Fluorescent light vs. LED for *Gloeothece* sp. biomass and bioactive compounds production – a promising approach from blue biotechnology?

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Abstract

Cyanobacteria are microscopic photosynthetic organisms with high metabolic diversity, in which are included some of the most efficient converters of solar energy into biomass. They have a great application in biotechnology due some metabolites production namely lipidic compounds, such as carotenoids and polyunsaturated fatty acids (PUFAs) – known for their bioactivities, e.g. antioxidant capacity. Their metabolism is controlled by abiotic and biotic factors, however, as photosynthetic organisms, light exerts a crucial role in biomass and metabolites production. Fluorescence light (FL) has been used as light source to indoor production; however, Light Emitting Diodes (LED) have been suggested as a feasible alternative with some advantages, which are related to their capacity of being fitted in different design and be shaped according to their use. Plus, LEDs are mercury-free and are about 5 times more durable than the fluorescent lamp.

The aim of this study was to ascertain the effects of different LED wavelengths in cyanobacterial production as an alternative to regular FL. Blue (B) and red (R) LEDs, and two combinations thereof (BR) were studied in terms of *Gloeothece* sp. biomass productivity (P_X), carotenoids and fatty acids (FA) production, as well as compounds with antioxidant capacity(AC). To the LEDs that presented better results in terms of bioactive compounds productivity, infrared (IR) LEDs were added, and their influence assessed on the various parameters studied. Finally, the best LEDs, previously defined in terms of AC, were optimized in terms of total light intensity (50, 100, 150 and 200 $\mu mol_{photon.}m^{-2}.s^{-1}$); and a more detailed study concerning bioactive compounds was performed.

As expected the use of different LEDs wavelengths allows a manipulation of the cyanobacterial metabolism. In terms of light quality, it was observed that B LEDs increases the P_X and PUFAs, while R and BR (40:60) increase the production of carotenoids and AC. Extra combination of BR and R with IR enhanced P(x) and FA, besides producing a change in carotenoid profile and AC over time. In terms of light intensity, 150 $\mu mol_{photon.}m^{-2}.s^{-1}$was the best condition for biomass production while 100 $\mu mol_{photon.}m^{-2}.s^{-1}$ causes a higher AC.

As major conclusion, LEDs will be an increasingly better choice for algal cultivation, although it still needs optimization – once even the LED technology itself keeps changing and improving its efficiency and energy savings. The LED use may allow the control of specific metabolites production by target use of particular wavelengths.

It is desired that the work developed along this dissertation will constitute a valid contribution to solve one of the bottlenecks found in cyanobacteria-based bioprocessing – specifically with regard to increase the intracellular concentration of bioactive compounds.

Keywords: Cyanobacteria, LED, antioxidant capacity, carotenoids, fatty acids, phenolic compounds, light quality, light intensity.

Resumo

As cianobactérias são organismos microscópicos fotossintéticos com grande diversidade metabólica, da qual fazem parte alguns dos mais eficientes conversores de energia solar em biomassa. Esses organismos têm uma elevada aplicação biotecnológica uma vez que produzem compostos lipídicos, tais como carotenoides e ácidos gordos polinsaturados (PUFAs) – que são conhecidos por possuírem várias bioatividades, como por ex., a capacidade antioxidante. O seu metabolismo é controlado por fatores abióticos e bióticos, contudo, tratando-se de organismos fotossintéticos, a luz exerce um papel crucial na produção de biomassa e metabolitos. A luz fluorescente (FL) tem sido utilizada como fonte de luz para a produção desses organismos em reatores fechados; no entanto, os Díodos Emissores de Luz (LEDs) têm sido sugeridos como uma alternativa viável com várias vantagens relacionadas com a capacidade de adaptação a diferentes conceções de acordo com a sua utilização. Além disso, os LEDs estão livres de mercúrio e têm uma durabilidade de cerca de 5 vezes mais comparativamente às FL.

O objetivo deste estudo foi verificar os efeitos de diferentes LEDs na produção de cianobactérias como alternativa às FL usualmente utilizadas. Assim, LEDs azuis (B), vermelhos (R), e duas combinações destes (BR) foram estudados em termos do seu efeito na produtividade mássica (P_x), da *Gloeothece* sp., na produção de carotenoide, ácidos gordos (FA), e compostos com capacidade antioxidante (AC). Aos LEDs que apresentaram os melhores resultados em termos da produção de compostos bioativos, foram adicionados LEDs infravermelhos (IR), e a influência da sua adição foi avaliada nos diversos parâmetros estudados. Finalmente, os LEDs em que foram obtidos melhores resultados, foram otimizados em termos de intensidade luminosa total, sendo assim testadas as intensidades de 50, 100, 150 e 200 $\mu mol_{photon.}m^{-2}.s^{-1}$, e um estudo mais detalhado sobre os compostos bioativos foi efetuado.

Tal como esperado, o uso de LEDs permitiu uma manipulação do metabolismo da cianobactéria em estudo. Em termos de qualidade de luz, observou-se que o LED B induz o aumento da P_X e dos PUFAs, enquanto os LEDs R e BR (40:60) induzem um aumento da produção de carotenoides e da AC. Além disso, observou-se que a adição de IR induz um aumento na produção de PUFAs, apesar de alterar a produção de carotenoides e da AC ao longo do tempo. Em termos de intensidade luminosa, 150 $\mu mol_{photon.}m^{-2}.s^{-1}$mostrou ser a melhor condição para a produção de biomassa, enquanto 100 $\mu mol_{photon.}m^{-2}.s^{-1}$ induziu um aumento na produção de compostos com AC.

Como principal conclusão, os LEDs são uma melhor opção para o cultivo de algas, embora ainda necessite de otimização - uma vez que mesmo a tecnologia LED continua permanentemente em processo de melhoramento da sua eficiência e poupança de energia.

Além disso, o uso de LEDs pode permitir um maior controlo na produção de compostos específicos, pelo uso de determinados comprimentos de onda específicos.

É desejável que o trabalho desenvolvido ao longo desta dissertação constitua uma contribuição válida para resolver uma das limitações encontradas no bioprocessamento baseado em cianobactérias - especificamente no que diz respeito ao aumento da concentração intracelular de compostos bioativos.

Palavras-chave: Cianobactéria, LED, capacidade antioxidante, carotenoides, ácidos gordos, compostos fenólicos, qualidade de luz, intensidade luminosa.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AC	Antioxidant Capacity
AL	Artificial Light
ALA	α-Linolenic Acid
B	Blue
BCA	Bicinchoninic Acid
Chl	Chlorophyll
DPPH	2,2- diphenyl-1-picrylhydrazyl
DW	Dry Weight
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FL	Fluorescent Light
FR	Far-red
G	Green
GAE	Gallic Acid Equivalent
GLA	γ-Linolenic Acid
HPLC	High-Performance Liquid Chromatography
IR	Infrared
LA	Linoleic Acid
LED	Light Emitting Diode
LHC	Light-Harvesting Complex
MUFA	Monounsaturated Fatty Acid
OA	Oleic Acid
ORAC-FL	Oxygen Radical Absorbance Capacity
P_X	Biomass Productivity
PAR	Photosynthetically Active Radiation
PS	Photosystem
PUFA	Polyunsaturated Fatty Acid
R	Red
ROS	Reactive Oxygen Species
SFA	Saturated Fatty Acid
t_d	Duplication Time
TE	Trolox Equivalent
UV	Ultraviolet
V	Violet
W	White
Y	Yellow
μ_{max}	Specific Growth Rate

Chapter 1

State of Art

1.1. Introduction

Cyanobacteria and microalgae are able to produce several compounds with a great importance to industry; among them are antibacterial, antifungal, antiviral or even anticancer compounds; such as carotenoids, fatty acids and peptides [1]. These compounds have different roles inside the cell; being usually associated to vital metabolism or to stresses adaptations mechanisms [2, 3].

These intracellular compounds production are influenced by abiotic factors such as salinity, pH, temperature and light or nutrients availability. However, once microalgae and cyanobacteria are photosynthetic organisms, under photoautotrophic cultivation, light exerts a crucial role, namely in terms of quality and intensity. It is along the photosynthetic activity and other pathways regulated by light that the main metabolic process occurs, from the change in density of biomass till the accumulation of compounds [4, 5].

The sunlight is used since the 60s for the large outdoor cultivation [6]. Besides being a low cost source of light, it wide spectrum of radiation allowing the absorption of energy by different pigments and compounds present in the algae, increasing the photosynthetic efficiency of the organism [5, 7]. Although, sunlight is composed by a large wavelength spectrum, a great part of it cannot be used as energy source by photosynthetic organisms – the visible light and photosynthesis active radiation (PAR) (Figure 1.1), represents only 15% of the sunlight spectrum [8]. Said part ranges from the violet (V) (380 nm) to the far-red (FR) (750 nm). But in cyanobacteria and microalgae, non-visible radiation, such as ultraviolet (UV) (280-315 nm) can also trigger the production of secondary metabolites, through activation of expression of UV-B protective genes that consequently lead to production of antioxidant compounds such as phenols [9, 10].

Nevertheless, the conversion efficiency of these organisms is reported to be higher under artificial and controlled treatments, this is because during the dark cycle the algae consume part of its products in a heterotrophic metabolism.

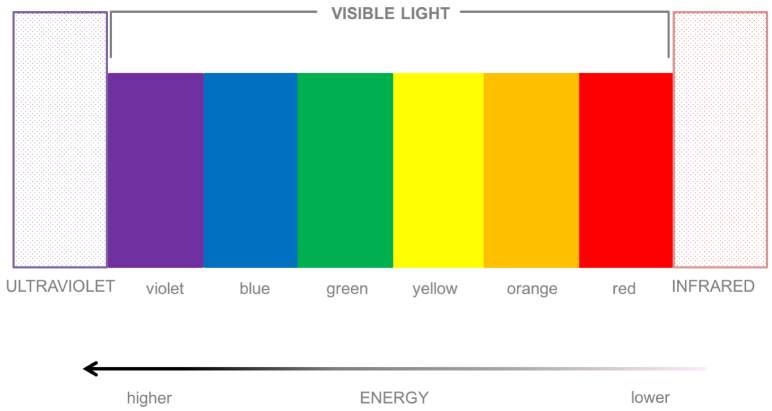

Figure 1.1. Light qualities used by photoshyntetic organisms.

The disadvantages that the use of sunlight may present in the production are mainly related to the reduced photoperiod and constant instability of light intensity caused by weather changes, either seasonable or daily (e.g., the lack of light in cloudy days), which may lead to a decrease in the microalgal production [11, 12].

Although sunlight is the most economic and strongest energy source, the artificial light may be more feasible when the biomass production is intended to obtain high value products due the possibility to control light intensity and time of exposure [4, 5]. Thus, with the beginning of the use of cultures in closed systems and the use of photobioreactors, the employment of lamps that simulate sunlight became frequent and effective, but in both cases the excess of light can cause overheating of the system and as a consequence, loss of biomass [13].

1.2. Artificial Light in Microalgae Production

While sunlight still is the more common light source in outdoors cultivation, in indoor systems, the artificial light appears as a more controllable source. So, the most widely used artificial light source is fluorescent light (FL), however, the use of light-emitting diodes (LEDs) seems to be more and more an effective option in artificial lightening [14].

Artificial light (AL) sources (Table 1.1) are used to promote growth by emitting a specific electromagnetic spectrum for photosynthesis. However, light spectrum of AL can be in a large range as is the case of FL (some of them almost similar to the sun), or either to provide a more narrow spectrum, as is the case of LEDs [4, 14]. In general, the AL source has as advantage the continuous and controlled illumination of the cultivation, which may lead to an improvement of the microalgae productivity, once the autotrophic metabolism is not dissipated during the night.

However, the feasibility of the use artificial light source should be evaluated by several factors, namely its electrical efficiency, heat dissipation, reliability, durability, compactness, cost and its spectral output [15, 16]. Besides, is necessary to take into account that the maintenance of the lamps/panels increases the production cost [14].

Furthermore, sometimes the supply of energy for lightening is acquired from fossil fuels, which increases the carbon systems footprint and reduces the ecological potential of this type of cultivation [17]. There are a few viable alternatives to energy supply, the use of solar-energy-excited, optical fiber systems, solar panels associated to LEDs systems and also wind power generators. The combination of these alternatives can lead to a greater potential for commercial development for microalgae and cyanobacteria cultivation [5, 18] – See Table 1.1.

With the discovery of new uses for microalgae and cyanobacteria metabolites, the industry needed to find new solutions to not only reduce energetic costs, but also increase productivity. Thereby, LEDs seems to be a good alternative to FL [4, 16, 19].

LEDs have several advantages, some of them are related to their size – due its small size they can be fitted in different design and be shaped according to their use and the association with the cultivation method [20, 21]. Plus, LEDs are mercury-free and are about 5x durable than the FL [19]. Nonetheless, LEDs systems are able to emit light in a very narrow emission peak, (10-30 nm), what may cause a compound specific production, due to the metabolic response depending of the photosynthetic adaptation of the microalgae and cyanobacteria to this spectrum [21].

Table 1.1. Features of several artificial light (AL) sources (adapted from [5, 22]).

Source	Feature	Operation stability
Conventional artificial light sources (FL)	Higher biomass productivity and stability, large illumination area, low construction cost	High
Light-Emitting Diodes (LED)	Lower energy consumption, lower heat generation, longer life-expectancy, toleration to higher frequency of on–off switching, higher stability, low constructing cost.	High
Optical fiber excited by metal–halide lamp	Lower energy consumption, lower area required, good light path, uniform light distribution, lower space requirement, low contamination risk.	Moderate
Optical fiber excited by solar energy	Low electricity consumption, good light path, uniform light distribution, lower space requirement, low contamination risk, low cost.	Low
LED/Optical fiber combined with wind power/solar panel	No electricity consumption, Good light path, uniform light distribution, lower space requirement, low contamination risk.	High

Changes in light conditions allow the synthesis of specific products, or a change on the processing capabilities of microalgae and cyanobacteria by indirectly manipulation of the metabolic pathways. Thus, first, it is essential the understanding how the photosynthesis process occurs in these organism, once it is their most important metabolic process.

1.3. Metabolic Control

Photosynthesis represents, directly or indirectly, the source of energy for the metabolisms of almost all living beings on the planet. The principle of photosynthesis is a light energy conversion process, where energy is converted to organic matter using the light energy to the reaction [23]. In photosynthesis, the two main systems responsible for the uptake and transformation of the light energy are photosystems I and II (PSI and PSII), these systems are large protein assemblies that contain a great amount of pigments, which will absorb light and transfer the energy to the photosynthetic reaction centres [24].

Due some light stress conditions, either by excess, low intensity or oscillation in light quality, several biological adaptations are triggered in photosynthetic organisms, including on tightly stacked thylakoids and light-harvesting antenna complexes (LHCs) [25]. All types of LHC are composed by a core and a reaction centre pigment – composed by chlorophyll (Chl) *a* and a light-harvesting antenna. The antennae allow an extended range of light absorption, what increase the efficiency of the photosynthetic activity. To harvest light energy, photosynthetic organisms use pigments, mainly Chls, but also carotenoids and phycobilins [24].

Despite their great importance on metabolism and in the photosynthetic activity, all Chls have absorption peaks both in blue (B) (450–475 nm) and red (R) (630–675 nm) spectrum and little absorption in the region of 500–600 nm, leading to a green spectrum gap, which is responsible for the green colour of most photosynthetic organism. In a way to fill this gap and absorb green light, microalgae and cyanobacteria use mostly carotenoids and phycobilins [26].

Carotenoids have an absorption range mostly between 400 and 550 nm. However, these pigments have other functions on the organism than as accessory components of LHCs, they also give protection to excess of irradiance, Chl triplets and reactive oxygen species (ROS) [23, 27].

Additionally, other pigments can be used by photosynthetic organisms to absorb green (G) light. Cyanobacteria and red algae produce phycobilins, that absorb between 550 and 680 nm [23]. Yet, the colour of the brown algae (Phaeophyta), diatoms (Bacillariophyta) is due to the presence of xanthophylls, (e.g. fucoxanthin), in the LHC [28].

Therefore, there is a great variety of antenna complexes, it appears in order to promote the evolutionary success of photosynthetic organisms in different environments, not only in the absorption of light, but also to supply the need in some organisms of balance the energy input in the two PS [29, 30].

In the same way that the environment led to evolutionary adaptations in the antenna, the change of the luminous or climatic conditions, forces the organism to react quickly with

metabolic changes, in a acclimation process [30]. Changes in light quantity and quality lead to modulation of the antenna in practically all organisms, what means that occurs a chromatic acclimation – i.e. an induction of the synthesis of different pigments for the maximum match to growth light [24].

However, light seems to have an influence in many other metabolic pathways, resulting in distinct physiological and morphological states of cells under different light quality conditions. It is believed that the metabolic pathways are related to two-component systems that use phytochrome-like photoreceptors with sensor-kinase domains to control response regulators that function as transcription factors [31]. Particularly the phytochrome is related to the response to R light, in a photo-reversible system. When exposed to R light, the molecule is converted to an active form that triggers signalling cascades, which lead to an adjustment on the metabolic pathways of the organism [30-32]. Withal other photoreceptors such as cryptochromes, phototropins, aureochromes, and neochromes are specific related to B light responses [33].

In the follows sections, it will be summarized the process optimization of the cyanobacteria and microalgae production with metabolic control through light quality, untangling the main metabolites whose concentration and composition were changed by the light quality.

1.4. Red Light

Photosynthesis and its components have been object of study for a long time. The studies, based on the ideas generated by plant scientists, were mainly based on the R and FR light effect in microalgae and cyanobacteria, due to their direct influence on phytochrome and consequently the responses mediated by it. In 1973, Lipps [34] has showed the effect of the FR light in 4 diatom species, which had their duplication time reduced when illuminated only for FR spectra. However, studies on the effect of light quality on the microalgae and cyanobacteria production appear only decades later, due to the progress of bioreactors and the possible commercial use of specific light wavelengths. Sanchez-Saavedra et al. [35] studies use FR supplementation to the artificial daylight in a way to verify the influence on the production of carotenoids and other pigments by *Dunaliella bardawil*. They found that the FR light induces an increase of the carotenoid content, and a decrease of Chl content.

Only on the last decade, with the appearance of LEDs, the research of light conditions optimization had increased, once this type of light source has several advantages. Some of them, as already mentioned, are related to their size, due its small size they can be fitted in different design and be shaped according to their use and the association with the cultivation method [20, 21]. However, the light response, both in growth

and in compounds production, varies according to the species (Table 1.2). Until now, studies have focused on the influence of R light on the production of biomass, lipids and pigments.

Light responses to cyanobacteria are very species dependent. R light increases the production of biomass on *Nostoc flagelliforme* [36] and *Synechocystis* sp. [37, 38]. While in *Gloeothece membranacea* induced the production of phycobiliproteins [39]; and in *Spirulina platensis*, induces a higher level of purity of phycocyanin [40], which could be an advantage if is necessary the extraction and purification of the pigment.

The different responses in green algae are also related to specific species. In *Picochlorum atomus,* R light treatment showed higher productivity when compared to other light qualities, including V, B, G and yellow (Y) LEDs and also better than FL [41]. Likewise, in *Tetraselmis chuii* and *Tetraselmis suecica* production, the use of R light induced higher growth rates when compared to B light, and also increase the eicosapentaenoic acid (EPA) content [42, 43]. To *Chlorella* sp. continuous R light stimulated higher growth when compared to FR or B [44-47]. Furthermore, R light treatment decrease the ROS activity, what may be explained to a higher amount of antioxidant compounds production, or a less stressful growth condition [47]. Overall, the use of R light in photobioreactor was the most efficient on growth and on an economic point of view, to *Chlorella* sp. [48, 49].

Some species have been reported to have high lipidic content, and R light treatments have shown to be promising on increasing specific lipids amounts, as in the case of oleic acid, increased both in *C. minutissima* [50] and in *Nannochloropis* sp. [43, 51]. Moreover, in *Ettlia oleoabundans,* known as capable to accumulate significant amount of triacylglycerides per dry weight (DW), R light treatment increased the lipidic content [52].

An interesting study had to do with the production of microalgae on wastewater. It can be used as low-cost nutrient medium, decontaminate the water, but also to enable the harvest of microalgal biomass or high-valued metabolites. Thus, the use of R light treatment on wastewater has shown to be very effective on the case of *C. vulgaris*, to purify synthetic sanitary sewage [46] and chemical fertilizer agricultural wastewater, removing more nutrient [53]. Furthermore, R was also the optimal light wavelength for *Chlorella* sp. growth, biogas upgrading, and nutrient reduction on residual agriculture water [12].

Besides that, the use of Y light treatments has shown similar results to R; this because the wavelengths are close. That was observed by Hultberg et al. [54], where *C. vulgaris* produced a higher amount of biomass in; and also by de Mooij et al. [55] where the productivity of *Chlamydomonas reinhardtii* was higher.

Table 1.2. Optimization of specific products of cyanobacteria and microalgae in different light qualities treatments.

Phylum	Specie	Processing Conditions	Light Conditions	Optimal Light Quality	Increased Productivity	Reference
Cyanobacteria	*Arthrospira platensis*	**T** = 30 °C **M** = Zarrouk	R, B, G, Y, BR and W LEDs **I** = 9000 lx	B R G	Lipids and carbohydrates Biomass Protein	[57]
	Gloeothece membranacea	**pH** = 7.1 **T** = 20 °C **M** = BG11	Xenon arc lamps with filters R, B, G, Y, W **I** = 150 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	R G	Phycobiliprotein Chlorophyll	[39] [73]
	Nostoc flagelliforme	**T** = 25 °C **M** = BG11	R, B, G, Y and P LEDs **I** = 60 µmol.m^{-2}.s^{-1}	B R	Extracellular polysaccharide Biomass	[36] [36]
	Pseudanabaena sp.	**T** = 25 °C **M** = ASN-III	Fluorescent lamps with filters R, B, G, Y and W **I** = 220 lx **LDC** = 12:12 h	G	Biomass	[72]
	Spirulina platensis	**T** = 30 °C **M** = Zarrouk **Ro** = 120 rpm	R, B, G, Y and W LEDs **I** = 0 – 3000 µmol.m^{-2}.s^{-1}	B	Phycocyanin and Chlorophyll	[18]
		pH = 8.5 – 9.5 **T** = 30 °C **M** = Zarrouk **Ro** = 120 rpm	Fluorescent lamps with filters R, B, G and Y **I** = 800 lx **LDC** = 12:12 h	R	Phycocyanin	[40]
	Synechocystis sp.	**pH** = 7.5 – 8.0 **T** = 19 – 25 °C **M** = BG11 **Ro** = 70 rpm	R, B and W LEDs **I** = 0 – 960 µE.m^{-2}.s^{-1} **LDC** = 16:8 h	R	Biomass	[38]
Rhodophyta	*Porphyridium cruentum*	**T** = 25 °C **M** = ASW	Fluorescent lamps with filters R and B **I** = 30 – 90 µE.m^{-2}.s^{-1}	B	Extracellular polysaccharide	[58]
Bacillariophyta	*Achnanthes* sp. *Amphora* sp. *Navicula* sp. *Nitzschia* sp.	**pH** = 8.2 **T** = 20 °C **M** = Sterile seawater	R, B, Y and W LEDs **I** = 10 – 400 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	B	Biomass	[60]

T – Temperature; M – Culture medium; Ro – Rotation; A – Aeration; LDC – Light:Dark Cycle; I – Intensity; R – Red; B – Blue; G – Green; Y – Yellow; P – Purple; BR – Mixture of blue and red; W – White; FR – Far-Red

Table 1.2. *Cont.*

Phylum	Specie	Processing Conditions	Light Conditions	Optimal Light Quality	Increased Productivity	Reference
Haptophyta	*Isochrysis galbana*	**T** = 20 °C **M** = F/2	R, B and W LEDs **I** = 52 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	B	Lipids	[61]
	Isochrysis sp.	**pH** = 7.2 **T** = 26 °C **M** = Conway	B and W fluorescent tubes **I** = 100 µmol.m^{-2}.s^{-1}	B	Protein	[62]
Ochrophyta	*Nannochloropsis oculata*	**T** = 25 °C **M** = Enriched seawater	R, B, G and W LEDs **I** = 400 – 1200 lx	B R	Biomass Lipids	[51] [51]
		T = 25 °C **M** = F/2 **Ro** = 150 rpm	Halogen lamps with filters R, B, G, P and W **I** = 100 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	B	Lipids	[64]
		T = 22 °C **M** = F/2 **A** = 0.5 L.min^{-1}	R, B, P, BR LEDs **I** = 100 µmol.m^{-2}.s^{-1}	R	Lipids	[43]
		T = 20 °C **M** = F/2 **A** = 2.5 L.min^{-1}	R, B, G, Y and P LEDs **I** = 40 – 130 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	G	Lipids	[74]
Chlorophyta	*Botryococcus braunii*	**pH** = 7.5 **T** = 20 °C **M** = C	R, B and G LEDs **I** = 30 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	B	Biomass	[71]
	Chlamydomonas reinhardtii	**pH** = 6.7 **T** = 25 °C **M** = Sueoka HS	R, B, and Y LEDs **I** = 1500 µmol.m^{-2}.s^{-1}	R	Biomass	[55]
	Chlorella minutissima	**pH** = 6.2 **T** = 23 °C **M** = Bold 3N **A** = 0.06 L.min^{-1}	R and W LEDs and fluorescent lamp **I** = 100 µE.m^{-2}.s^{-1} **LDC** = 15:9 ; 12:12 ; 24:0 h	R	Oleic acid	[50]
	Chlorella vulgaris	**T** = 25 °C **M** = Synthetic sanitary sewage	R, B, G, P and W LEDs **I** = 500 – 3000 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	R	Nutrient uptake	[46]
		T = 20 °C **M** = 3N-BBM+V	Xenon arc lamps with filters R, B, G, Y, W **I** = 150 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	G G	Chlorophyll content Biomass	[39] [73]

T – Temperature; M – Culture Medium; Ro – Rotation; A – Aeration; LDC – Light:Dark Cycle; I – Intensity; R – Red; B – Blue; G – Green; Y – Yellow; P – Purple; BR – Mixture of blue and red; W – White; FR – Far-Red

Table 1.2. *Cont.*

Phylum	Specie	Processing Conditions	Light Conditions	Optimal Light Quality	Increased Productivity	Reference
Chlorophyta	*Chlorella vulgaris*	**T** = 20 °C **M** = Z8 **A** = 0.3 vvm	R, B, G, Y, BR and W LEDs **I** = 100 µmol.m^{-2}.s^{-1} **LDC** = 16:8 h	G	PUFAs	[54]
	Chlorella sp.	**T** = 20 °C **M** = BBM	B and R cool white lamp **LDC** = 12:12 h	B	Lipids	[66]
	Ettlia oleoabundans	**T** = 25 °C **M** = BBM **Ro** = 100 rpm **A** = 1.2 vvm	Cool white lamp supplemented with R or FR LED **I** = 95 µmol.m^{-2}.s^{-1}	R	Lipids	[52]
	Haematococcus pluvialis	**pH** = 6.8 **T** = 20 °C **M** = Kobayashi	R, B, G and P LEDs and fluorescent lamp **I** = 2 – 12 µmol.m^{-2}.s^{-1}	B	Astaxanthin	[69]
	Picochlorum atomus	**T** = 20 °C **M** = F/2 **A** = 2.5 L.min^{-1}	R, B, G and P LEDs **I** = 70 µmol.m^{-2}.s^{-1} **LDC** = 12:12 h	R G	Biomass Lipids	[41]
	Tetraselmis chuii	**T** = 22 °C **M** = F/2 **A** = 0.5 L.min^{-1}	R, B, P, BR LEDs **I** = 100 µmol.m^{-2}.s^{-1}	R	Biomass	[43]
	Tetraselmis suecica	**pH** = 7.6 – 7.8 **T** = 25 °C **M** = ASW **A** = 0.4 L.min^{-1}	R, B, G and W LEDs **I** = 160 µmol.m^{-2}.s^{-1}	R	EPA	[42]

T – Temperature; M – Culture Medium; Ro – Rotation; A – Aeration; LDC – Light:Dark Cycle; I – Intensity; R – Red; B – Blue; G – Green; Y – Yellow; P – Purple; BR – Mixture of blue and red; W – White; FR – Far-Red

1.5. Blue Light

As already referred, all Chl have absorption peaks both in B and R zone of the spectra. Moreover, B light has proven to influence gene expression and several metabolic pathways in photosynthetic organisms, via photoreceptors such as cryptochromes, phototropins, aureochromes, and neochromes [33]. Plus, this same light is also responsible to lead the organism to endogenous breakdown of carbohydrate reserves [4, 56].

For cyanobacteria, the effects of this light treatment were generally related to the production of metabolites, such as lipids, carbohydrates, phycocyanin, Chl and polysaccharides (Table 1.2).

Chen et al. [18] saw that to *S. platensis*, B light in high intensities allows the enhancement of both phycocyanin and Chl contents. Furthermore, under B light *N. flagelliforme* showed higher extracellular polysaccharide production, and these cyanobacteria derived biopolymers have advantages over other polysaccharides extracted from plants or marine microalgae [36]. Likewise, *Arthrospira* (*Spirulina*) *platensis* both lipidic and carbohydrates content had increased under B LED treatments [57]. Still, for red algae, You and Barnett [58] observed that B light increased the production of extracellular polysaccharides and the growth rate of *Porphyridium cruentum*.

On the other hand, for diatoms, B light increases either the production of secondary metabolites and the biomass. Costa et al. [59] saw that *Phaeodactylum tricornutum* under B light was found to be in an acclimation state with an increased photoprotective potential when compared to R light; and it was also found an up-regulation of proteins involved in photoprotection. Moreover, benthic microalgae used to phytoremediation (*Achnanthes* sp., *Amphora* sp., *Navicula* sp. and *Nitzschia* sp.) have their growth, productivity and removal efficiency increased under B light [60].

Isochrysis are small marine microalgae (Prymnesiophyceae) commonly used in shellfish hatcheries. The effect of B light illumination to *Isochrysis* sp. was an enhancement of the biomass productivity and metabolites production. Yoshioka et al. [61] observed that the amounts of neutral lipids, glycolipids, and phospholipids were higher under this light quality. Likewise, Marchetti et al. [62] observed, in *Isochrysis* sp., an increase of photosynthetic activity and Chl content when compared to white (W) light, resulting in higher carbon fixation rates. Moreover, it was found higher protein content under B light illumination.

Moreover, *Nannochloropsis* sp. seem to have similar effects to *Isochrysis* sp. – an increase on the biomass productivity and growth rate [43, 51, 63, 64], and also an enhancement of the lipidic content, namely in fatty acids [51], crude oil [63] and pigments

[64]. Teo et al. [63] also observed a specific production focused on palmitic acid and stearic acid production.

Lastly, green algae have shown to be adapted to B light illumination, having great results to this kind of light treatment. *Chlorella* sp. exhibited higher photosynthetic activity [65] and also an increase of lipid production and accumulation [66-68]. In parallel, Katsuda et al. [69] saw that in *Haematococcus pluvialis*, astaxanthin concentration was higher in B and V lights, this effect, has been also seen by Beltran et al. [70], which also verify a change of colour in the culture, from green to red, due to the production of the compound. Furthermore, Okumura et al. [71] show that the *Botryococcus braunii* presented an increase of biomass production efficiency.

As already mentioned, R and B are the most studied light qualities due to their direct relation with photosynthesis, and their influence is closely related to the growth of the culture. However, the light response is not limited to these wavelengths.

1.6. Green Light

As already referred, the low absorption in the region of 500–600 nm leads to a green spectrum gap. And also that, photosynthetic organisms have other compounds/pigments that fill in some way the gap and absorb G light [26].

Studies about G light are not common, but the few ones have shown various effects derived from light treatment in different groups of microalgae and cyanobacteria. Gutu et al. [31] said that cyanobacteria, in general increase the production of phycoerythrin, due to chromatic acclimation on these organisms; however, other green-light-associated events may vary according to the species. In the case of *Pseudanabaena* sp. both growth rate and phycoerythrin content were higher and the carotenoid content increased after 15 days of production, while with all other light treatments start to decrease the production [72]. Likewise, in *G. membranacea*, the pigment content increased [73].

In *Nannochloropsis* sp. the main response to G light is the increase of the production of lipidic compounds [51, 74]; namely an enhancement on the production and purity of palmitic acid and oleic acid [74]. The same was observed on the green algae *P. atomus* [41]. Furthermore, in *C. vulgaris* occurred an increase in the biomass productivity [73] and Chl content [39], along with the concentration of polyunsaturated fatty acids in this specie [54].

1.7. Light Qualities Combinations

The use of more than one wavelength, or light quality, can bring benefits to the production, since microalgae and cyanobacteria are originally designed to sunlight, and have different pigments and different absorption peaks, which may increase the biomass productivity or specific compound production, when illuminated with more than one specific

light quality. Usually it is made a composition of R and B, due to the photosynthetic requirements of these organisms.

Scenedesmus sp., when illuminated by R and B light mixed, had its production rate about 50% higher than the rate of the culture with W light. Also, increased the removal rates of nitrogen and phosphorus compared to a single wavelength of light [47]. Likewise *Chlorella* sp. showed higher productivity in dichromatic R and B (1:1) treatments [75]. The same ratio was found to be the most appropriate for biogas upgrading and biogas slurry nutrient removal [76]. However Kula et al. [77] said that the addition of far-red light is necessary to reduce the stress related to a R and B illumination on the production of *C. vulgaris*, increasing the biomass production along time.

Furthermore, Kim et al. [47] propose that *C. vulgaris* biomass and lipid productivity could be significantly increased when alternated between B (increase cell size) and R light (increase production) in a two-phases production. On the other hand, *C. vulgaris*, *S. obliquus*, and *Neochloris oleoabundans*, had higher growth rate and nutrient removal from wastewater when under R and B mixed LEDs [78].

Lastly, *D. salina* have its carotenoid content increased when illuminated with R and B (3:1) LEDs [49] and *B. braunii* produced more biomass under the mixture of R, G and B LEDs [71].

The W LEDs, as well as FL, may have fluctuations ratio between the different wavelengths, thus it is speculative to take effect and the efficiency of each component of this type of light source.

1.8. Radiation Non-Photosynthetic Active

UV radiations in the bands A (315–400 nm) and B (280–315 nm) are able to reach the Earth's surface. UV radiation is known to cause DNA damage, resulting in mutation in most organisms and has also been reported to inhibit growth and enzymatic activity in many microalgal species[10]. Wang and Chai [79] showed that different microalgae (*Thalassiosira pseudonana*, *Skeletonema costatum*, *Chaetoceros calcitrans*, *Phaeodactylum tricornutum*, I. *galbana, Pavlova lutheri*, *Chroomonas salina*, and *Prorocentrum micans*) have similar effect on omega 3 content, that decrease on the presence of UV-B light. This effect is also confirmed by Guihéneuf et al. [80], where *P. lutheri* had its fatty acidic content decreased. However, the diatom *Odontella aurita* seems to be resistant to the exposure of cells to UV radiation, once it did not change the fatty acid composition of the total lipids, and EPA levels remained high during all treatment.

However, several studies suggested that UV radiation increases the production of lipids in *D. bardawil* [81], *N. closterium, and I. zhangjiangsensis* [82]. To *C. pyrenoidosa*, the

exposures to short times of UV radiation increased biomass yield and also enhance the production of triacylglycerol [83].

As already mentioned, the UV-B radiation can be used to trigger the production of different compounds (e.g. phenols) in some organisms. Kumar et al. [9] saw that targeted low influence rate of UV-B treatment may enrich the content of proteins, vitamins, and antioxidants on *N. muscorum*, *P. foveolarum*, and *A. platensis*. After UV-B exposure, the antioxidant potential of the studied cyanobacteria increased more than 70%.

UV radiation of the range C (UV-C, 100–280 nm) possesses the highest energy per photon and could be useful as a stress induction technique for inducing lipidic accumulation in microalgae. UV-C stress leads to cellular lipid biosynthesis to improve survival of microalgal cells, once UV-C leads to an oxidative stress causing damage to DNA and cellular membranes and the cell needs to produce higher amount of antioxidant compounds.

Sharma et al. [84] demonstrate that UV-C stress not only led to doubling of cellular lipid contents on *Tetraselmis* sp. M8, but also led to the loss of flagella and subsequent settling, facilitating the microalgae harvesting. Likewise, Ahmed et al. [85] found an increase of lipidic content in *T. suecica*. Moreover, higher accumulation of EPA was observed in *P. lutheri* [86]. Therefore, UV-C radiation also increased the production of phytosterol by *P. lutheri* [87].

Besides that, very energetic light, such as infrared (IR), can also trigger to some effects inside the cell. However, a few studies reports the ability of absorb light at IR (860 nm) [88]. Furthermore, Behrendt et al. [89] saw that the cyanobacterium *Acaryochloris marina* was well adapted to a biofilm growth mode under both visible and near-IR radiation (700-730 nm).

1.9. Overview

After compiling the information presented above, it was possible to associate some general responses regarding light quality – e.g. R light mainly induces increase of biomass. However, the responses are intrinsic to the unique metabolism of each species, and the determination of a specific response must be made with studies specific to each specie. Still, the State of Art allows a greater direction for the works of this dissertation and for future works, since it gives an indication of methodologies to be used for the chosen purpose.

1.10. Dissertation aims

Taking into account the above information, the main aim of the proposed dissertation is the optimization of light conditions for the production of *Gloeothece* sp. as source of bioactive compounds. The dissertation plan was divided into three fundamental and

sequential topics for light conditions optimization in the chosen cyanobacterium *Gloeothece* sp.:

(i) Study of the effects of LEDs as an alternative to source compared to FL, on the cyanobacterium growth and biochemical composition, namely in production of bioactive compounds.

(ii) Study of the effect of addition of IR, to the best LED conditions found in the first topic, on the cyanobacterium growth and biochemical composition, namely in bioactive lipidic compounds production.

(iii) Study of the effect light intensities, selected in the previous tasks, for *Gloeothece* sp. biomass and, bioactive compounds production.

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Chapter 2

LED vs. fluorescent light for *Gloeothece* sp. biomass and high value-metabolite production

2.1. Introduction

Despite some species being able to undergo mixotrophic metabolism, cyanobacteria and microalgae are essentially photosynthetic organisms – so light is the essential form of energy needed for their existence. Light is composed by a large spectrum, but only the visible part apparently contains PAR. Said part ranges from the V (380 nm) to the FR (750 nm), but the energy is inversely proportional to the wavelength, what means that the B light (about 400 nm) is more energetic than of R light (around 700 nm).

To fully understand how light affects cyanobacteria growth and biochemical composition, it is essential to understand the metabolic starting point of photoautotrophic microorganism – photosynthesis.

To harvest light energy, photosynthetic organisms possess three major classes of pigments: Chl, carotenoids and phycobilins – organized in LHC. All types of LHC are composed by a core and reaction centre pigment – composed by Chl *a* (a subtype of Chl present in all oxygenic photoautotrophs with absorption peaks at 665 nm and 465 nm); and light-harvesting antennae (composed by pigments such as other subtypes of Chl (*b* or *c*) and carotenoids), (Figure 2.1) [1]. Carotenoids entail a large group of biological chromophores, with an absorption range from the 400 nm to 550 nm. They possess numerous roles in the photosynthetic apparatus, operating as: I) accessory light-harvesting pigments transferring excitation to Chl *a*; II) structures in the light-harvesting and reaction centre pigment–protein complexes; and III) protection of molecules against excess of irradiance, Chl triplets and ROS. In cyanobacteria, the major antennae are composed by phycobilins (phycoerythrobilin, phycocyanobilin and phycourobilin). Hence, cyanobacteria are able to utilize R, Y and G light – and, to a lesser extent, B light [2].

The thylakoid membrane, where photosynthesis occurs, holds five major complexes: light-harvesting antennae, PSI and PSII (both containing a reaction centre), cytochrome and ATP synthase. The antenna systems primary function is light-harvesting and energy transfer to the photosynthetic reaction centres [1]; here two major classes of light-harvesting pigment-protein complexes can be identified: a) hydrophilic phycobiliproteins, usually found in cyanobacteria attached to the protoplasmic side of the thylakoid membrane; and b) hydrophobic pigment-protein complexes, such as LHC II and LHC I, that are composed by Chls and carotenoids, as depicted in Figure 2.1.

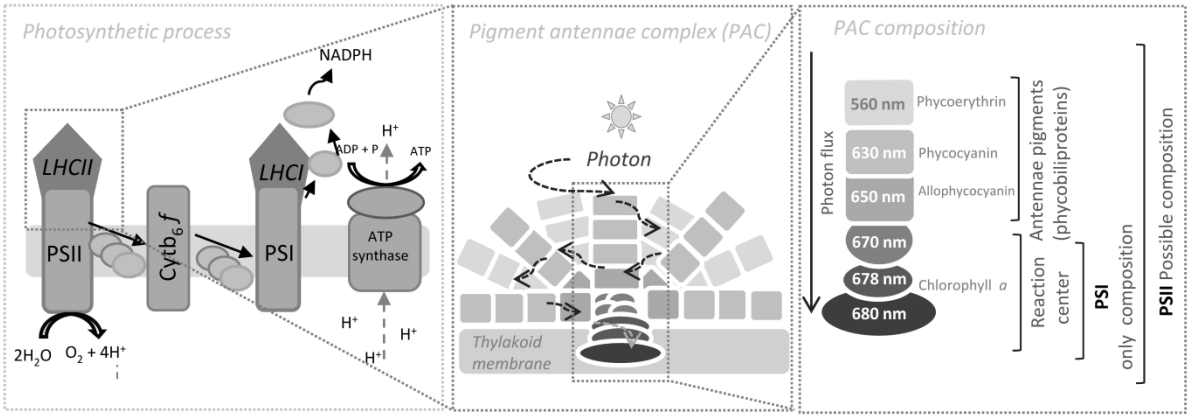

Figure 2.1. Schematic representation of cyanobacteria pigment antenna complex, and its composition in terms of pigment and photosystems I (PSI) and II (PSII) (Adapted from Govindjee and Amaro et al. [3, 4]).

In cyanobacteria, while light-harvesting antenna of PS I is exclusively constituted by Chl *a*, PS II is mainly composed of phycobilisomes. Moreover, PSII possess a relatively larger optical absorption cross-section excited by Chl *a*. when compared to PS I. So, in order to balance the electron flow between PS II and PS I, cyanobacteria generally contain more PS I reaction centres than PS II, which is a deed prompted both by light intensity and spectral distribution.

B and R lights are the light bands most effectively absorbed by photosynthetic pigments, however, in microorganims photoregulation is not limited to the photosynthetic apparatus. Several other photoreceptors, i.e. pigments that absorb light and transduce light signals, are also actively involved in triggering numerous light responses that are independent from the photosynthetic apparatus – as is the case of those first detected in prokaryotic genes encoding phytochrome-like proteins in cyanobacteria, as depicted in Figure 2.2 [5, 6]. Among all types of photoreceptors, one stands outs due its unusual photoreversibility, the R/FR light absorbing phytochrome. This blue protein pigment, with an absorption peak in the red region of the 650–680 nm spectrum, can switch between two interconvertible forms, Pr (inactive form), and Pfr (active form) [6]. When Pr is exposed to red light it is converted to Pfr, blue-green form, thus triggering several biochemical responses. Also, in algae and plants, B light has also proven to have influence in gene expression and in several metabolic pathways via photoreceptors such as cryptochromes, phototropins, aureochromes, and neochromes [7]; besides, it also shown to be responsible for endogenous breakdown of carbohydrate reserves [2, 8]. Thus, as briefly summarized in Figure 2.2, is possible to see how light quality can determine several biophysical and physiological properties.

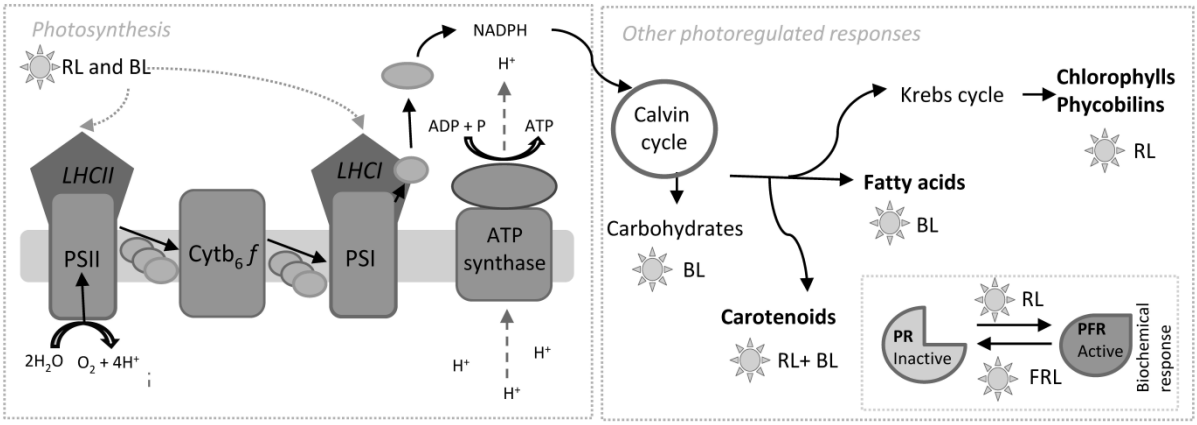

Figure 2.2. Schematic representation of selected metabolic pathways of cyanobacteria that are affected by light spectrum in BL- blue light, FRL- far-red light and RL- red light (Adapted from Amaro et al. [4]).

As found in the specialized literature, B light also showed to promote Chl synthesis and chloroplast formation in *Chlorella* [6], and to induces nitrate and nitrite uptake in *Monoraphidium braunii* [9]. Furthermore, R and FR lights revealed to affect growth, cell size, and photosynthesis rate of microalgae [10]. For example, R LED showed to reduce cell volume of *C. vulgaris* without changing the total biomass yield when compared to FL [10]. In other way, in *D. bardawil* production FR light, when supplemented to a daylight fluorescent lamp, induced much larger cell volume but lower cell population and Chl concentration than one cultivated under single daylight lamps [11]. Other study with *Chlorella pyrenoidosa* showed that continuous red lighting enhanced growth and ethylene production, whereas long-term FR lighting inhibited both [12].

Therefore, light quality appears as a key point for cyanobacteria growth and biochemical composition optimization. If the most proper light source is elected, it will be possible to manipulate the cyanobacteria biomass in terms of optimum biomass productivity, as well as content of high value metabolites for specific uses – particularly for high-end markets [2]. Fluorescent lighting is the most common light source employed in microalgae and cyanobacteria production, but it possesses a width light spectrum – and (as seen before) the range of photosynthetic active radiation is more restricted, making fluorescent lightening energetically inefficient (besides their energy cost and unwanted heat production). Thus, the use of a light source with a narrow spectral output that overlaps the photosynthetic absorption spectrum will improve the overall energy conversion [13]. Among the currently available light sources LEDs are the only ones that meet the previous criteria – it have a light emission spectra between 20 and 30 nm, besides it are small enough to fit into almost any photobioreactor; it have longer life-expectancy, lower heat generation and higher conversion efficiency [14, 15].

For the reasons already mentioned, in recent years have witnessed an increased interest in replacing fluorescent lighting by LED for cyanobacteria and microalga production, but still are important gaps in the knowledge of how these organisms respond to specific light. The combined use of LEDs for cyanobacteria production or general metabolic response patterns was only partly investigated to date, and very few studies have focused on cyanobacteria [2]. To help fill in the gaps found in the current state of the art, the present study was aimed at understanding whether the use of LED lighting is a feasible alternative to FL. The effects of light quality, particularly B and R LEDs and FL, upon growth rate and biochemical composition, in terms of lipidic components (carotenoids and fatty acids), of *Gloeothece* sp., were accordingly ascertained. Since these compounds, particularly the former, have been described as potent antioxidant agents, with proved health and industrial applications, antioxidant compound production was assessed [16-20]. Moreover, the effect of IR light on growth and biochemical composition is essentially unknown, so this study is innovative and will bring ground-breaking information to cyanobacteria production.

2.2. Material and Methods

2.2.1. Cyanobacterium source and growth conditions

Gloeothece sp. (ATCC 27152) obtained from ATCC (American Type Culture Collection, USA), was maintained at 25 ºC in Blue Green (BG11) medium [21]. A pre-inoculum was cultivated for 10 days, with an initial optical density of 0.1 at 680 nm, in 800 mL of BG11, set at pH 8 buffered with Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) 25 mM. A continuous illumination with fluorescent Osram BIOLUX lamps with intensity of 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ was assured, as well as air bubbling at a flow rate of 0.5 $L.min^{-1}$.

Light conditions assays, conducted in batch biological triplicates, were performed in 1800 mL of culture, also with an initial optical inoculum density of 0.1 at 680 nm. Medium, bubbling conditions and continuous illumination were the same as with the pre-inoculum, except the light source.

2.2.2. Light conditions

B LED (peak at 440 nm with a range of 420–470 nm) and R LED (peak at 660 nm with a range of 600-700 nm) were tested, as well as two dichromatic LED percentage compositions of B and R – BR 40:60, % and BR 50:50, %. The influence of a near-IR LED (peak at 862 nm, with a range of 800-900 nm) was tested with the most promising LED conditions at an intensity of 7.14 $W.m^2$.

For a better understanding of how LED affects the cyanobacterium production, a common FL source was used for comparison.

In all experiments, the same light intensity was used either in monochromatic or dichromatic light source – 100 $\mu mol_{photon}.m^{-2}.s^{-1}$, measured with a spherical light sensor WALZ (US-SQS/L), equipped with a logger WALZ (ULM-500).

Cyanobacterium growth LED assays were performed in climate chambers 600 S (Aralab, Portugal), equipped with removable LED panels containing B, R and infrared LEDs. In each assay, a uniform light distribution was provided by spot lights, as well as by establishing the correct distance of cultures to the light source, as depicted in Figure 2.3. The study conducted with FL was performed with Osram BIOLUX lamps in climate chamber 750 E (Aralab).

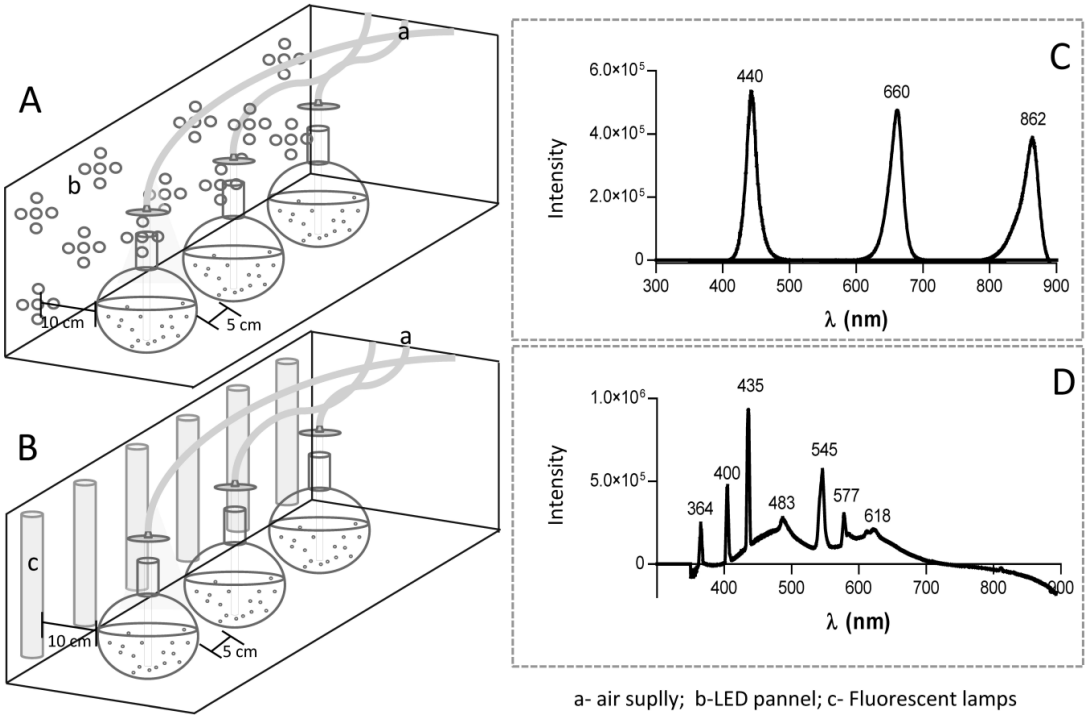

Figure 2.3. Schematic representation of experimental set-up – one of two lighting parallel panels used for *Gloeothece* sp. production under LED light **(A)** and fluorescent light (FL) **(B)**, and their respective wavelengths peaks, LEDs **(C)** and fluorescent lamps Osram Biolux (D).

2.2.3. Biomass quantification

For each biological triplicate, cultivated under the light conditions mentioned above, biomass growth was monitored (in duplicate) along time (at 0, 1, 4, 6, 8, 11, 13, 15, 18, 20, 22, 25, and/or 27, and/or 32) by DW; this means *n* = 6 pseudo-replicates were considered for each set of experimental conditions. The DW was ascertained by filtering a certain volume of culture through preconditioned GF/C glass fiber filters (Whatman, UK), and further drying at 100 ºC till constant weight. The specific growth rate (μ_{max}) was also found by a numerical regression of experimental data, and the biomass doubling time (t_d) was calculated as t_d = (ln

2)/μ_{max}. Biomass productivity (P_X) was discovered using the variation between initial and final values of DW ($g.L^{-1}$) obtained in the exponential phase (EP), $P_X = (DW_f - DW_i)/\Delta d_{EP}$, referred to the underlying exponential phase period, according to P_X (t) = $X_0[(\exp^{(\mu max.t)}-1)/t]$, where t denotes actual sampling time and X_0 denotes initial biomass concentration at start-up.

2.2.4. Antioxidant capacity assessment

Two millilitres of each batch (in triplicate) was centrifuged, at 4,000 rpm for 5 min, and the pellet was resuspended and homogenized in 2 mL of a mixture of ethanol and water (1:1, v/v). Cells were then crushed in an Ultra Turrax T 18 basic homogenizer (Ika, Germany) at 14,000 rpm for 30 s, centrifuged at 4000 rpm for 5 min; and the supernatant (intracellular extract) was collected separately.

The radical-scavenging capacity of the intracellular extracts was evaluated, in triplicate, via the ABTS radical cation ($ABTS^{\bullet+}$) assay, following the method described elsewhere [22, 23]. For quantification, a calibration curve using a known antioxidant – Trolox, was established, so antioxidant capacity (AC) was expressed as trolox equivalents (TE) per dry DW of biomass ($\mu g_{TE}.g_{DW}^{-1}$).

2.2.5. Compounds Identification

Carotenoids identification

High-performance liquid chromatography (HPLC) system was employed as before [19], in order to identify and quantify carotenoids produced by *Gloeothece* sp. (particularly β-carotene and lutein).

Under all light conditions, cyanobacterium cell-free extracts were prepared from each biological triplicates using 20 mL of centrifuged culture sampled over time. The pellet was then resuspended in the same volume of acetone (99.6% purity), and 1 mg of sodium sulfate (Sigma, USA) and β-apo-carotenol (Sigma) was added as internal standard. Cells were then disrupted by sonication for 15 min; and the extract filtered and evaporated in a rotavapor. The residue was then resuspended in a mixture of acetone and ethyl acetate at 9:1 (v/v) prior to injection.

The carotenoids profile was obtained via a Merck-Hitachi HPLC system, equipped with a Diode Array Detector (DAD) Merck-Hitachi L-7450 to resolve, detect, and identify the various chemical compounds of interest. The absorption spectra were recorded between 270 and 550 nm, and the stationary-phase was a Purospher Star RP-18e (5μm, 4 x 250 mm) column (Merck). The mobile-phase was constituted by solvent A – ethyl acetate, and solvent B – acetonitrile/water at 9:1 (v/v), both from (VWR, Portugal), under a flow rate of 1 $mLmin^{-1}$. The following gradient was used: 0–31 min (0–60% A); 31–46 min (60% A); 46–51 min (60–100%

A); 51–55 min (100% A); 55–60 min (100-0% A); and 60–65 min (0% A). The carotenoids elution times of the chromatographic standards were: neoxanthin 7.4 min, violoxanthin 8.4 min, lutein 14.4 min, and β-carotene 34.4 min. Standards were purchased in CarotNature, Lutein (No. 0133, Xanthophyll, (3R,3'R,6'R)-β,ε-Carotene-3,3'-diol with 5% Zeaxanthin and purity of 96%), β-carotene ((No. 0003, β, β -Carotene) with 96% purity) and β-apo-carotenol ((No. 0482, 8'-Apo- β -caroten-8'-al) with 97%, purity). Identification was achieved by comparison of retention time and UV–visible photo-diode array spectra, following the procedure detailed elsewhere [24]

Determination of fatty acid profile

By the end of each light condition experiment, biomass triplicates were harvested, by sedimentation and then centrifuged at 4,000 rpm for 5 min, prior to lyophilisation.

Fatty acid methyl esters were generated from 100 mg of previously lyophilized biomass by direct transesterification according to the acidic method adopted previously [25], using heptadecanoic (C17:0) acid as internal standard and acetyl chloride as catalyst. Esters were analysed in a GC Varian Chromapack CP-3800 gas chromatograph, using a flame ionization detector, and quantified with the program Varian Star Chromatography WorkStation (Version 5.50). A silica CP-WAX 52 CB (Agilent) column was used, and helium was employed as carrier gas in splitless mode. Injector and detector were maintained at 260 and 280 °C, respectively, and the oven heating program was as described in Table 2.1.

Table 2.1. Oven heating program for fatty acids identification.

T (ºC)	Rate (ºC.min^{-1})	Holding time (min)	Time (min)
100	-	5	5
180	6	0	18.33
200	2	0	28.33
205	0.5	0	38.33
230	1	0	63.33
233	0.5	0	69.33
240	4	14.3	90

Chromatographic grade standards of fatty acids in methyl ester form CRM47885 (Supelco) were used for tentative identification, based on comparison of retention times: C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1 n9-cis + trans, C18:2 n6, C18:2 n6 c, C18:3 n6, C18:3 n3, C20:0, C20:1, C20:5 n3, C21:0, C22:0, C22:2, and C22:1 n9. The mean of the results from the aforementioned chemical assays was used as a datum point.

2.2.6. Statistical analysis

Obtained data were analysed using GraphPad Prism V. 5.0. Firstly, Shapiro-Wilk test of normality was done, and then 1-way ANOVA with Tukey's multi-comparison test was used to assess variances between different light conditions on growth parameters. Two-way ANOVA with the same multi-comparison test was employed to found differences between carotenoids, FA content and AC between each light condition tested.

This analysis permitted one to ascertain whether light quality influenced the production of biomass and bioactive compounds. Since each datum point had been replicated, a representative measure of variability was available in all cases to permit statistical analyses.

2.3. Results and discussion

2.3.1. Effects of light source on biomass production

Light is an essential factor for cyanobacteria growth, and particularly light spectral quality and intensity must be considered when choosing the purpose of their production. As said before, spectral quality is defined by the absorption spectrum of Chls and other photosynthetically active pigments, such as phycobilins and carotenoids; and its energy absorption is dependent on their chemical nature [26-28]. Chls, particularly Chl *a,* have two major spectrum absorption bands at blue (450–475 nm) and red (630–675 nm). However, it is important to notice that each specie has its particular preference on growth for a particular balance of absorption bands, so there is not a universal formula of light spectra that can be applied to all organisms towards optimum growth or metabolite synthesis [2, 13]. This difference over the effect of a wavelength on the growth between species has been often described in the literature, thus suggesting that the influence of light wavelength on the production of biomass is species-dependent [14, 29]. Nevertheless, studies indicated that blue and red wavelengths are the chief responsible for different metabolic and physiological responses as those described before in *C. vulgaris* [30].

For an optimum growth, light intensity should be delivered equally over the culture surface and with adequate amount of PAR to enable photons to reach the cell in the culture [28]. An excessive intensity may lead to photooxidation and photoinhibition, while low light levels will become growth-limiting [31]. Thus, the light intensity at which culture growth becomes saturated is an essential factor in determining light utilization efficiency; cyanobacteria light saturation usually begins at an incident light intensity around 200 $\mu mol_{photon}.m^{-2}.s^{-1}$[27], so a light intensity was chosen for this study that would avoid this situation, i.e. 100 $\mu mol_{photon}.m^{-2}.s^{-1}$.

In this study, the cyanobacterium biomass production was monitored by culture DW. As expected, the cyanobacterium *Gloeothece* sp. exhibited different behaviours under

different light conditions in terms of biomass production along time – as depicted on Figure 2.4A. Following inspection of the growth parameters tested (Figure 2.4B), the shorter duplication time (td) – 2.54 d, higher specific growth rate (μ_{max}) – 0.2735 d^{-1}, and higher P_X – 0.132 $g.L^{-1}.d^{-1}$ indicated that B LED is the more suitable for biomass production ($p < 0.05$), even overrating the ones obtained under FL. The other values for all the parameters are shown on Table 2.2.

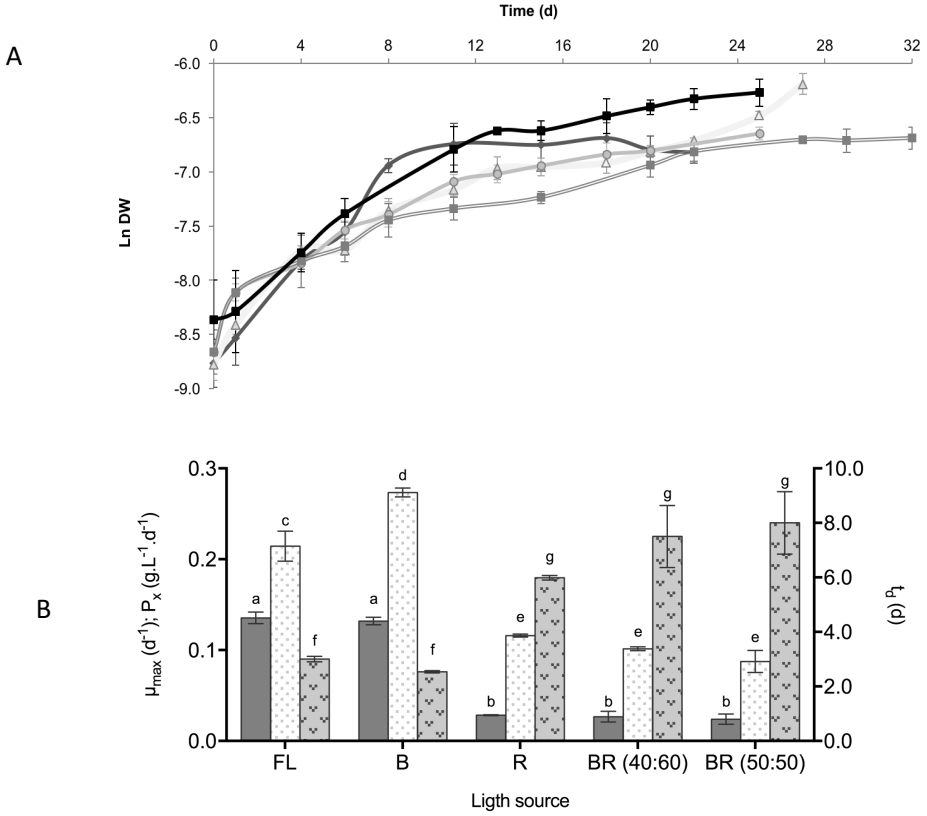

Figure 2.4. (**A**) Variation of biomass concentration (average ± standard deviation) of *Gloeothece* sp., expressed as natural logarithm of dry weight, Ln DW, with incubation time, at several light condition (n = 6) FL (Fluorescent light) B (Blue LED), R (Red LED), BR (Blue:Red LED) (40:60), BR (Blue:Red LED) BR (50:50). (**B**) Characteristic growth parameters (average ± standard deviation), viz. Biomass productivity (P_X) Specific growth rate (μ_{max}) and Duplication time (t_d), obtained for each light condition. Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

Table 2.2. Biomass parameters calculated (average ±standard deviation) on the exponential phase for the different light conditions (n=3).

Light Quality	Exponential Phase (d)	P_x ($g.L^{-1}.d^{-1}$)	μ_{max} (d^{-1})	t_d (d)
FL	1 - 11	0.14 ± 0.01^{a}	0.21 ± 0.02^{c}	3.24 ± 0.24^{f}
B	1 - 8	0.13 ± 0.00^{a}	0.27 ± 0.01^{d}	2.54 ± 0.40^{f}
R	1 - 8	0.28 ± 0.00^{b}	0.12 ± 0.02^{e}	5.99 ± 0.08^{g}
BR (50:50	1 - 11	0.28 ± 0.10^{b}	0.08 ± 0.02^{e}	8.00 ± 1.13^{g}
BR (40:60)	1 - 11	0.29 ± 0.02^{b}	0.10 ± 0.00^{e}	7.50 ± 1.14^{g}

Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

2.3.2. Effects of light source on the biochemical composition

Effects of light source on carotenoids production

Evidence has shown that some carotenoids can be overproduced in response to stressful light conditions [31]; hence, a well-designed LED lighting may lead to an efficient and sustainable production of carotenoids, such as β-carotene and lutein.

Carotenoids biosynthesis is a complex and coordinated process with the biogenesis of Chls and proteins that composes photosynthetic apparatus [32]. As emphasized before, carotenoids such as β-carotene and lutein play a central role in PS II, they harvesting B LED and transfer energy to photosystem reaction centres, at the same time that protects the photosynthetic apparatus against photo-oxidative damage caused by deactivating ROS [31, 33].

In this study *Gloeothece* sp. carotenoids production was strongly affected by the light source, as can be seen from Figure 2.5. Xanthophylls, such as neoxanthin, violoxanthin, lutein and β-carotene, were quantified along time under the different light spectrum conditions. All of them exhibit two peaks of production, the first in the early exponential phase (from day 1 to 13) and another in the stationary phase (from day 13 to 25), as observed in Figure 2.5. This may be due the stressful conditions that cells are submitted to in said stages. In the exponential phase, cells are at a very low density, and thus very exposed to light – so they trigger their secondary metabolism, i.e. carotenoids production, in order to stabilize the cell structure and aid in the function of photosynthetic complex.

Conversely, the culture attains a state of nutrients starvation in the stationary phase, and the cell density reaches such values that cells self-shading areas inside the culture vessel increase, so cells stresses for light harvesting. Consequently, cells responded again by increasing carotenoids production in attempt to improve light harvesting [19, 34]. This behaviour is fairly typical, and may explain the presence of a peak in carotenoids production in the plain stationary phase – as perceived in Figure 2.5 around 20 –25th day of culture.

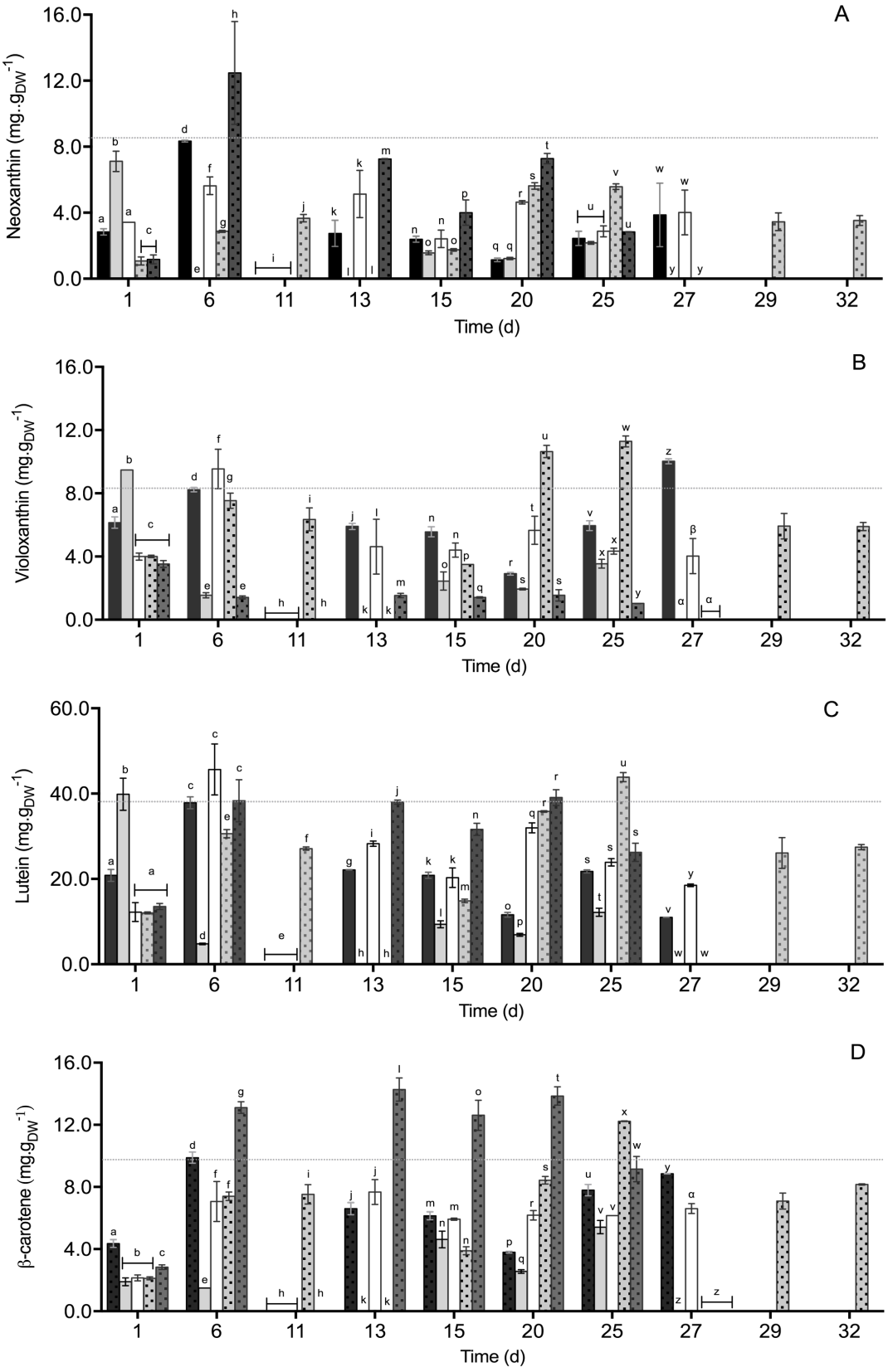

Figure 2.5. Variation of selected carotenoids production (average ± standard deviation) by *Gloeothece* sp. (**A**) Neoxanthin, (**B**) Violoxanthin, (**C**) Lutein and (**D**) β- carotene, with incubation time; under the different light sources (n = 3): ■ FL(fluorescent light) ■ B (blue LED), ■ R (red LED), ■ BR (Blue:Red LED) (50:50) and ■ BR (Blue:Red LED) (40:60). Maximum carotenoids production attained in exponential phase under fluorescent lamps is marked (with a line) for comparison with use of LEDs. Different lowercase letters in bars for the same time show statistically significant differences ($p < 0.05$) between averages.

Since one of main goals of this study was to assess whether the use of LED can replace FL in cyanobacteria production, the maximum content of carotenoids at the exponential phase under FL is marked to facilitate comparison of results. Observing Figure 2.5, it is possible to witness that *Gloeothece* sp. culture – under monochromatic or dichromatic LED, attained a higher production of carotenoids than under FL. Furthermore, R LED(as expected) seems to play an important role upon all carotenoids production, either as monochromatic LED – particularly on violoxanthin (9.54 ± 1.24 $mg.g_{DW}^{-1}$) and lutein (45.66 ± 5.98 $mg.g_{DW}^{-1}$) at day 6; or in conjugation with 40% B LED in production of neoxanthin (12.5 ± 3.2 $mg.g_{DW}^{-1}$) at day 6 and lutein (38.31 ± 4.92 $mg.g_{DW}^{-1}$) at day 6. As detected in plants (although not been fully studied), the mechanism of action of the monochromatic R LED may affect terpenoid production (the basis molecule of carotenoids) in the chloroplast through phytochrome activation [35]. In particular, the dichromatic conjugation of RB (40:60) seems to induce synthesis of β-carotene (from 6 to 20th day, between 9.06 ± 1.12 and 14.27 ± 0.75 $mg.g_{DW}^{-1}$), as observed before in *D*. *salina* [31]; and additional R or B LED caused stress, by activating the xanthophyll cycle – although B light is less stressful than R light. Production of violoxanthin attained values as high as those produced under R LED ($p < 0.05$), but under dichromatic RB (50:50) in plain stationary phase, at day 20 (10.63 ± 0.39 $mg.g_{DW}^{-1}$) and day 25 (11.27 ± 0.35 $mg.g_{DW}^{-1}$) ($p < 0.05$).

As observed on growth under monochromatic LED, there is not a consensus on which light spectrum is more suitable to improve carotenoids production. Some studies claim that B LED induces production of astaxhantin in *Haematococcus pluvialis* [36]; others show production of a larger pool of xanthophylls and higher Chl *a* content compared to R LEDs, at low light intensities, in the case of *Phaeodactylum tricornutum* [37]; still others invoked a higher carotenoid/Chl ratio under R LED when compared to B or G LEDs in *Botryococcus braunii* Bot-144 cultures [38]. Other authors refer that β-carotene and lutein accumulation is increased when R light is supplemented with B in *Dunaliella salina* [31] – in agreement with our results. A justification of such phenomena lies on an analogy with plants, they have different photoreceptors/domains – some B light and others R light-regulated, and thus B light signal transduction might be different from R light one. However, these photoreceptors could overlap and thus distinct functions may explain disparate responses [39].

Effects of light source on PUFA production

Interest in cyanobacterial fatty acids has emerged in many fields in recent years, for their potential for therapeutic uses or nutritional applications – e.g. omega 3 and 6 like C18:3 n3 (α-

linolenic acid, ALA), C18:2 n6 (Linoleic acid, LA), C18:3 n6 (ϒ -linolenic acid, GLA), or even omega 9 C 18:1 n9 (Oleic acid, OA) [18].

Light is one of the key factors that affects fatty acids (FA) production by photosynthetic microorganims, so it can be used as a tool to enhance production and increase the potential of cyanobacteria exploitation [26, 40].

In an attempt to ascertain how light spectrum affects fatty acids cyanobacterial content, in all light conditions, the biomass was collected and freeze-dried when the culture reached the 4th day of the stationary phase. Fatty acid methyl esters (FAMES) were then generated and quantified by GC-FID, and the main results are depicted in Figure 2.6. In a general way, under FL, *Gloeothece* sp. cultures have a higher neutral lipid content. However, comparing only the LED conditions tested under the monochromatic B LED, this cyanobacterium produces more fatty acids relative to other LEDs tested – e.g. 1.7-fold more C16:0 (34.26 ± 3.08 $mg_{FA}.g_{DW}^{-1}$) and 1.9-fold more C18:2 n6 *trans* (0.21 ± 0.01 $mg.g_{DW}^{-1}$) than dichromatic LEDs, and 1.6-fold more C18:3 n3 (24.31 ± 3.58 $mg.g_{DW}^{-1}$) than all other LEDs tested ($p < 0.05$).

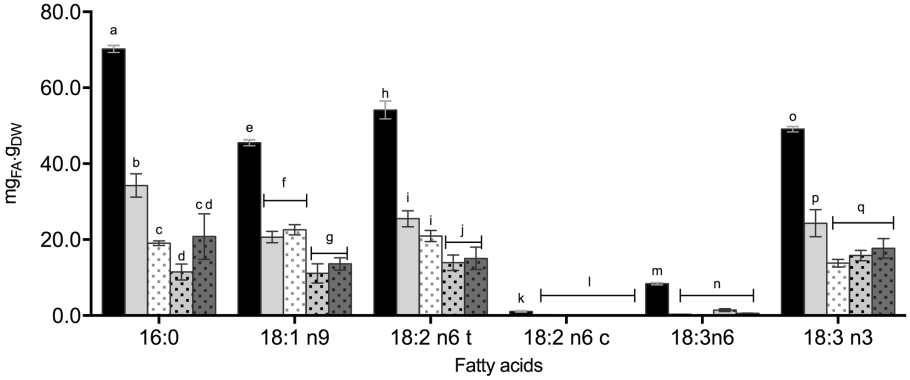

Figure 2.6. Fatty acids production of *Gloeothece* sp. under the different light sources ($n = 9$) FL (fluorescent light), B (blue LED), R (red LED), BR (Blue:Red LED) (50:50) and BR (Blue:Red LED) (40:60), at stationary phase. Different lowercase letters in bars for the same FA show statistically significant differences ($p < 0.05$) between averages.

These effects of B LED were observed before in *Tetraselmis* sp. and in *Nannochloropsis* sp. by Teo et al. [26]. Yoshioka et al. [41] also found that *Isochrysis galbana* attained maximum lipid content under B LED, probably because in microalgae the enzymes affecting the carbon dioxide rates are mostly under control of B light. Therefore, under B light, higher the enzyme activity is associated with an increased accumulation of triglycerides [42].

Effects of light source on antioxidant capacity of intracellular extracts

Antioxidant compounds have received in recent decades a growing interest in the market due to their several roles: e.g. in human health as therapeutic aids (i.e. prevention or

control of several diseases), as an ingredient in functional food, or even as food preservative [18].

Hydrogen peroxide (H_2O_2) is the most common oxidative product of photosynthesis, photorespiration, respiration and other metabolic processes in plants, microalgae and cyanobacteria. Therefore, light may contribute to the increase of cyanobacterial oxidative stress for being a result of the photosynthetic process. Hence, production of antioxidant is triggered to scavenge free radicals, chelate catalytic metals and act as oxygen scavengers. Some cyanobacteria contain several enzymatic and non-enzymatic antioxidant protection systems to constrain the concentration of ROS, in attempts to protect themselves from damage. Compounds like phenolic acids, tocopherols, terpenoids, alkaloids, phycobilin pigments and carotenoids were accordingly described to overcome said harmful effects and restore intracellular equilibrium [18, 19, 43].

To evaluate the effect of light spectrum on antioxidant compounds production, for each light condition assay, samples were taken along time as biological triplicates, and AC was assayed by $ABTS^{\bullet+}$ assay. Upon inspection of the results in Figure 2.7, it is possible to notice that light spectrum has a significant effect on the AC. Again, for easier comparison of results, the maximum AC of *Gloeothece* sp. in the exponential phase under FL is marked. *Gloeothece* sp. under R LED attained a better AC, particularly in days 8, 18 and 20, with a maximum of 2.95 ± 0.14 $mg_{TE}.g_{DW}^{-1}$. Higher results ($p < 0.05$) than under FL were as well found when using dichromatic LED illumination in days 4, 18, 25 and 32 at BR (50:50) – with values between 2.27 ± 0.08 and 2.73 ± 0.08 $mg_{TE}.g_{DW}^{-1}$, and days 8, 18 and 20 days under BR (40:60), 2.34 ± 0.11 and 2.44 ± 0.18 $mg_{TE}.g_{DW}^{-1}$, respectively. Recalling Figure 2.4, it is possible to conclude that these days correspond to the exponential phase (days 4 and 8) and the stationary phase (days 18, 20, 25 and 32). As seen before in section 2.3.2, cells are under stress in these two growth phases, either due the excess or limitation of light energy; hence, an internal cell oxidative stress may enhance the antioxidant compound mechanism of production in attempts to restore oxidative equilibrium. Under B LED, *Gloeothece* sp. production of antioxidant compound exhibited a different behaviour compared to other light conditions; besides its content being lower, it exhibited an almost constant production profile along time, with an average of 1.25 ± 0.12 $mg_{TE}.g_{DW}^{-1}$ – with the exception of the first and last day of cultivation, with 2.31 ± 0.11 and 1.72 ± 0.06 $mg_{TE}.g_{DW}^{-1}$, respectively. Another point worthy of notice is the very low values of AC under R and BR (40:60) until the 4^{th} day of culture, probably because it takes more time to the culture to respond to these light conditions. The same did not happen in BR (50:50), so a higher ratio between BR may induce more stress, and more AC with consequently be produced at start-up.

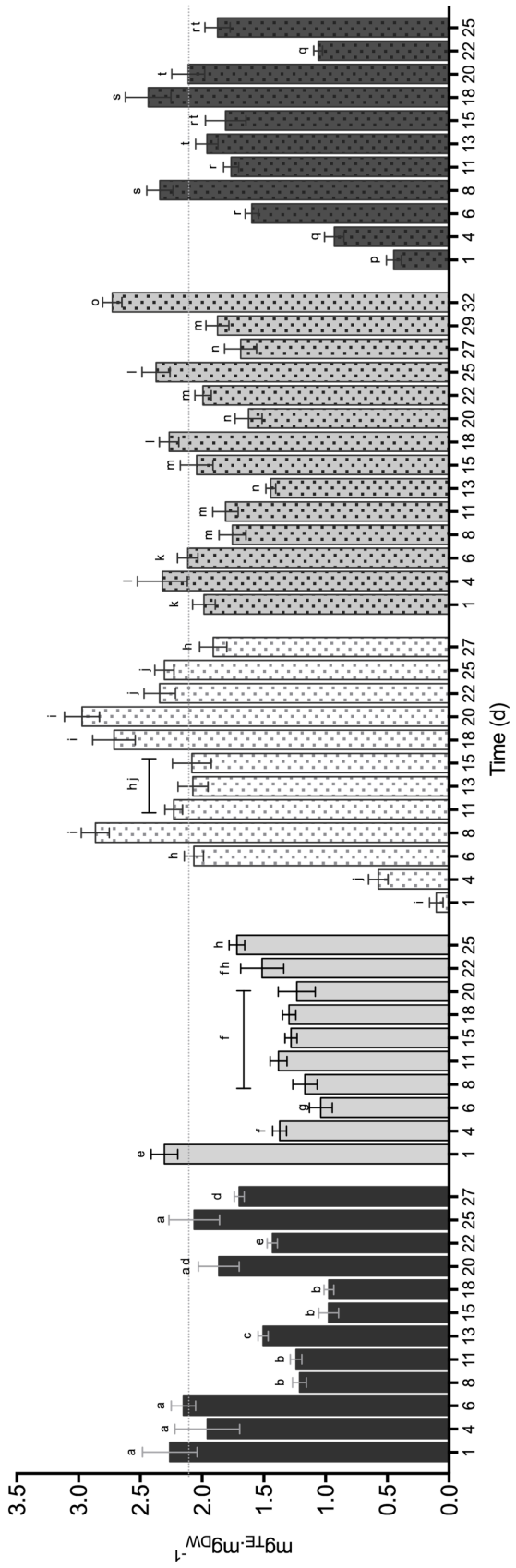

Figure 2.7. Variation of the specific antioxidant capacity (average ± standard deviation) of *Gloeothece* sp. extracts (mg$_{TE}$.mg$_{DW}^{-1}$) with incubation time and under different light sources (n = 9) FL (fluorescent light), B (blue LED), R (red LED), BR (Blue:Red LED) (50:50) and BR (Blue:Red LED) (40:60). Different lowercase letters in bars for the same time show statistically significant differences ($p < 0.05$) between averages.

Among their several functions in light harvesting, carotenoids contribute to cell structure stabilization by neutralizing ROS and dissipating excess energy. As observed before (Figure 2.5), lutein is the major carotenoid produced by *Gloeothece* sp.. In attempts to consubstantiate the antioxidant properties of the *Gloeothece* sp., HPLC analyses of its intracellular extract were performed; the contents in lutein, overlaid with the antioxidant power produced during the same period of time, are shown in Figure 2.8.

Higher lutein contents were in fact recorded at the same time, with higher antioxidant power (namely on FL and BR (50:50) – Figures 2.8A and D) – which suggests that the antioxidant capacity could be due in part to this compound; as described before in *Scenedesmus obliquus* [19]. However, under R LED (Figure 2.8C) and slightly in B (Figure 2.8B) and BR (40:60) (Figure 2.8E), a weaker relation was found; despite the similarity between profiles. They present different times for peak production, thus leading to the conclusion that other compounds besides carotenoids (namely lutein), bearing AC, are synthesized. For example, phycocyanin, under R LED, as observed long ago in *Synechococcus* sp. by Tanako et al. [44], or phenolics compounds observed in lettuce leaves [45].

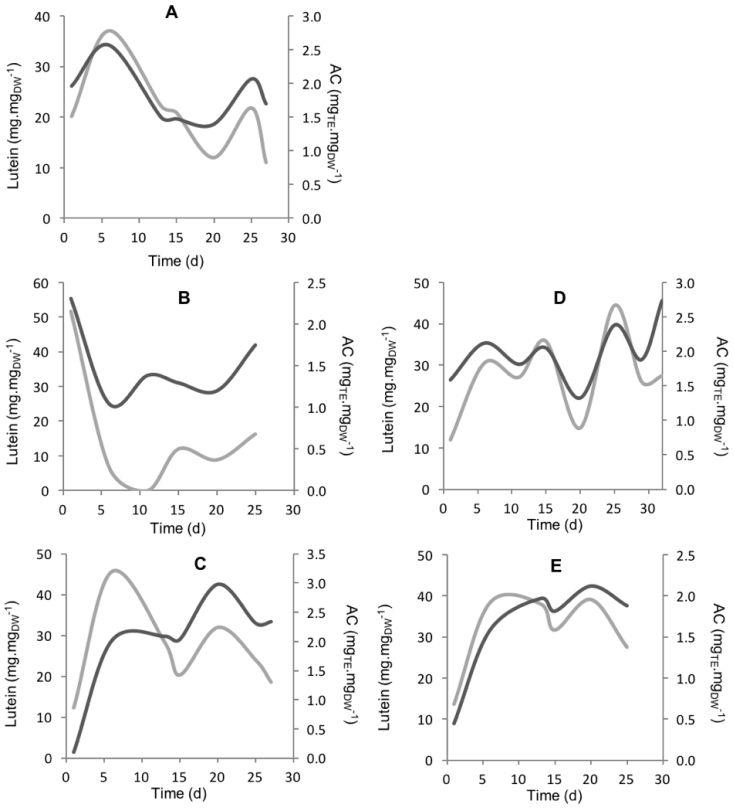

Figure 2.8. Variation of the specific antioxidant capacity (—) (average ± standard deviation) of *Gloeothece* sp. extracts, (expressed as mg_{TE}. mg_{DW}^{-1}), and variation of the lutein production (—) (average ± standard deviation) of *Gloeothece* sp. (expressed as mg.mg_{DW}^{-1}), with incubation time, under different light conditions (**A**) FL (fluorescent light) (**B**) B (blue LED), (**C**) R (red LED), (**D**) BR (Blue:Red LED) (50:50), (**E**) BR (Blue:Red LED) (40:60).

2.3.3. Influence of infrared (IR) LED on the cyanobacterium growth and biochemical composition

As seen before, only the visible range of light spectrum is photosynthetically active. Up to now, the farthest wavelengths studied on photosynthetic organisms were in the range of far-red (630-750 nm); beside its low energy, it seems to exert some effects on *D. bardawil* [11]. Far red photons appeared to induce high growth rates and smaller cells, by accelerating the cell cycle in many microalgae of diverse evolutionary lines. They can suppress volumetric biomass production when supplementing a broadband light source, because they regulate light-harvesting mechanisms [2, 11].

However, effects of IR (750-1000 nm) on photosynthetic performance have remained quite unexplored. A few studies reported on the ability of a photosynthetic bacterium, *Rhodopseudomonas capsulate,* to harvest monochromatic light at 860 nm; photons are apparently absorbed by bacteriochlorophyll, a pigment with a higher affinity for light than carotenoids [1]. More recently, it was found that the cyanobacterium *Acaryochloris marina* is the only known prototroph harbouring Chl *d* – which permits a good adaptation to growth under both visible and near infrared irradiance [46].

In this study, *Gloeothece* sp. growth under LEDs proved that this light source can be a good alternative to fluorescent lighting; it indeed promotes growth and increases carotenoids and antioxidant production, particularly under monochromatic R or dichromatic BR (40:60) LEDs. Hence, the effect of an extra near IR (800-900 with a peak at 862 nm) was tested, when added to the aforementioned LEDs – upon *Gloeothece* sp. growth, carotenoids, AC and FA production.

2.3.3.1. Influence of IR LED on *Gloeothece* sp. growth

IR caused different effects when coupled with different LEDs – as per observation of Figure 2.9. When combined with R, the P_X in DW decreased 4.0-fold and t_d increased 2.4-fold. When added to BR (40:60), P_X was enhanced 2.0-fold and t_d decreased 2.0-fold. However, IR light produced an increase in μ_{max} in conjugation with BR (40:60), i.e. 1.9-fold, as already observed with cyanobacterium *A. marina* [46]. Therefore, IR may be used in conjugation with BR (40:60) to enhance biomass production. The other values for all the biomass parameters are shown on Table 2.3.

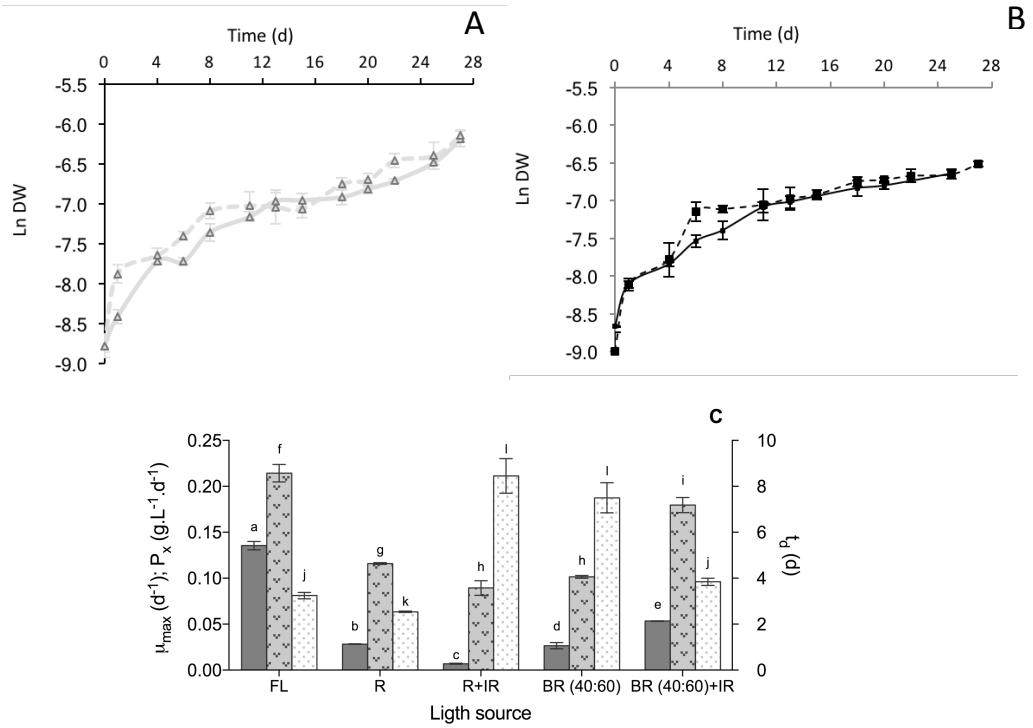

Figure 2.9. Variation of biomass concentration (average ± standard deviation) of *Gloeothece* sp., expressed as natural logarithm of dry weight, Ln DW, with incubation time, at several light condition (n = 6) (**A**) R (red LED), R+IR (red LED + infrared), (**B**) BR (Blue:Red LED) (40:60), BR (40:60)+IR (Blue:Red LED + infrared). (C) Characteristic growth parameters (average ± standard deviation), viz. Biomass productivity (P_X), Specific growth rate (μ_{max}) and Duplication time (td), for each light condition. Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

Table 2.3. Biomass parameters calculated (average ±standard deviation) on the exponential phase for the different light conditions (n=3).

Light Quality	Exponential Phase (d)	μ_{max} (d^{-1})	P_x ($g.L^{-1}d^{-1}$)	t_d (d)
FL	1 - 11	0.14 ± 0.01^{a}	0.21 ± 0.02^{f}	3.24 ± 0.24^{j}
R	1 - 8	0.28 ± 0.00^{b}	0.12 ± 0.00^{g}	5.99 ± 0.08^{k}
R + IR	1 to 11	0.01 ± 0.00^{c}	0.01 ± 0.01^{h}	8.45 ± 1.30^{l}
BR (40:60)	1 - 11	0.03 ± 0.00^{d}	0.10 ± 0.00^{h}	7.50 ± 1.14^{l}
BR + IR	1 to 6	0.05 ± 0.00^{e}	0.18 ± 0.01^{i}	3.84 ± 0.22^{j}

Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

2.3.3.2. Influence of IR LED on *Gloeothece* sp. biochemical composition

Recent results are scarce about the influence of IR on carotenoids production; and the farthest wavelength tested was FR radiation, which proved to significantly increase carotenoids content in *Dunaniella bardawil* [11]. The effect of IR on each carotenoids production along time was tested in conjugation with R and BR (40:60), as depicted in Figure 2.10. Unlike *D. bardawil* with IR (860 nm) provoked a decrease of *Gloeothece* sp. carotenoids production when conjugated with R or BR LEDs. The profile of production of all carotenoids was different when IR was added; both R+IR and BR+IR only had a peak of production in the plain exponential phase, at days 6 and 15 (for R+IR) and in the plain exponential phase, day 25 (for BR+IR).

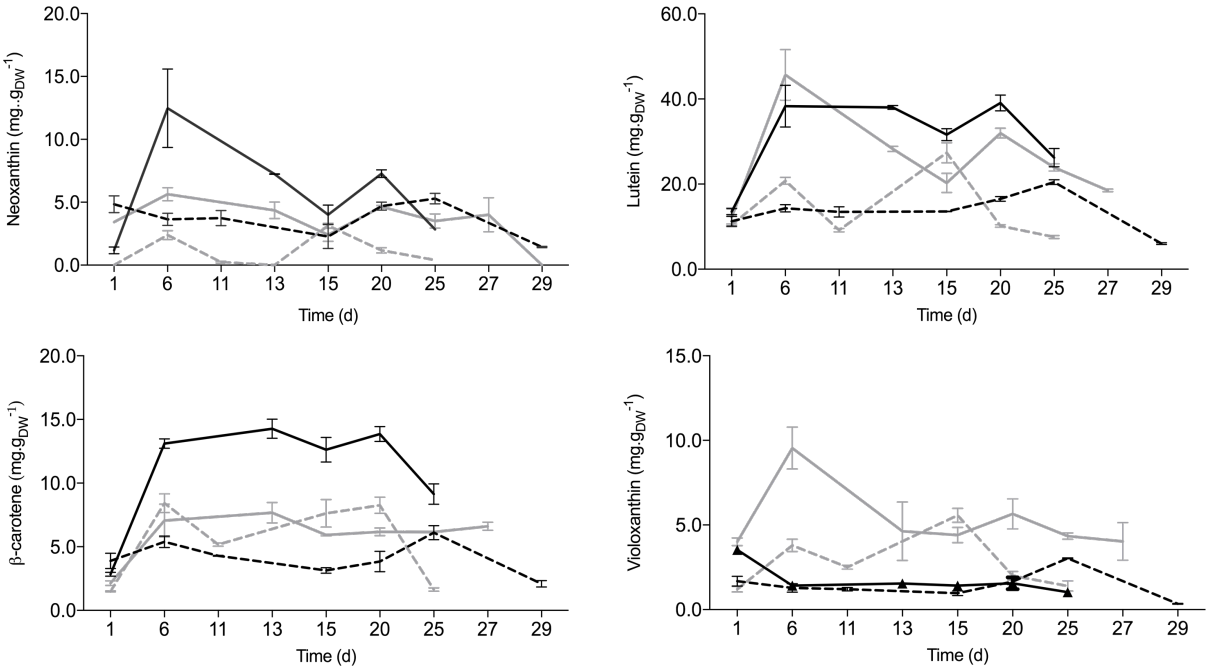

Figure 2.10. Effect of infrared (IR) LED on carotenoids production (average ± standard deviation) of *Gloeothece* sp., with incubation time, when conjugated with the R (red LED); R+IR and BR (Blue:Red LED) (40:60), BR (40:60) + IR.

However, it was observed that IR affects differently the production of fatty acids, as depicted in Figure 2.11. When this radiation is added to R or BR in *Gloeothece* sp. cultures, the lipid production is increased, but this phenomenon is more pronounced in conjugation with BR – where it increases (on average) 1.7-fold each FA production. On the other hand, the effect of IR when added to R LED is statistically significant ($p < 0.05$) in terms of increase on C16:0, C18:3n3 and C18:3n6. Note that IR had an apparently significant role in the stimulation of production particularly of C18:3 n6.

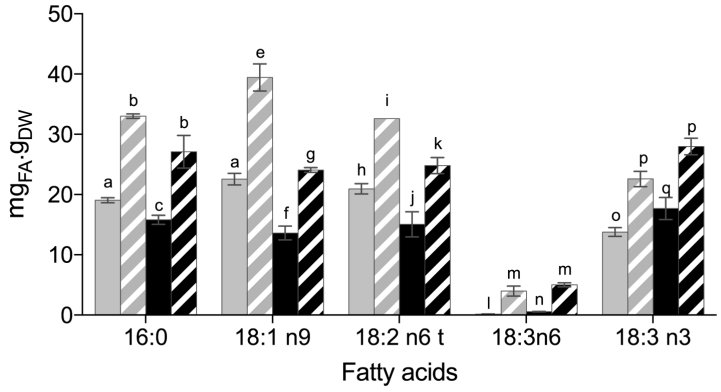

Figure 2.11. Effect of infrared (IR) LED on fatty acids production (average ± standard deviation) of *Gloeothece* sp., under R (Red LED) and BR (40:60) (Blue:Red LED) ($n = 9$), on stationary phase.s R, R+IR BR (40:60) and BR+IR (40:60). Bars for same fatty acid without a common superscript, are significantly different ($p < 0.05$).

In terms of AC production (Figure 2.12), R+IR and BR (40:60)+IR presented a different profile of production along time when compared to their counterparts without IR. In conjugation

with R LED, IR seems to increase their production in the stationary phase. Together with BR, IR induced an apparently constant production of AC only at day 4 and 25, corresponding to the plain exponential and stationary phases of *Gloeothece* sp. growth, respectively. Unlike previous results, encompassing single monochromatic or dichromatic B and/or R illumination, it was not possible to correlate carotenoids to AC production when IR is added, although a peak of production of carotenoids and AC arose by 25th day of cultivation under BR+IR. These findings indicate that IR may induce production of other AC than carotenoids.

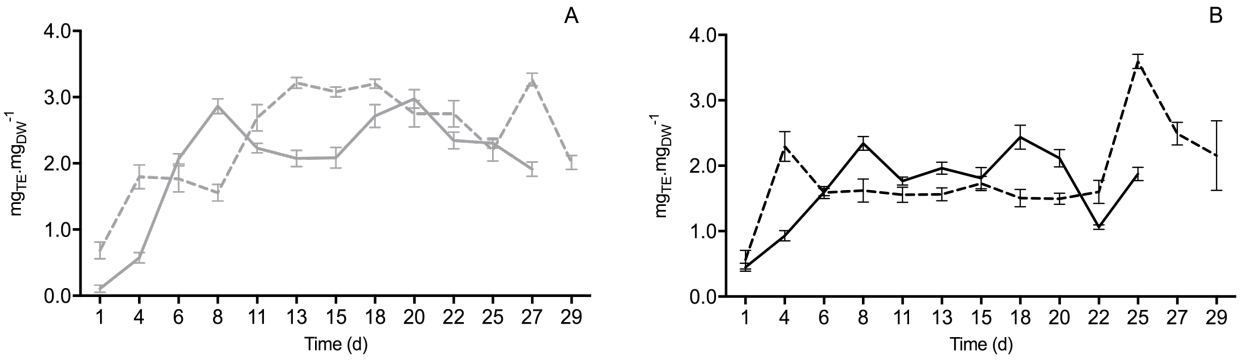

Figure 2.12. Effect of infrared (IR) LED on the antioxidant capacity (average ± standard deviation) of *Gloeothece* sp. extracts with incubation time (*n* = 9), under R (Red LED) and BR (40:60) (Blue:Red LED). R; R+IR and BR (40:60), BR+IR (40:60).

2.4. Conclusion

LEDs appear promising as light source alternative toward biomass and metabolites production by *Gloeothece* sp. For biomass production, the B LED is the most appropriate; once the cyanobacterium grow faster than under FL, and accumulates a higher content in FA than in the other LEDs tested. When using BR (40:60) LED, it is possible to obtain biomass rich in carotenoids, particularly lutein and β-carotene, besides AC; however, only a slightly higher content in antioxidants was obtained with R, although its P_X was lower.

When added to BR (40:60), IR LEDs enhanced biomass production as well as fatty acids content. On the other hand, the IR seems to change carotenoids profile and even reduce it production, as well as AC.

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Chapter 3

Effect of light intensity on *Gloeothece* sp. biomass and high value-metabolite production

3.1. Introduction

As already mentioned, the compounds produced by cyanobacteria have a great importance to industry, mainly due to the already know bioactivity related to them [1]. Once cyanobacteria are photosynthetic organisms, under photoautotrophic conditions, light exerts a crucial role, mainly in terms of quality or intensity. Furthermore, these organisms exhibit adaptive responses to oxidative stresses, via stimulation of their antioxidant defence system [2], that consists of both enzymatic and non-enzymatic mechanisms: superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase are key enzymes in the former, whereas the non-enzymatic counterpart includes such mediator compounds as, carotenoids, polyphenols, peptides and PUFAs [3, 4]. These compounds have different roles being usually associated to vital metabolism or to stresses adaptations mechanisms in the referred microorganisms [5, 6].

The light intensity represents the amount of energy given to the environment, on the case of a controlled production to the culture. When photosynthetic organisms are exposed to optimal luminous intensity values, biomass productivity increases. However, when the availability of light intensity is extremely high or low, these organisms may present different mechanisms and strategies to optimize the rate of photosynthesis and growth [7]. For example, it is known that, in a low light intensity treatment, the amount of light actually available to the cells is affected by mutual shading [7, 8] due to the lack of light; this affects negatively both their growth rate and biochemical composition. On the other hand, excess of light can cause photoinhibition, thus wasting energy and promoting cell death. Therefore, assessment of the light available for photosynthesis throughout culture time is an important step toward accurate control of light intensity [9], for best use thereof.

The amount of light used by the photosynthetic organism varies according to it species and culture concentration, however, once the light quantity reaches the saturation limit, this organism won't be able to use the additional limit, wasting the extra light. Nonetheless, on large scale cultures, the culture depth and density may cause shading to itself, and an higher light intensity may allow the better intake of energy to the production [10].

This chapter has as main objective to verify the influence of light intensity on the production of biomass and high-value compounds from *Gloeothece* sp., in order to point the metabolic changes and evaluate the possible applications for its bioactive compounds.

3.2. Material and Methods

3.2.1. Cyanobacterium source and growth conditions

As already described on Chapter 2, *Gloeothece* sp. (ATCC 27152) obtained from ATCC (American Type Culture Collection, USA), was maintained at 25 ºC in Blue Green (BG11) medium [11]. A pre-inoculum was cultivated for 10 days, with an initial optical density of 0.1 at 680 nm, in 800 mL of BG11, set at pH 8 buffered with Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) 25 mM. A continuous illumination with fluorescent Osram BIOLUX lamps with intensity of 100 $\mu mol_{photon.}m^{-2.}s^{-1}$ was assured, as well as air bubbling at a flow rate of 0.5 $L.min^{-1}$.

Light conditions assays, conducted in batch biological triplicates, were performed in 1800 mL of culture, also with an initial optical inoculum density of 0.1 at 680 nm. Medium, bubbling conditions and continuous illumination were the same as with the pre-inoculum, except for the light source.

3.2.2. Light emission conditions

Monochromatic R light (peak at 660 nm) and dichromatic BR (40:60) - (peaks at 440 nm and 660 nm) were selected, based on the results obtained previously, in Chapter 2. In a way to understand the influence of light intensity on *Gloeothece* sp. production, four different intensities were tested: 50, 100, 150 and 200 $\mu mol_{photon.}m^{-2.}s^{-1}$, measured with a spherical light sensor WALZ (US-SQS/L), equipped with a logger WALZ (ULM-500).

The cyanobacterium growth assays were performed in climate chambers 600 S (Aralab), equipped with removable LED panels containing B and R LEDs. In each assay, a uniform light distribution was provided by spot lights, as well as by establishing the correct distance of cultures to the light source.

3.2.3. Biomass quantification

For each biological triplicate, cultivated under the light conditions mentioned above, biomass growth was monitored (in duplicate) along time (at days 0, 1, 4, 6, 8, 11, 15 and 18) by DW; this means n = 6 pseudo-replicates were considered for each set of experimental conditions. The DW was ascertained by filtering a certain volume of culture through preconditioned GF/C glass fiber filters (Whatman), and further drying at 100 ºC till constant weight. The specific growth rate (μ_{max}) was also found by a numerical regression of experimental data, and the biomass doubling time (t_d) was calculated as $t_d = (\ln 2)/\mu_{max}$. Biomass productivity (P_X) was discovered using the variation between initial and final values of DW ($g.L^{-1}$) obtained in the exponential phase (EP), $P_X=(DW_f-DW_i)/\Delta d_{EP}$, referred to the underlying exponential phase period, according toP_x (t)

= X_0 [(exp $^{(\mu max\ t)}$ -1)/ t], where t denotes the actual sampling time and X_0 the initial biomass concentration at start-up.

3.2.3. Effects of light source on the biochemical composition

Protein content quantification

Protein content was quantified by bicinchoninic acid (BCA) based in the PierceTM BCA Protein Assay Kit (Thermo Scientific, Rockford, USA), using bovine serum albumin as standard. The results are expressed as mg of protein per DW of biomass $mg.g_{DW}^{-1}$.The assays were performed spectrophotometrically in a FLUOstar Omega from BMG LABTECH and the extracts tested were obtained as follows: 1.5 mL of each batch (in triplicate) was centrifuged, at 4,000 rpm for 5 min, and the pellet was re-suspended and homogenized in 1.5 mL of a mixture of ethanol and water (1:1, v/v). Cells were then crushed in 5 cycles of 30 seconds, with a rest time between cycles of 10 seconds in a Precellys® Evolution cell homogeneizer (Bertin Corp., Rockville, USA).

Phenolic content quantification

Quantification of total phenols was performed by the spectrophotometric Folin-Ciocalteu method described elsewhere [12]. The extracts tested were obtained by the same method mentioned above. Gallic acid was used as reference, and the results are expressed as Gallic acid equivalents (GAE) per DW of biomass $mg_{GAE}.g_{DW}^{-1}$.

Determination of fatty acid profile

By the end of each light condition experiment, biomass triplicates were harvested, by sedimentation and then centrifuged at 4000 rpm for 5 min, prior to lyophilisation.

Freeze-dried samples (ca. 45 mg) were transmethylated *in situ* with methanol/acetyl chloride (95:5), as described elsewhere [13, 14]. The undecanoic acid (C11:0) was used as internal standard, in the form of triglyceride (triundecanoin, Larodan, USA).

Gas chromatographic analysis was performed in an Agilent 7890A gas chromatograph, with FID detection, equipped with a Select FAME (50 m; 0.25 mm ID; Agilent, USA) and using Helium as carrier. The temperature program started at 80 ºC (1'), with a 20 ºC/min increase up to 160 ºC (0'), a 1 $ºC.min^{-1}$ increase up to 198 ºC and a later 5 $ºC.min^{-1}$ increase to 250 ºC (5') for elution of retained contaminants, in a total of 58 minutes. The injection was performed in the split mode (1:10; 1 µL) at 250 ºC, with the detector at 300 ºC.

Identification was achieved by comparison with authentic commercial standards from diverse suppliers (Sigma-Aldrich, Supelco, Larodan and Nu-Check). Quantification was based on

a previous calibration of the detectors response using a certified reference mixture (Supelco 37 FAME mix) followed by direct quantification on the basis of the internal standard added. Results are expressed as $mg_{FA}.g_{DW}^{-1}$.

3.2.4. Antioxidant capacity assessment

The antioxidant scavenging activity was ascertained via three different assays for total activity: two synthetics ($DPPH^{\bullet}$ and $ABTS^{+\bullet}$), and one that measure the biological reactive specie (ORAC−FL). The extracts tested were obtained by the same method mentioned above (section 3.2.3).

The radical-scavenging capacity of the intracellular extracts was evaluated, in triplicate, via the ABTS radical cation ($ABTS^{\bullet+}$) assay, following the method described by Guedes et al. [4, 15]; via the DPPH radical ($DPPH^{\bullet}$) assay following the method as described by Ferreres et al. [16]; and via the ORAC assay, following the method described by Dávalos et al. [17].

For quantification, a calibration curve using a known antioxidant – Trolox, was established, so antioxidant capacity was expressed as TE per DW of biomass $mgTE.g_{DW}^{-1}$.

3.2.5. Statistical analysis

Obtained data were analysed using GraphPad Prism V. 7.0. Firstly, a Shapiro-Wilk test of normality was done, and then a one-way analysis of variance (ANOVA), if normal distribution of the residuals was confirmed. For mean comparison, Tukey's multi-comparison test was used to assess variances between different light conditions on the several parameters. Two-way ANOVA with the same multi-comparison test was employed to found differences between protein e phenolic compounds content, FA composition and AC. Since each datum point had been replicated, a representative measure of variability was available in all cases to support said statistical analyses.

3.3. Results and Discussion

3.3.1. Effects of light intensity on biomass production

Light is one of the most important factors to take in account on a cyanobacteria production, and the amount of energy is limiting to the growth rate and metabolites accumulation of photosynthetic organisms. Also, it is know that light intensity changes the production of specific metabolism in these organisms, shaping the biochemical profile and the metabolism itself [10, 18]. In order to examine the effect of light intensity upon growth of *Gloeothece* sp., the biomass was quantified by DW, under all conditions light intensity considered for both, R and BR (40:60).

The variation of biomass during all experiments – and represented in a logarithmic scale versus incubation time, is depicted in Figure 3.1A and 3.1B. Results were grouped by type of LED R and BR (40:60), so as to facilitate comparative interpretation, and consequent detection of trends. The biomass increased exponentially with incubation time until it reached a plateau (as expected); the time required to reach that status was a function of both quality and intensity of light.

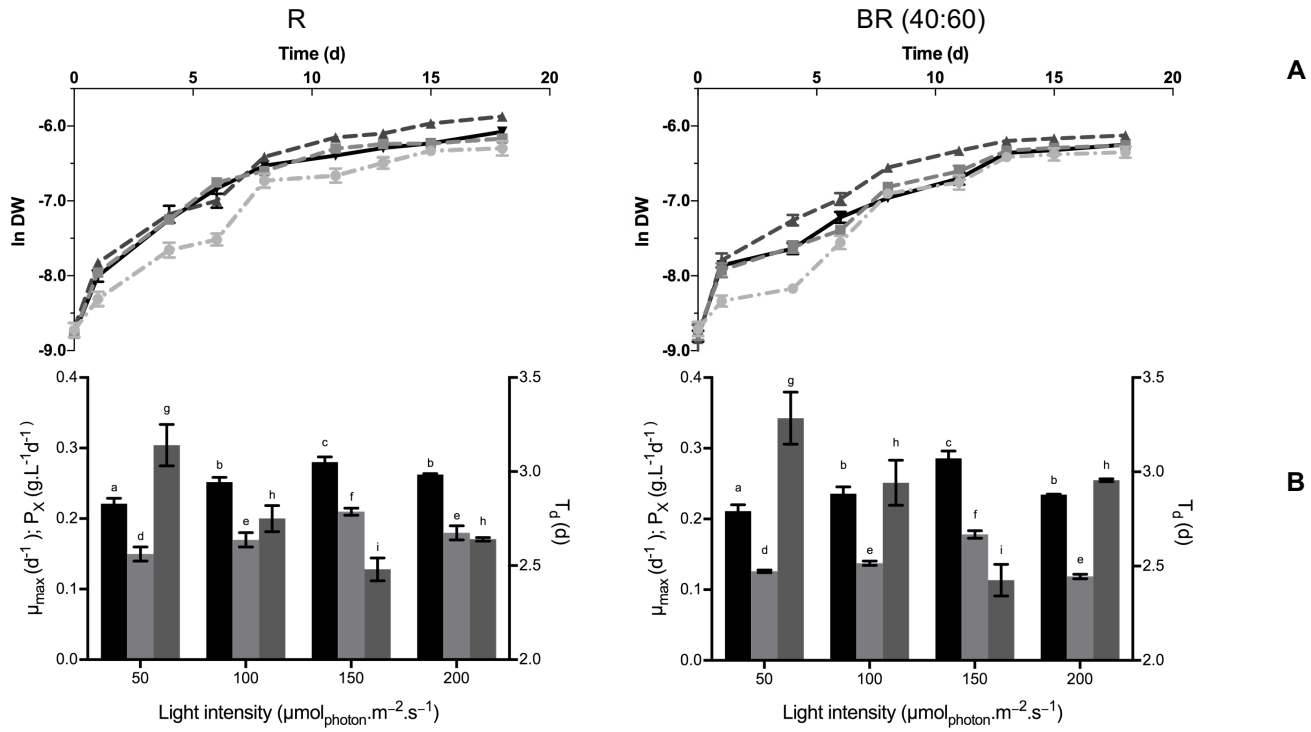

Figure 3.1. (A) Variation of biomass concentrations (average ±standard deviation) of *Gloeothece* sp., expressed as natural logarithm of dry weight, Ln (DW), with incubation time, for each light intensity (*n*= 6) 50 100 150 and 200 µmol$_{photon}$.m^{-2}.s^{-1}, to R (Red LED) and BR (40:60) (Blue:Red LED); and (B) Characteristic growth parameters (average ± standard deviation), viz. Specific growth rate (μ_{max}), Biomass productivity (P_X) and Duplication time (t_d) to each light intensity (*n*= 3), to R LED and BR (40:60) LED. Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

The cyanobacterium *Gloeothece* sp. exhibited similar behaviours under different light conditions in terms of production of biomass along time (Figure 3.1A) with an exponential phase from day 1 to day 8, and a stationary phase from day 11. However, the parameters measured for biomass, showed a pattern on the different intensities. As shown on Figure 3.1B, both R and BR (40:60) treatments had better growth in a 150 µmol$_{photon}$.m^{-2}.s^{-1} intensity, with the higher specific growth rate (μ_{max}) – 0.28 ± 0.01 and 0.29 ± 0.01 d^{-1}, respectively. From the two different light qualities, R induced the higher P_x – 0.21 ± 0.01 g.L^{-1}.d^{-1} while the mixture BR (40:60) induces to the shorter duplication time (t_d) – 2.42 ± 0.08 d^{-1}($p < 0.05$). The other values for all the parameters are shown on Table 3.1.

Table 3.1. Biomass parameters calculated (average ±standard deviation) on the exponential phase (from day 1 to 8) for the different light conditions(*n*=3).

Light Quality	Light Intensity ($\mu mol_{photon}.m^{-2}.s^{-1}$)	μ_{max} (d^{-1})	P_x ($g.L^{-1}.d^{-1}$)	T_d (d)
R	50	0.22 ± 0.01^{a}	0.15 ± 0.01^{d}	3.14 ± 0.11^{g}
	100	0.24 ± 0.01^{b}	0.17 ± 0.01^{e}	2.75 ± 0.07^{h}
	150	0.28 ± 0.01^{c}	0.21 ± 0.01^{f}	2.48 ± 0.06^{i}
	200	0.26 ± 0.00^{b}	0.18 ± 0.01^{e}	2.64 ± 0.01^{g}
BR (40:60)	50	0.21 ± 0.01^{a}	0.13 ± 0.00^{d}	3.28 ± 0.14^{g}
	100	0.24 ± 0.01^{b}	0.14 ± 0.00^{e}	2.94 ± 0.12^{h}
	150	0.29 ± 0.01^{c}	0.18 ± 0.01^{f}	2.42 ± 0.08^{i}
	200	0.23 ± 0.00^{b}	0.12 ± 0.00^{e}	2.95 ± 0.01^{h}

Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

It is noticeable that in 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, the cultures start to decrease the productivity, reaching values in the same order of magnitude as in 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ – possibly due to a photoinhibition; and that in 50 $\mu mol_{photon}.m^{-2}.s^{-1}$the culture shown the lower production than the other intensities, maybe due to the lack of light.

The growth profile observed in this study follows the found in other ones. For example, Wang et al. [19] found that *Spirulina platensis* had a better growth rate in higher intensities, and that in low light intensity the amounts of biomass were similar to the dark treatment. The low light intensity may lead to a lower productivity, because the organism consumed more oxygen during photorespiration leading to a high oxidative stress [20, 21].

However, from a certain amount of light, an excess of light intensity may damage or kill the organism due to an overcharge on the photosystem [21]. This was also found by several studies [20-23], which define a “moderate intensity range” as optimal condition to growth.

3.3.2 Effects of light source on the biochemical composition

It is known that variations in light conditions alter not only the growth and productivity of the culture, but also the biochemical composition of the organism. In order to evaluate the influence of light intensity, different groups of chemical compounds with recognized bioactive capacity were quantified, specifically, phenols, proteins, FA and carotenoids.

Effects of light source on the protein content

Becker [24] says that photosynthetic microorganism can be a high quality source of protein; comparable and even superior to conventional plant proteins.

In this study, for almost all intensities, the protein content was constant over time (Figure 3.2). However, for BR (40:60) LED at 50 $\mu mol_{photon}.m^{-2}.s^{-1}$, there was a variation of the protein

content over time, i.e. in the first 11 days, the protein content was below the method detection limit, probably due to the low cellular concentration (see Figure 3.1A). Furthermore, at BR (40:60) LED 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ some variation in protein content was observed over time, probably due to the stress caused by the excess of light. So, as said before, protein content of all R LED intensities studied was constant, in that other, the protein average from different days at the same light intensity, thus there were no statistical significant differences ($p > 0.05$). The same was verified for BR (40:60) LED between 100 and 150 $\mu mol_{photon}.m^{-2}.s^{-1}$.

Although quantification of total proteins was not enough to observe the effects of light on the protein content, it is possible that there are differences in terms of composition, since the organism, under stress situations, tends to produce antioxidant enzymes [3]. This result was consistent to other studies, e.g. in the case of *Isochrysis* sp. and *N. oculata* that under different light intensities, had the same protein content [25].

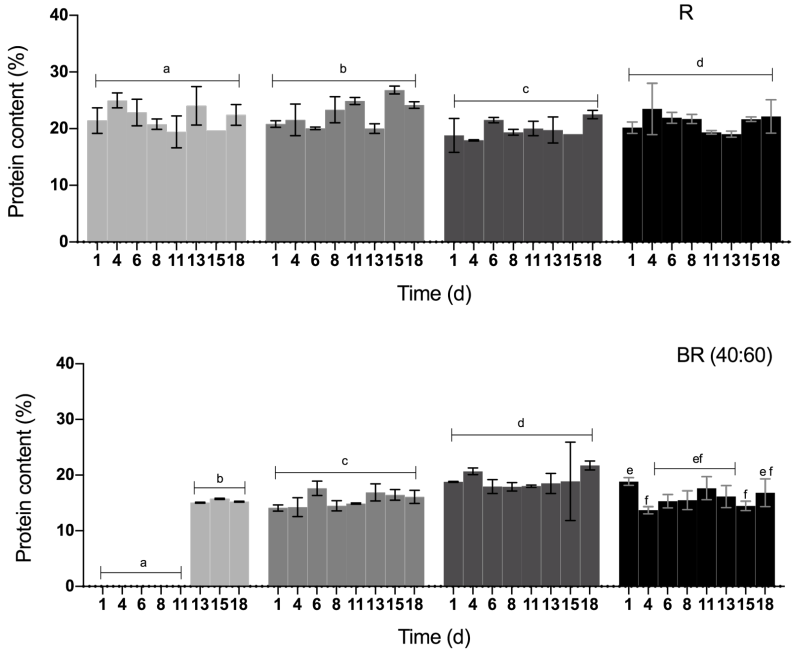

Figure 3.2. Variation of protein content (average ± standard deviation) of *Gloeothece* sp. with incubation time, to each light intensity (*n*=9) 50, 100, 150 and 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, to R (Red LED) and BR (40:60) (Blue:Red LED). Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

Effects of light source on the production of phenolic compounds

Phenolic compounds are well known as great natural antioxidants, more specifically, polyphenols act as antioxidant through single electron transfer and hydrogen atom transfer.

In cyanobacteria and microalgae, studies with an approach to polyphenolic components are rare, but some studies showed that several classes of flavonoids, such as isoflavones,

flavanones, flavonols, and dihydrochalcones could also be found [26, 27]. The role of phenols in the photosynthetic microorganism is not clearly elucidated yet; however, Duval et al. [28] and Kováčik et al. [29] showed that the content of phenolic compounds increases upon exposure to UV-light, which suggests that they play a role in the antioxidative response to this type of stress. However, there is no consensus on the importance of phenolic constituents for the antioxidant capacity of these organisms [26].

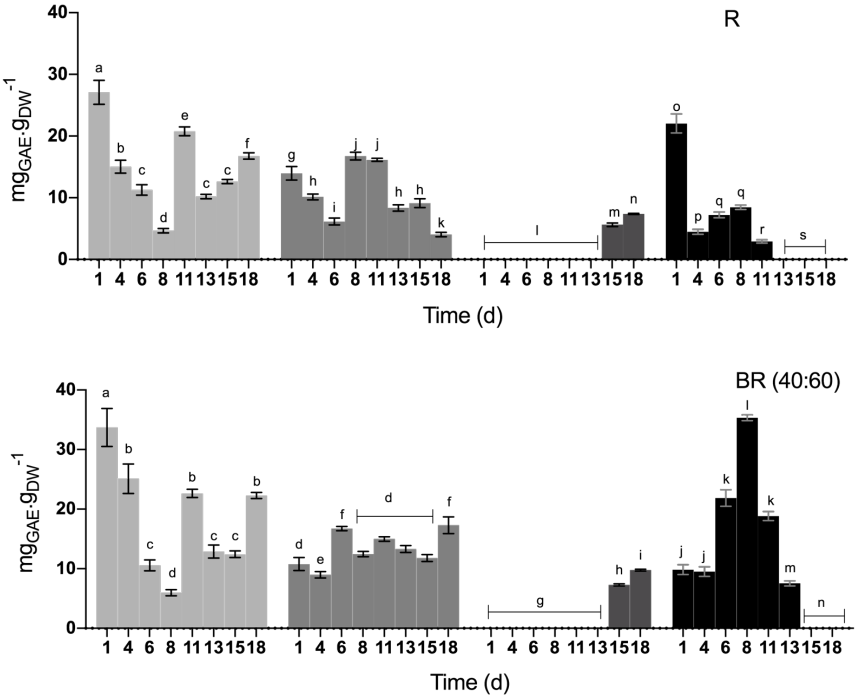

Figure 3.3. Variation of phenolic content (average ± standard deviation) of *Gloeothece* sp. with incubation time, to each light intensity (*n*=9) 50, 100, 150 and 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, to R (Red LED) and BR (40:60) (Blue:Red LED). Different lowercase letters in bars for the same parameters show statistically significant differences ($p < 0.05$) between averages.

In *Gloeothece* sp., the production of phenolic compounds (Figure 3.3), that may have a role in cells of protection against oxidative stress, was higher on both extremes of light intensities range tested (low and high). Under BR (40:60) LED, the higher content ascertained was 35.35 ± 0.49 $mg_{GAE}.g_{DW}^{-1}$ at the day 8th of the culture under 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ when compared to other intensities ($p < 0.05$), however, under 50 $\mu mol_{photon}.m^{-2}.s^{-1}$the culture showed a high production during all the production time. Under R LED, the culture showed higher results both in 50 and 100 $\mu mol_{photon}.m^{-2}.s^{-1}$, and at 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, only on the first day of culture showed a higher production – what may mean a stressful adaptation from the start of production.

The lower content was found in 150 $\mu mol_{photon}.m^{-2}.s^{-1}$ compare to other intensities ($p < 0.05$), that doesn't even show any detectable amount until 15 days of culture, this can be explained once that intensity provides higher growth, and it is culture condition that causes the

less stress. Also, it is notable the increase of the phenols production with the addiction of B LED to the culture, what means that BR (40:60) LED induced to a higher production of phenolic compounds than R LED ($p < 0.05$). The production profile is not constant, and it varies both between intensities and between different qualities.

Effects of light intensity on FAs production

Unlike the majority of higher plants, where ALA is the predominant constituent of thylakoid lipids, microalgae and cyanobacteria show a great diversity in their FA composition [30]. The role of FA, specially PUFAs, as antioxidant compounds is also already evidenced in human aortic endothelial cells, where the supplementation with omega 3 PUFAs resulted in lower formation of ROS, as compared with cells supplemented with omega 6 [31]. Therefore, as great producer of PUFAs, these organisms appear as promising organisms to show the antioxidant capacity of this class of compounds [32].

The main results for the FA quantification, to the four different light intensities studied and for the two different kinds of LEDs (R and BR (40:60)), are depicted in Table 3.2. As already referred, the FA quantifications it was be done at the end of each light condition experiment, in the stationary phase. For total FA, R light was better than BR light, and the best intensity was 150 $\mu mol_{photon}.m^{-2}.s^{-1}$ (94.38 ± 3.03 $mg_{FA}.g_{DW}^{-1}$) ($p < 0.05$).

More specifically for PUFAs, R LED increased the production, at the intensity of 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, ($53.69 \pm 2.37$ $mg_{FA}.g_{DW}^{-1}$). While under BR light, the intensities of 100, 150 and 200 are significantly identical. The production of PUFAs in high intensities may cause because these compounds are related to the protection against photo-oxidation [33].

Moreover, in both R and BR conditions, the higher intensities seem to increase the production of some fatty acids. In R LED C18:1n9 (OA) is higher in 150 $\mu mol_{photon}.m^{-2}.s^{-1}$ (25.9 ± 1.4 $mg_{FA}.g_{DW}^{-1}$) and under both light qualities the C18:3n3 (ALA) is higher in 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ (24.9 ± 1.8 $mg_{FA}.g_{DW}^{-1}$ to R and 28.6 ± 3.5 $mg_{FA}.g_{DW}^{-1}$ to BR (40:60)). It was also observed that R light induces the production of C18:2n6cc (LA) more than BR LED, but with no significant difference between intensities ($p>0.05$).

It is also notable the presence of C28:0 in the samples on every condition. This unusual FA have been identified in some microalgae [34-36]; and also, diverse aquatic animals have been found to contain these unusual FA [36]. Long chain FA may be derived from the elongation and desaturation of shorter chain fatty acids in some higher aquatic organism. Also, the production of C28:0 may be due to inhibition of chain shortening (β -oxidation) resulting from the stress of nutrient limitation [36].

Table 3.2. Effect of light intensity on fatty acid production (average ± standard deviation) by *Gloeothece* sp. grown under the different light qualities R (R LED) and BR (40:60) (Blue:Red LED).

Fatty Acid	Amount of Fatty Acid ($mg_{FA}.g_{DW}^{-1}$)							
	R Intensities ($\mu mol_{photon}.m^{-2}.s^{-1}$)				BR (40:60) Intensities ($\mu mol_{photon}.m^{-2}.s^{-1}$)			
	50	100	150	200	50	100	150	200
C 16:0	18.09 ±0.92^{ab}	16.13 ± 0.91^{b}	19.47 ± 1.06^{a}	18.14 ± 0.52^{a}	17.52± 0.62^{a}	16.15 ± 1.64^{a}	17.71 ± 1.92^{a}	15.81 ± 1.14^{a}
C18:1n9c	22.12 ±1.87^{a}	14.15 ± 1.24^{b}	25.96 ± 1.45^{c}	12.78 ± 1.41^{b}	14.49 ± 1.14^{a}	9.20 ± 0.76^{b}	14.78 ± 3.85^{a}	8.30 ± 1.42^{b}
C18:1n7c	2.30 ±0.19^{a}	0.90 ±0.08^{b}	2.18 ± 0.15^{a}	0.97 ± 0.03^{b}	1.84 ± 0.18^{a}	0.73 ± 0.04^{b}	2.32 ± 0.18^{a}	0.70 ± 0.07^{b}
C18:2n6cc	23.59 ±2.22^{a}	23.07 ± 2.17^{a}	22.09 ± 0.67^{a}	23.02 ± 0.72^{a}	19.03 ± 0.47^{a}	17.70 ± 1.67^{ab}	16.52 ± 1.67^{ab}	14.63 ± 1.18^{b}
C18:3n6	3.48 ±0.21^{a}	2.23 ±0.22^{b}	3.32 ± 0.30^{a}	2.27 ± 0.25^{b}	2.19 ± 0.09^{a}	1.83 ± 0.16^{a}	1.75 ± 0.28^{a}	1.65 ± 0.23^{a}
C18:3n3	11.29 ± 0.61^{a}	16.87± 0.99^{b}	13.54 ± 0.87^{c}	24.91 ± 1.88^{d}	15.30 ± 1.74^{a}	23.12 ± 1.18^{b}	20.80 ± 1.27^{b}	28.59 ± 3.53^{c}
C18:4c + C20:1n9	1.95 ± 0.17^{a}	2.18 ±0.13^{a}	3.16 ± 0.30^{b}	3.13 ± 0.17^{b}	2.26 ± 0.17^{a}	3.29 ± 0.14^{b}	3.76 ± 0.17^{b}	4.84 ± 0.65^{c}
C20:3n6	0.05 ± 0.02^{a}	0.04 ±0.01^{a}	0.04 ± 0.02^{a}	0.03 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
C22:2	0.03 ± 0.02^{a}	0.03 ±0.01^{a}	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}	0.13 ± 0.03^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
C28:0	1.20 ± 0.11^{a}	0.96 ±0.24^{a}	0.99 ± 0.16^{a}	1.01 ± 0.11^{a}	0.65 ± 0.19^{a}	0.58 ± 0.04^{a}	0.61 ± 0.13^{a}	0.49 ± 0.05^{a}
Σ SFAs	23.33 ± 1.38^{a}	21.43 ± 1.69^{a}	24.68 ± 1.48^{a}	24.01 ± 0.65^{a}	22.24 ± 1.08^{a}	20.99 ± 1.84^{a}	22.69 ± 2.62^{a}	20.35 ± 1.40^{a}
Σ MUFAs	29.53 ± 2.72^{a}	18.26 ± 1.52^{b}	31.90 ± 1.66^{a}	16.68 ± 1.35^{b}	21.60 ± 1.65^{a}	12.65 ± 0.97^{bc}	20.73 ± 4.16^{ab}	11.37 ± 1.38^{c}
Σ PUFAs	40.74 ± 2.25^{a}	44.69 ± 2.85^{a}	42.62 ± 1.95^{a}	53.69 ± 2.37^{b}	39.21 ± 2.09^{a}	47.48 ± 3.46^{ab}	43.26 ± 1.87^{ab}	50.02 ± 5.54^{b}
Σ FA	93.72 ± 3.54^{ab}	84.38 ± 5.83^{b}	99.20 ± 2.96^{a}	94.38 ± 3.03^{a}	83.06 ± 1.95^{a}	82.39 ± 7.11^{a}	86.68 ± 8.19^{a}	81.75 ± 6.37^{a}

Different lowercase letters in a row for each light quality (R or BR (40:60)) show statistically significant differences ($p < 0.05$) between averages (n= 12).

3.3.4. Effects of light intensity on antioxidant capacity of intracellular extracts

As observed in the previous chapter, the AC presented for R and BR (40:60) could be due to compounds other than carotenoids, due to the lack of correlation between the results obtained for lutein and AC. In order to elucidate the AC of the culture grown under these conditions, it was extended the range of AC assays to be able to assess other compounds that could be influencing the AC.

Thus, different methods were used to assess the antioxidant capacity of the extract of *Gloeothece* sp. in a way to avoid a misinterpretation of the total antiradical capacity of extracts, once the methods have different sensibility to different compounds. $ABTS^{+\bullet}$ and $DPPH^{\bullet}$, the most used assays, have radicals that are foreign to biological systems. Comparatively, $ABTS^{+\bullet}$ is more sensible to carotenoids, while $DPPH^{\bullet}$ may be more sensitive to phenolic antioxidants and PUFAs [32]. Finally the ORAC assay measures the scavenging activity of a compound against peroxyl radicals, that is the most abundant radicals in biological systems [17] – with special emphasis to peptides and phenolic compounds [37].

The AC profile is not constant along time (Figure 3.4), and the culture has a greater AC on the exponential phase (days 1 to 8), probably due to a bigger oxidative stress on this phase. Also, it seems to be a trend to the response to the intensity, once both R and BR (40:60) LED showed similar trend to each light intensity.

The cultures grown at the intensity of 50 $\mu mol_{photon}.m^{-2}.s^{-1}$ showed the best AC for ABTS and DPPH assays when compared to other intensities ($p < 0.05$), with two peaks of production, one at day 4, on the exponential phase, where for R LED, the values to ABTS and DPPH were 12.15 ± 0.15 and 5.04 ± 0.58 $mg_{ET}g_{DW}^{-1}$ respectively; while for BR LED were 19.19 ± 0.68 $mg_{ET}g_{DW}^{-1}$ and 11.44 ± 0.94 $mg_{ET}g_{DW}^{-1}$. The second peak was already on the stationary phase, but all values were significantly lower than the first peak ($p < 0.05$).

Moreover, at the intensity 100 $\mu mol_{photon}.m^{-2}.s^{-1}$, both R and BR (40:60) have the peak of AC are restricted to the exponential phase. However, specifically to ORAC assay, R LED seemed to be the best condition, with a constant AC with a medium value of 43.86 ± 4.81 $mg_{TE}g_{DW}^{-1}$. As already seen (section 3.3.2), the low intensities induced a higher production of phenolic compounds, what may explain the higher AC in these cultures. Thus, under the intensity of 150 $\mu mol_{photon}.m^{-2}.s^{-1}$, the values of AC are the lowest, that occurs probably due to this intensity being the optimal for the *Gloeothece* sp. growth.

Finally, the intensity of 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, the cultures has also showed high and constant values for ORAC assay, but not significantly higher than the already mentioned R LED 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ ($p > 0.05$), not being worthy the use of a high intensity for this purpose.

In general, 50 and 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ seemed to be the best intensities to produce antioxidant compounds, BR light was better at the ABTS and DPPH assays and R LED at the ORAC assay.

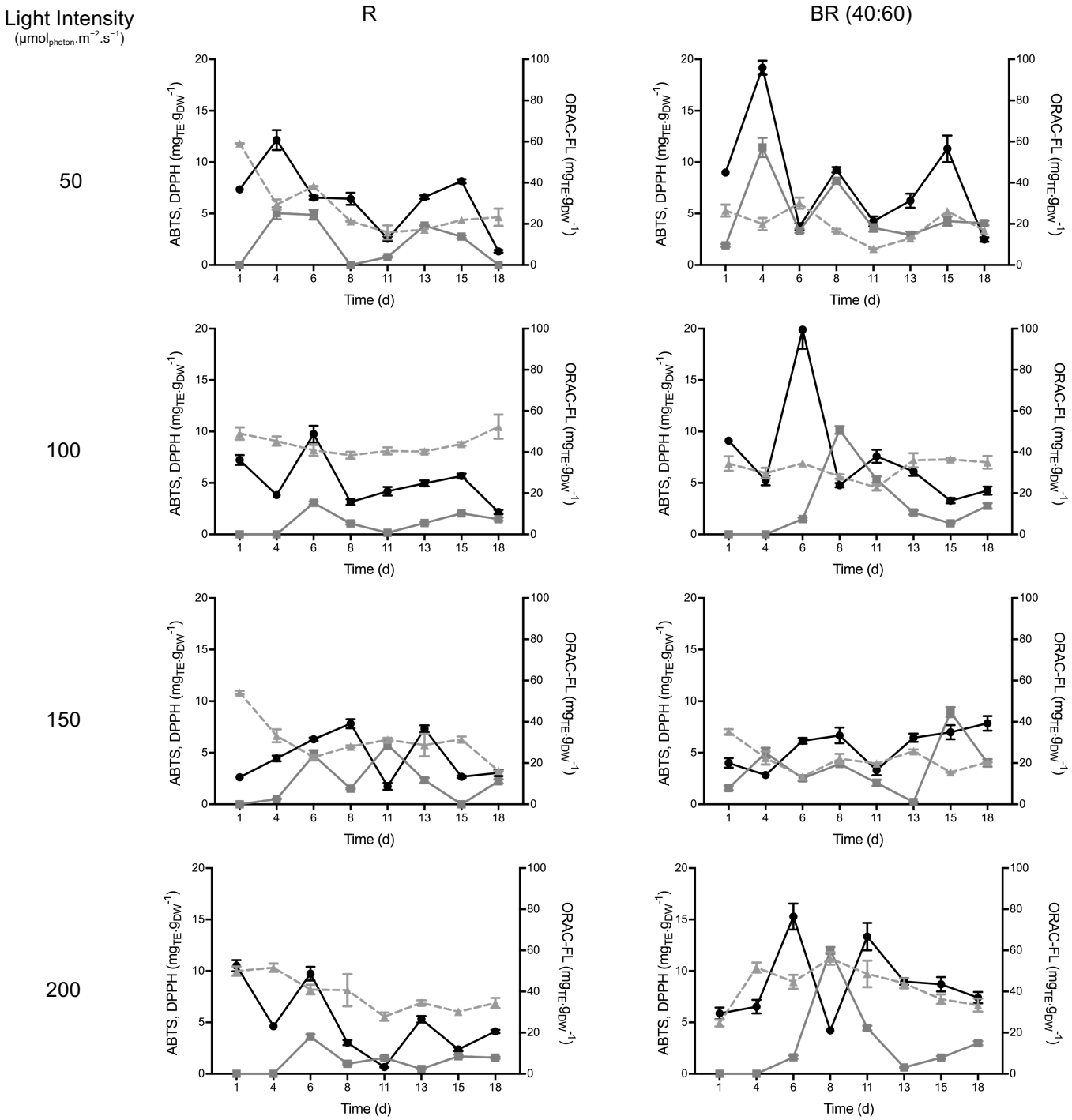

Figure 3.4. Variation of antioxidant capacity (average ± standard deviation) with incubation time, of the extracts of *Gloeothece* sp. grown at the different light intensities and qualities treatments R (Red LED) and BR (40:60) (Blue:Red LED) for the different antioxidant assays (n= 9): ABTS, DPPH and ORAC.

3.4. Conclusions

Light intensity represents a key factor to cyanobacterial production either for biomass or bioactive compounds. In this study it was found that for biomass production, the use of R LED at an intensity of 150 $\mu mol_{photon}.m^{-2}.s^{-1}$ showed to be the most appropriate; and in the opposite, higher intensity of 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ showed to promotes culture photoinhibition.

In terms of total FA, R LED was better than BR (40:60) LED, also in MUFA and PUFA production. Regarding to the light intensity, 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ was the optimal condition to promote production of PUFAs, particularly under R LED. Also, this light intensity also was favourable to produce more specifically C18:3n3 (ALA), in both light qualities.

At last, regarding the AC, 50, 100 and 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ seemed to be the best intensity, on both light qualities, to induce the production of compounds with AC. Under BR (40:60) LED the culture of *Gloeothece* sp. exhibited better results in total antioxidant capacity achieved by ABTS and DPPH assays than under R LED, although, in ORAC assay, cultures under R LED showed the best results.

Annotation: The characterization of the carotenoid content is intended, to the different conditions tested above in this chapter, so the procedure will be carried out shortly, according to the method presented in Chapter 2.

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Chapter 4

Concluding remarks and Future perspectives

4.1. General Discussion

The biotechnology of cyanobacteria has gained considerable importance in last years, with a wide range of potential applications – from simple biomass production for food and feed to valuable products for pharmaceutical/nutraceutical uses.

As discussed along this dissertation, light quality interferes with many metabolic mechanisms (including obviously photosynthesis), and may stimulate the production of bioactive compounds under certain narrow wavelengths. Note that the utilization of a light source that emits wavelengths beyond the range of radiation harvested by the photosynthetic organisms means a waste of energy, and undesirable heat production when powering the most common forms of culture lightning – – fluorescent light (FL). LED technology is more common nowadays, and their advantages to large scale production are well seen – it is small enough to fit into virtually any photobioreactor, holds a longer life-expectancy, reduces heat generation and enhances conversion efficiency. The use of its narrow light emission spectra is also applicable to the target and specific metabolite production.

Albeit several studies made available in recent years, that effects of light are species-dependent (as discussed on Chapter 1); a thorough study of such effects upon synthesis of carotenoids and PUFA by our elected specie is thus essential for eventual further exploitation at large scale.

For *Gloeothece* sp. biomass production, B LED is the most appropriate – and growth is even faster than under FL. B LED, in comparison to other LEDs tested, also provides higher cellular content of FA. If the goal is to obtain *Gloeothece* sp. biomass rich in carotenoids, BR (40:60) and R LEDs are the most indicated; they particularly enhance the content of lutein and β-carotene, and concomitantly the AC.

An attempt to better understand the role of IR LED was also pursued; this LED was tested only in addition to BR (40:60) and R, once they proved to be the most promising regarding AC. Together with BR (40:60), it enhanced biomass production, as well as FA content – while it induced changes in AC profile of production along time, yet reaching similar maximum concentrations.

Finally, regarding the light intensity, it was observed similar responses on both BR and R LEDs, with the optimal condition for biomass production at 150 $\mu mol_{photon}.m^{-2}.s^{-1}$, while for phenolic compounds, the low intensities are greater. Also, light intensity doesn't seem to induce a response at the protein content, however, it might have on the composition, once light stress induces the production of specific enzymes.

In the case of FAs composition, it is notable that 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ increased the production of C18:3n3 (ALA), in both light qualities. On the other hand, the optimal condition for AC was at 100 $\mu mol_{photon}.m^{-2}.s^{-1}$.

Besides being a promising alternative to FL, particularly B, R and BR (40:60), LEDs have proven a useful tool to improve (desired) metabolite concentration.

4.2. Concluding Remarks

The work developed in this dissertation was part of an effort to find the best light conditions, either in terms of quality and intensity, for bioactive compounds production by *Gloeothece* sp. The main conclusions drawn may be summarized as follows:

- Light quality, use of B, R and/or a combination thereof BR (40:60) LEDs for *Gloeothece* sp. production:
    I) B LED enhanced biomass productivity,
    II) R and BR (40:60) LEDs enhanced intracellular concentration of carotenoids.
    III) R and BR (40:60) LEDs improved AC

- Light quality, with addition of IR LED on *Gloeothece* sp. production:
    I) the extra addition IR to R and BR (40:60) LEDs enhanced carotenoid and PUFAs concentrations.

- Light intensity, its influence on *Gloeothece* sp. production:
    I) an intensity of 150 $\mu mol_{photon}.m^{-2}.s^{-1}$ of R LED enhanced biomass productivity.
    II) an intensity of 50, 100 and 200 $\mu mol_{photon}.m^{-2}.s^{-1}$ provided the best condition for AC compounds production on both light qualities (R and BR (40:60)).
    III) at an intensity of 200 $\mu mol_{photon}.m^{-2}.s^{-1}$, R LED increased the production of PUFAs.
    IV) Both phenolic and PUFA content might be related to the AC, once the conditions of best production of this compounds are the ones with better AC.

- As major conclusion, it can be said that the best conditions for the production of biomass and bioactive compounds for *Gloeothece* sp. are Red and Blue:Red (40:60), at the intensity of 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ once at this intensity, the cyanobacterium would have the greatest production with the lowest energetic cost.

4.3. Future Perspectives

Current implementation of production systems of cyanobacteria-based lipidic compounds has been economically constrained by poor volumetric efficiency, that leads to

excessively high costs. Technological improvements of such processes are thus critical and will require a multiple approach at production, extraction and purification levels.

For future works, a more comprehensive understanding of the involved biochemical pathways could be a starting point to a more rational operational strategy. That should improve the bioprocess competitiveness, being also evaluated the influence of light on transcription changes on genes related to carotenoids synthesis.

Moreover, other classes of compounds should be studied in more detail – e.g. peptides and phenolic compounds. Some studies have also showed a relation between peptides and antioxidant activities in some marine organisms. However, for cyanobacteria only a few studies have carried out to target on bioactive peptides and some more remaining until being explored.

Since the responses are species-dependent, an increase in studies of different species under different conditions is required to cover as many situations as possible. Wavelengths corresponding to R and B lights are the major factor affecting photosynthesis and other metabolic pathways; however, only two combinations of said spectrum bands were considered. Therefore, a study of the influence of other combinations of such bands may be useful toward understanding and optimizing production of carotenoids and PUFA. Since the influence of IR spectrum on said compound production was found to be favourable, further efforts should be developed to study its influence upon the metabolism. Also, other monochromatic LEDs might be studied, such as G, Y and V, as seen on Chapter 1, these light qualities also induce responses to light that lead to the enhancement of high value products.

Regarding the continuation of the bioprocess, extraction costs of cyanobacteria intracellular metabolites remain high; the downstream separation stages often account for 50%–80% of the total production costs, depending on the biochemical characteristics of the target metabolite and purity required for the intended use – thus limiting commercial exploitation. Also, due to the variability of cyanobacterial metabolites, there is no standard method for extraction and purification of the bioactive extracts.

For the extraction, legislative restrictions require the removal of traces of toxic solvents. Electro-technologies are gaining considerable interest for several biotechnological applications. Ohmic Heating due to the presence of a Moderate Electrical Field and different ways of delivering electric energy at high (> 25 kHz) or low electrical frequency (< 50 kHz), assembles a great potential for the extraction of bioactive and valuable compounds from biological matrices, such as cyanobacteria. The presence of an electric field may cause permeation of cellular tissues allowing an enhanced and selective extraction of bioactive compounds. The selection of the proper extraction conditions to recover compounds such as

pigments, carbohydrates, proteins and lipids can be very challenging due to the intrinsic nature of the cyanobacteria cell walls, which can limit the mass transfer through it.

Therefore, there is an urgent need to combine appropriate, quick, selective, cost-effective, and environment-friendly extraction procedures abiding to legal requirements, including the use of food-grade solvents, and processes that allow their incorporation in food and health industries.

In parallel, the nutraceutical applications of cyanobacteria extracts should be deepened. The optimization of the extract should be based on a series of biological activities of the extracts, in particular, anti-inflammatory, antihypertensive and anti-tumour. These assays may allow a better understanding on the applications and value of the cyanobacteria extract. Also, the synergetic effects of the compounds may be responsible to the bioactivity and skipping the purification step, and the using of an extract as a final product may increase the bioactivity and at the same time decrease the cost of the production.

Finally, the objective of increasing the process of production to an industrial scale should be improved, since this is the best way to contribute and meet a demand to society.