photosynthetic activity. The same positive effect of pH occurred in *S. obtusiusculus*, which showed a higher photosynthetic activity in neutral to alkaline environment, with optimal pH of 8.0 (Cabello et al., 2015). Regarding [NaCl], *Cyanobium* sp. seems to have higher photosynthetic activity under low concentration of salt. Likewise, in *A. platensis,* the antennae modulation was also highly affected by salinity (NaCl concentration) and affected directly the photosynthetic efficiency (Cepoi, 2019).

### 3.2.4. Antioxidant capacity

Cyanobacteria are extremely flexible organisms, capable to acclimate to the different kind of stresses. In nature, these organisms are exposed to variations on light, temperature, salinity, pH, heavy metals and nutrient availability. The response to those changes in environmental conditions is made mainly by changing the metabolic content and functioning of the cell. The first defence against abiotic stress are antioxidant components, both enzymatic and non-enzymatic (Babele et al., 2019). To evaluate the potential of the antioxidant capacity of *Cyanobium* sp., two intracellular extracts were made, the first with acetone for lipophilic components, and the second with water, for hydrophilic components.

#### 3.2.4.1. Acetonic extract

Due to results in section 3.1.2., acetone was chosen for the preparation of extracts to evaluate the antioxidant capacity of *Cyanobium* sp. grown in different temperatures, pH and salinities. The antioxidant capacity was measured every sampling day and the maximum antioxidant equivalents productivity was used for the factorial modelling. The response surface plot from the quadratic model (Table 5) is represented in Fig. 8.

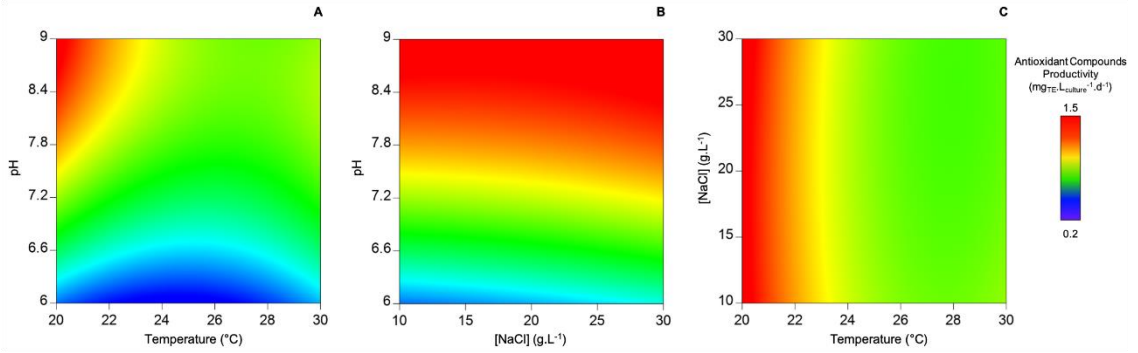


Figure 8. Response surface plots for maximum antioxidant compounds productivity ($mg_{TE}.L^{-1}.d^{-1}$) of acetonic extract of *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 $g.L^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (20 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

The antioxidant productivity ranged from 0.26 ± 0.01 and 1.54 ± 0.15 $mg_{TE}.L^{-1}.d^{-1}$, being the higher productivity values found in the middle exponential growth phase. The optimal condition, found with the quadratic model (Table 5) for this parameter, was at T = 20 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$, the same optimal conditions for the biomass productivity. The predicted value for optimal antioxidant productivity was 1.55 ± 0.19 $mg_{TE}.L^{-1}.d^{-1}$, which is 3.16-fold higher than productivity obtained with the best condition for maximal cell content in antioxidant compounds (T = 30 °C; pH = 6.0; [NaCl] = 20 $g.L^{-1}$). Thus, the antioxidant compounds productivity seems to be influenced in the same way as in biomass, by the three abiotic factors, but in a more effective way by temperature and pH. The antioxidant capacity of acetonic extracts is probably due to carotenoids and other lipophilic compounds (such as fatty acids and some phenolic compounds). These compounds allow a greater maintenance of the main metabolism in the cyanobacterium, helping on the growth of the culture.

Lipophilic antioxidant compounds, such as PUFAs and carotenoids, have been shown to be influenced in different way by abiotic factors. In most cases, low temperatures are related to an increase of lipid accumulation and the activation of superoxide dismutase (SOD) (Cepoi, 2019). On *Anabaena flos-aquae*, the optimal temperature for the production of linolenic acid, a known antioxidant PUFA was at 20 °C, in a range from 9 to 32 °C (Nalley et al., 2018).

Regarding pH, lightly alkaline conditions are favorable for the accumulation of carotenoids, as in *A. platensis*, which had the highest amount of carotenoid in a pH 8.5 in a range from 7.5 to 11.0 (Ismaiel et al., 2016).

Furthermore, Verma *et al.* (2019) have observed that in *Synechococcus* PCC 7942, an increase in [NaCl] increased the total amount of lipids, although the production of PUFAs with 15 $g.L^{-1}$ was superior to the one with 30 $g.L^{-1}$. Additionally, lower [NaCl] have induced

to a higher carotenoid content in *Nostoc ellipsosporum* and *Nostoc piscinale*, the same study indicates the induction of antioxidant defenses in lower [NaCl] (Rezayian et al., 2019).

### 3.2.4.2. Successive aqueous extract

The successive aqueous extract using water after the acetonic extraction is based on the principle found in 3.1, where successive extractions allow the obtaining of different groups of compounds by solvent affinity. The antioxidant capacity was measured every sampling day and the maximum antioxidant equivalents productivity was used for the factorial modelling. The response surface plot from the quadratic model (Table 5) is represented in Fig. 9.

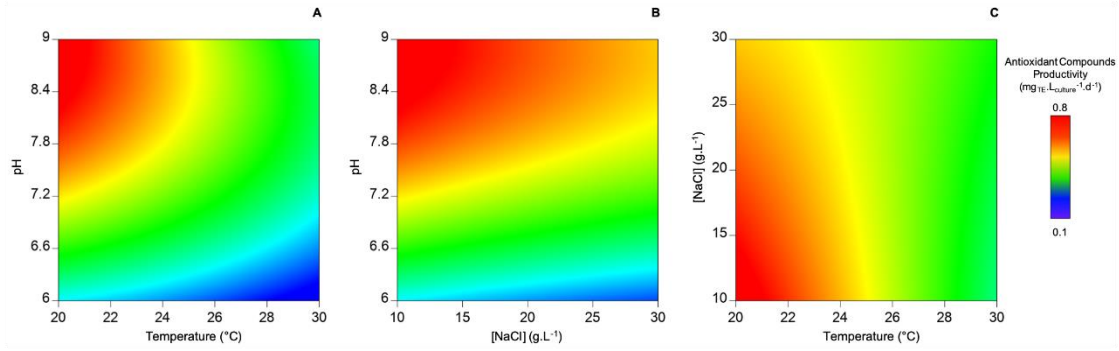


Figure 9. Response surface plots for maximum antioxidant compounds productivity ($mg_{TE}.L^{-1}.d^{-1}$) of aqueous extract of *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 $g.L^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (20 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

The antioxidant productivity of aqueous extracts ranged from 0.11 ± 0.01 and 0.82 ± 0.12 $mg_{TE}.L_{culture}^{-1}.d^{-1}$, being the higher productivity values found in the middle exponential growth phase. The optimal value obtained from the factorial optimization was found at T = 20 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$, also the same optimal condition for the acetonic extracts and the biomass productivity, with a possible correlation between these parameters. The predicted value for maximum antioxidant productivity in aqueous extracts was 0.84 ± 0.11 $mg_{TE}.L^{-1}.d^{-1}$. This productivity value was 4.2 fold-higher than the one obtained with the optimal condition for hydrophilic antioxidant compound cell content (T = 30 °C; pH = 6.0; [NaCl] = 30 $g.L^{-1}$). The antioxidant capacity of aqueous extracts comes mainly form phycobiliproteins, phenolic compounds and possibly, peptides. Valuta et al. (2015) found a correlation between the content of phycobiliproteins and ABTS assay values for aqueous extracts of *Nostoc linckia*.

Among the three factors, temperature and pH seems to have more influence than [NaCl], although the effect of this factor be also significative. Cepoi (2018) observed that in *A. platensis* the antioxidant capacity of aqueous extracts decreased under high

temperatures. Besides, Ismaiel et al. (2016) saw that *A. platensis* increase the radical scavenging activity at pH 9.0, with 2.0-fold higher activity than in pH 7.5. The same study also showed that the highest amount of total phycobiliprotein was found at pH 9.0.
Regarding [NaCl], *A. platensis* and *N. linckia* showed a higher ABTS inhibition with lower concentrations of NaCl (10 and 20 $g.L^{-1}$) in a range from 10 to 50 $g.L^{-1}$ (Cepoi, 2019).

### 3.2.5. Pigment productivity

As photosynthetic organisms, cyanobacteria are dependent on the light harvesting complex to obtain energy and be able to perform photosynthesis. Changes on growth conditions led to different types of acclimation and consequently to changes in the pigment composition, in order to become more photosynthetically efficient. Carotenoids and phycobiliproteins are the main pigments with industrial potential, and the optimization of the production of these two groups of pigments will be discussed in the following sections.

#### 3.2.5.1. Carotenoids

Carotenoids are lipophilic pigments present in photosynthetic organisms and are part of the light-harvesting complex. These compounds have two main roles inside the cell, one in the photosynthesis metabolism, but also as non-enzymatic antioxidant defence. Carotenoids are an important defence for the cell in stress situations, the induction of carotenogenesis comes mainly through light conditions, but also through changes on the photosynthetic apparatus, since those compounds are responsible for light harvesting and also energy dissipation in form of heat.
The carotenoids content and profile were measured every two sampling days, and the maximum productivity of total carotenoids was used for the factorial modelling. The response surface plot from the quadratic model (Table 5) is represented in Fig. 10.

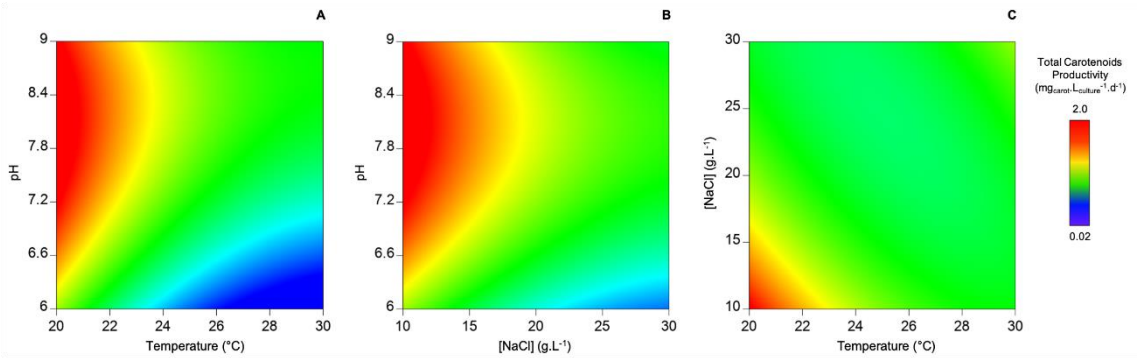


Figure 10. Response surface plots for maximum total carotenoids productivity ($mg_{carot}.L_{culture}^{-1}.d^{-1}$) of *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 $g.L^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (20 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

The productivity of carotenoids ranged from 0.27 ± 0.02 and 2.80 ± 0.08 $mg_{carot}.L_{culture}^{-1}.d^{-1}$, being the higher carotenoids productivity values found in the late exponential growth phase. From the quadratic equation, the optimal condition would achieve a carotenoid productivity of 2.17 ± 0.69 $mg_{carot}.L_{culture}^{-1}.d^{-1}$ at T = 20 °C; pH = 9; [NaCl] = 10 $g.L^{-1}$. The predicted optimal value for carotenoids productivity was lower than the found experimentally in the tested conditions, this difference may be due to statistical fit of the model. Nevertheless, the optimal value for carotenoids productivity was 2.0-fold higher than the productivity obtained in the optimal condition for maximum cell content in carotenoids (T = 30 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$). The three factors seem to have great influence on the productivity of carotenoids, but it is notable that the interaction of temperature and [NaCl] had less impact on the productivity of carotenoids.

Like most responses to environmental changes, carotenoid production varies from specie to specie. For example, *Arthronema africanum* seems to be more tolerant to temperature variations, so in a range from 15 to 50 °C, the content of carotenoids was kept constant (Chaneva et al., 2007). In other way, in the case of *A. platensis*, the carotenoid content was maximal at 35°C in a range of 20-40 °C (Kumar et al., 2011).

Concerning pH, general alkaline conditions appear to increase the productivity of carotenoids. For example, Touloupakis et al. (2016) observed a higher production of echinenone and β-carotene under pH 11 in *Synechocystis* sp. PCC 6803. Likewise, *A. platensis* showed higher carotenoids production at pH 8.5 (Ismaiel et al., 2016). Furthermore, NaCl concentration have been recorded as key factor for the composition of carotenoids in cyanobacteria. In *Synechocystis* sp. CCNM2501, grown in a range of [NaCl] from 0 to 60 $g.L^{-1}$, the optimal condition for carotenoids production was 10 $g.L^{-1}$. Also, any increase in salt concentration reduced the amount of zeaxanthin and echinenone (Paliwal et al., 2015). The same effect was observed in *Nostoc* spp. that had higher production of carotenoids in lower NaCl concentrations (Rezayian et al., 2019).

### 3.2.5.2. Phycobiliproteins

Phycobiliproteins are the main group of pigments in cyanobacteria, and in some cases, these compounds can represent more than half of the cell proteins (Bryant et al., 1979). In the cell, phycobiliproteins are responsible for light energy transfer to the photosystem and, as same as carotenoids, have an important role on the antioxidant defence of the cell.

The content of phycobiliproteins were measured every two sampling days and the maximum productivity of total phycobiliproteins was used for the factorial modelling. The response surface plot from the quadratic model (Table 5) is represented in Fig. 11.

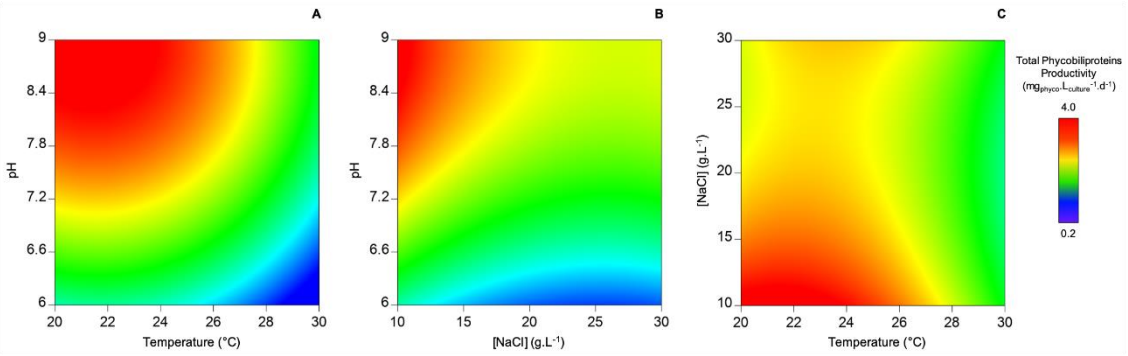


Figure 11. Response surface plots for maximum total phycobiliproteins productivity ($mg_{phyco}.L_{culture}^{-1}.d^{-1}$) of *Cyanobium* sp., showing (A) pH *versus* Temperature in constant [NaCl] (10 $g.L^{-1}$); (B) pH *versus* [NaCl] in constant Temperature (20 °C); (C) [NaCl] *versus* Temperature in constant pH (9.0).

Phycobiliproteins productivity ranged from 0.14 ± 0.02 and 4.26 ± 0.03 $mg_{phyco}.L_{culture}^{-1}.d^{-1}$, being the higher values of phycobiliprotein productivity found in the late exponential growth phase. The optimal condition was obtained at T = 20 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$ according to the quadratic model, with a maximum productivity of 4.14 ± 0.71 $mg_{phyco}.L_{culture}^{-1}.d^{-1}$. Yet, this productivity value is 1.30-fold higher than the one obtained in the optimal condition for a maximium cell content in phycobiliproteins (T = 25 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$). All the three factors had great influence in the production of phycobiliproteins. As the main pigment on cyanobacteria, these compounds are highly regulated by changes in the environmental conditions in order to guarantee the best metabolic fitting in stress conditions. The increase of the amount of phycobiliproteins can be considered an response to increase the light energy transfer efficiency on the light-harvesting complex (Rezayian et al., 2019). Responses trends for phycobiliprotein production, similar to the ones found in *Cyanobium* sp. have been suggested to other cyanobacteria; in special, *A. platensis*, which showed a higher phycobiliprotein content in a 30 °C than in 40 °C, with a reduction of 0.3-fold in the higher temperature (Cepoi,

2019). Besides, Ismaiel et al. (2016) reported that the optimal pH for phycobiliprotein production in *A. platensis* was 9.0 in a range from 7.5 to 11.0.

In regard to NaCl concentration, *A. platensis* had its photosynthetic metabolism inhibit in concentration higher than 40 $g.L^{-1}$ due to degradation of phycobiliproteins (Cepoi, 2019). The relation of high concentrations of NaCl and a decrease on phycobiliprotein content is related to a detachment of phycobilisomes induced by accumulation of intracellular sodium ions ionic and inhibition of the energy transfer from the phycobilisomes to the photosystem (Kannaujiya et al., 2017; Chentir et al., 2018).

### 3.2.6. Model Validation

In order to validate the quadratic model and the optimal conditions found in the Box-Behnken factorial, the optimal condition for simultaneal maximization of biomass, carotenoids and phycobiliproteins productivity was performed. This resulted in a triplicate batch in the following conditions: Temperature = 20 °C, pH = 9.0 and [NaCl] = 10 $g.L^{-1}$. The predicted and observed values are shown in Table 8.

Table 8. Responses for optimal condition for the culture of *Cyanobium* sp. based on the Box-Behnken design for: maximal quantum yield (Fv/Fm), antioxidant compounds for acetonic extract (AOX – A:), antioxidant compounds for aqueous extract (AOX – W), total carotenoids (TC) and total phycobiliproteins (TPB). Predicted and observed values were compared in a ANOVA analysis and the statistical validation is present in form of p value (identical when $p > 0.05$).

| Parameter | Predicted value | Observed value | p value |
|---|---|---|---|
| Biomass ($mg_{DW}.L_{culture}^{-1}.d^{-1}$) | 122.67 ± 17.71 | 127.12 ± 1.30 | 0.676 |
| Fv/Fm | 0.257 ± 0.009 | 0.255 ± 0.003 | 0.724 |
| AOX – A ($mg_{TE}.L_{culture}^{-1}.d^{-1}$) | 1.55 ± 0.19 | 0.92 ± 0.06 | 0.001 |
| AOX – W ($mg_{TE}.L_{culture}^{-1}.d^{-1}$) | 0.84 ± 0.11 | 0.97 ± 0.07 | 0.122 |
| TC ($mg_{Carot}.L_{culture}^{-1}.d^{-1}$) | 2.04 ± 0.51 | 2.09 ± 0.25 | 0.986 |
| Lutein | 0.05 ± 0.02 | 0.06 ± 0.00 | 0.929 |
| Zeaxanthin | 0.25 ± 0.07 | 0.28 ± 0.01 | 0.789 |
| Echinenone | 0.15 ± 0.05 | 0.12 ± 0.01 | 0.537 |
| β-carotene | 0.72 ± 0.17 | 1.00 ± 0.13 | 0.101 |
| TPB ($mg_{phyco}.L_{culture}^{-1}.d^{-1}$) | 4.14 ± 0.71 | 3.98 ± 0.07 | 0.708 |
| Phycocyanin | 2.94 ± 0.50 | 2.79 ± 0.03 | 0.841 |
| Allophycocyanin | 1.20 ± 0.22 | 1.18 ± 0.05 | 0.978 |

From the obtained results on the optimal condition, it is possible to validate the model to biomass, carotenoids and phycobiliprotein productivity, and also to photosynthetic activity and aqueous extract antioxidant capacity. The only parameter that had statistical

differences was the antioxidant capacity from acetonic extract. The limitation of statistical methods for biological optimization is dependent to the high variability in biological systems. However, the optimal condition found in this study was validated for most measured parameters.

Additionally, and using as a comparison the results obtained with standard conditions used to produce *Cyanobium* sp. biomass in LEGE-CC (Leão et al., 2013) and in the first part of this work (2.1.1. section) (T = 25 °C; pH = 7.5; [NaCl] = 30 $g.L^{-1}$), is possible to observe the improvement attained in each parameter, as depicted in Table 9.

Table 9. Comparison between maximal values of productivity of biomass ($P_X$), antioxidant compounds (AOX-W for aqueous extract and AOX-A for acetonic extract), total carotenoids (TC)**,** total phycobiliproteins (TBP), obtained with the optimal condition (20 °C, pH = 9.0 and [NaCl] = 10 g.L-1) and the standard condition (T = 25 °C; pH = 7.5; [NaCl] = 30 $g.L^{-1}$).

| Parameter | Standard Condition | Optimized Condition | Fold change |
|---|---|---|---|
| $P_X$ ($mg_{DW}.L_{culture}^{-1}.d^{-1}$) | 74.25 ± 17.71 | 127.12 ± 1.30 | 1.71 |
| AOX - A ($mg_{TE}.L_{culture}^{-1}.d^{-1}$) | 0.54 ± 0.19 | 0.92 ± 0.06 | 1.80 |
| AOX - W ($mg_{TE}.L_{culture}^{-1}.d^{-1}$) | 0.92 ± 0.11 | 0.97 ± 0.07 | 1.00 |
| TC ($mg_{Carot}.L_{culture}^{-1}.d^{-1}$) | 0.77 ± 0.51 | 2.09 ± 0.25 | 2.70 |
| TPB ($mg_{phyco}.L_{culture}^{-1}.d^{-1}$) | 2.71 ± 0.71 | 3.98 ± 0.07 | 1.47 |

In the optimal conditions, *Cyanobium* sp. showed an increase for biomass and pigments productivity when compared to the standard one. Regarding antioxidant capacity in acetonic extracts, it was improved by 1.80-fold while the aqueous extracts showed no statistical differences between conditions ($p>0.05$). Thus, the optimal condition shows an overall improvement when compared to the standard one, representing a new strategy for the production of *Cyanobium* sp. biomass and pigments.

# 4. Final Remarks

The biotechnological potential of cyanobacteria has been gaining attention in the last years, due to the wide range of applications that these microorganisms can have for industries, from production of biomass for food and feed industries to isolation of valuable compounds for pharmaceutical uses. Although the cyanobacteria potential as source of pigments for industrial applications is increasing, still are constrains regarding the economic aspects of the production of these organisms. Some of the main issues contemplate the high costs of production, difficulties on controlling the culture growth and contamination by other microorganism (Rizwan et al., 2018). For industrial-scale cultivation, a deep understanding of the growth and metabolic characteristics of cyanobacteria is still needed (Chen et al., 2019).

To overcome these constrains, solutions like screening for new strains with better composition, based on the desired product, and optimization of culture conditions are usually attempted in order to increase the pigment productivity and to reduce production costs (Rizwan et al., 2018). Therefore, this dissertation contributed on the exploitation of the biotechnological potential of a poorly studied but promising cyanobacteria strain, *Cyanobium* sp. LEGE 06113, first by evaluating and characterizing the bioactive potential of *Cyanobium* sp. pigments-rich extracts and secondly by optimizing pigment and biomass production in terms of temperature, pH and [NaCl].

## 4.1. General Conclusions

As discussed along this dissertation, cyanobacteria produce a wide range of pigments with different chemical properties, hence, several single and successive extractions aiming the extraction of several pigments were obtained from *Cyanobium* sp. and its profile and content in pigments was determined, as well as, its antioxidant capacity and cytotoxic effects. As for the second part, optimization of temperature, pH and [NaCl] for *Cyanobium* sp. biomass, carotenoids and phycobiliproteins production was achieved by a Box-Behnken factorial design. The main conclusions of this work are summarized as follows:

Evaluation and characterization of bioactive potential of *Cyanobium* sp. pigment-rich extracts:

Extraction yields

- Among the organic solvents **E** was the one that lead to the highest extract yield, 26.98 ± 2.49 %.

- Aqueous extractions lead to higher extract yields than organic extractions.
- Among the aqueous extracts **W** and EA-**W** were the ones that lead to the highest extract yields, 47.04 ± 5.12 % and 45.74 ± 1.66 %, respectively.
- The extraction combinations of **EA** followed by EA-**W,** and of **W** followed by W-**A,** lead to the highest combined yield percentages, 54.40 ± 1.07 % and 55.94 ± 5.29 %, respectively.

Biochemical profile

- The carotenoid profile of *Cyanobium* sp. extracts is composed by echinenone, zeaxanthin, lutein, β-carotene and derivates of those.
- **A** and W-**A** extracts showed the higher content in total carotenoids, 4.37 ± 0,73 and 3.97 ± 0.94 $mg_{carot}.g_{extract}^{-1}$, respectively.
- The phycobiliprotein profile of *Cyanobium* sp. is composed mainly by allophycocyanin but also by phycocyanin.
- The E-**W** extract revealed to have the highest contend in total phycobiliproteins, 341.29 ± 4.72 $mg_{phyco}.g_{extract}^{-1}$.

Antioxidant capacity of pigments-rich extracts

- W-**A** showed the best antioxidant capacity results in $ABTS^{\bullet+}$ and $^{\bullet}NO$ scavenging assays.
- Among the aqueous extracts the differences in antioxidant capacity is less accentuated.
- Successive extractions had a beneficial effect on the antioxidant capacity of the extracts.

Cytotoxicity of pigments-rich extracts

- W-**A** extracts showed cytotoxic effects for all the concentration tested, being more suitable for pharmaceutical industries
- E-**W** did not show any cytotoxic effects, in the range of concentrations tested, being suitable for food and feed industries.

Optimization of pigments production:

- The interaction of the three abiotic factors tested on *Cyanobium* sp. cultures was significant for all parameters studied.
- In general, T and pH seemed to take a more important role on *Cyanobium* sp. metabolism than [NaCl].

- Overall the best condition for *Cyanobium* sp. biomass and pigment production is T = 20 °C; pH = 9.0; [NaCl] = 10 $g.L^{-1}$.
- In the optimal set conditions, biomass, carotenoid and phycobiliprotein productivity reached a maximum of 127.12 ± 1.30 $mg_{DW}.L_{culture}^{-1}.d^{-1}$, 2.09 ± 0.25 $mg_{carot}.L_{culture}^{-1}.d^{-1}$and, 3.98 ± 0.07 $mg_{phyco}.L_{culture}^{-1}.d^{-1}$, respectively.
- The quadratic model for all parameters, except for antioxidant capacity of acetonic extract was confirmed by the model validation under the optimal condition.
- The factorial optimization can represent a valid tool to improve the cyanobacteria-based bioprocess.

## 4.2. Future prospects

This study tackled two commonly suggested solutions for some constrains of cyanobacteria production. The bioactivity screening for promising strains, in this case *Cyanobium* sp. and optimisation of culture conditions, here being temperature, pH and salinity (Rizwan et al., 2018). The results obtained were important for the biotechnological valorisation of *Cyanobium* sp., however further studies would be necessary for its application on industries since the production of cyanobacteria still faces other restrictions.

The lack of knowledge on cyanobacteria basic biology is pointed as one of the main drawbacks in the industrial production; therefore, fundamental research of the different metabolic pathways using genome, post-genomics, proteomic, and metabolomics technologies would be a significant step forward (Chen et al., 2019).

Genetic engineering is also suggested as a possible solution to increase the pigment production, e.g. successful introduction of *H. pluvialis* genes on *Saccharomyces cerevisiae* strain lead to an astaxanthin-producing yeast (Ye et al., 2019). This technology could be applied in cyanobacteria strains to increase the production of pigments or to promote the production of pigments with economic interest.

Regarding extraction and purification, there are no standard methods for cyanobacteria pigments products, mainly due to the variability of cell composition and biochemical profile. Also, the costs of extraction can be up to 60 % of total costs (Molina Grima et al., 2003). The choice of methodology may determine the final price and the use of the pigments. When used for food industries, extracts with less purity are allowed depending also on the toxicity. On the other hand, when used for pharmaceutical applications it is necessary a high purity of the compound and the cost can be a hundred times higher (Morais et al., 2018).

Furthermore, in an optimal scenario, it would be possible to use the biomass to a range of products, in a biorefinery scenario. The total revenues generated by the different coproducts in a biorefinery could increase the economic feasibility of cyanobacteria-based applications (Bastiaens et al., 2017). However, theoretical concepts that have been reported are mostly related to small-scale experiments and there is still a gap in technical feasibilities. So, the upscaling of biorefinery is needed to evaluate the viability of the process and its practical implementations (Bastiaens et al., 2017).

After all, both optimization in technical process and biological advances in cyanobacteria production must be tested by economic assessments in large-scale production systems by integrating life cycle analysis into the evaluation process (Chen et al., 2019).

Regarding all the mentioned restrains, future works in order to characterize *Cyanobium* sp. possible applications and to optimize its production are presented as follows:

- Due the antioxidant capacity of the extracts could not be directly correlated with their carotenoid, phycobiliprotein and phenolic content, a characterization of other groups of know antioxidant molecules like fatty acids could be elucidative (Stengel et al., 2011).
- Performing an anti-inflammatory assay, e.g. Cox Human inhibitory assay, could potentially add biotechnological value to this cyanobacterium, since, as referred before, antioxidant and anti-inflammatory capacities are usually related.
- Different extraction methodologies could be tried for the obtention of aqueous extracts in order to decrease the phycocyanin degradation (Sarada et al., 1999).
- Due the aqueous extracts showed a high content in allophycocyanin and this pigment already showed to have antiviral and potential antitumoral activities, a deeper bioactivity screening could add value to the aqueous extracts (Shih et al., 2003; Hou et al., 2006).
- Concerning the second part of the work, optimization of growth, futures works could contemplate the optimization of other parameters important for biomass and pigments production e.g. light quality and intensity (Fernández-Sevilla et al., 2010; Pagels et al., 2019).
- A scale-up experiment, e.g. in a bioreactor, would be interesting in order to try to approach production conditions similar to the ones used in industrial production in order to better evaluate its economic feasibility (Acién et al., 2012; Chen et al., 2019).

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# Annex

## List of publications

### Papers:

**D Salvaterra,** F Pagels, HM Amaro, AC Guedes. Evaluation and characterization of the bioactive potential of *Cyanobium* sp. pigments-rich extracts. – *In writing process*

F Pagels, **D Salvaterra**, HM Amaro, AC Guedes. Optimization of biomass and bioactive pigments production by *Cyanobium* sp. – *In writing process*

### Book Chapter:

F Pagels, **D Salvaterra**, HM Amaro, AC Guedes. Pigments from microalgae. Handbook of Microalgae-Based Processes and Products. Elsevier (Academic Press). – *Submitted and accepted.*

### Posters:

**D Salvaterra**, F Pagels, HM Amaro, I Sousa-Pinto, AC Guedes. Cytotoxicity of *Cyanobium* sp. pigment-rich extracts. APAA Conference 2019, 21-22th October, 2019, CIIMAR, Porto, Portugal.

**D Salvaterra,** F Pagels, HM Amaro, I Sousa-Pinto, AC Guedes. *Cyanobium* sp. optimization of biomass production. DCE 2019, 27-28th June, 2019, FEUP, Porto, Portugal. – *Awarded with Best Poster presentation on the Symposium on Chemical and Biological Engineering.*

**D Salvaterra,** F Pagels, HM Amaro, V Vasconcelos, I Sousa-Pinto, AC Guedes. *Cyanobium* sp. extracts - a potential source of natural antioxidants. IJUP 2019, 13-15th February, 2019, Porto, Portugal.