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Investigating the effects of temperature on the physiology and production of commercially relevant biomolecules in *Rhabdoderma lineare*.

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biotecnologia do Programa de Pós-Graduação em Biotecnologia da Universidade Federal de São Carlos.

Orientador:

Profa. Dra. Ana Teresa Lombardi

São Carlos - SP

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Abstract

Cyanobacteria are unicellular, prokaryotic, photosynthetic organisms of vital importance to the functioning of the biosphere. The morphological, physiological, and metabolic diversity within this group accompanies its diverse range of applications. Large-scale cultures are often conducted in open systems subject to climate fluctuations, such as temperature increases. These, in turn, can affect the physiology of these organisms, impacting photosynthesis, biochemical composition, and growth. *Rhabdoderma lineare* is a common species in freshwater environments, characterized by high protein content (~75% of dry biomass) and absence of toxins, making it a potential candidate for biotechnological applications. Considering global temperature rise and the fact that a significant portion of commercial cultures occur in natural environments, understanding the physiological effects of different temperatures on this species is important. In this study, we standardized a protocol to obtain maximum photosynthetic yield in cyanobacteria (Chapter 1) and subsequently conducted cultures at 25, 30, and 35 °C, revealing significant differences in growth rate, photosynthetic yield, and the content of proteins, carbohydrates, lipids and pigments (Chapter 2). This research contributes to microalgae biotechnology by exploring the effects of temperature on *R. lineare*.

Keywords: Cyanobacteria, temperature stress, photosynthesis, Rhabdoderma lineare.

Resumo

As cianobactérias são organismos unicelulares, procariontes e fotossintéticos de importância vital para o funcionamento da biosfera. A diversidade morfológica, fisiológica e metabólica dentro deste grupo acompanha sua ampla gama de aplicações. Culturas em grande escala são frequentemente realizadas em sistemas abertos sujeitos a flutuações climáticas, como o aumento da temperatura. Estes, por sua vez, podem afetar a fisiologia desses organismos, impactando a fotossíntese, a composição bioquímica e o crescimento. *Rhabdoderma lineare* é uma espécie comum em ambientes de água doce, caracterizada por alto conteúdo de proteínas (~75% da biomassa seca) e ausência de toxinas, tornando-a uma candidata potencial para aplicações biotecnológicas. Considerando o aumento global da temperatura e o fato de que uma parte significativa das culturas comerciais ocorre em ambientes naturais, entender os efeitos fisiológicos de diferentes temperaturas sobre essa espécie é importante. Neste estudo, padronizamos um protocolo para obter o rendimento fotossintético máximo em cianobactérias (Capítulo 1) e, posteriormente, realizamos culturas a 25, 30 e 35 °C, revelando diferenças significativas na taxa de crescimento, no rendimento fotossintético e no conteúdo de proteínas, carboidratos, lipídios e pigmentos (Capítulo 2). Esta pesquisa contribui para a biotecnologia de microalgas, explorando os efeitos da temperatura sobre *R. lineare*.

Palavras-chave: Cianobactérias, stress termico, fotossíntese, *Rhabdoderma lineare*.

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Table 1. Results obtained in the validation of the Ogawa & Sonoike (2016) protocol with *Synechocystis* sp. and *Rhabdoderma lineare*. Fo' and Fm values result from cell acclimationto dark conditions, Fo from acclimationto weak blue light, Fm from white light with DCMU in the sample, f is the value calculated based on equation [2], and Fv/(Fm-f) is based on equation [3].

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LIST OF ACRONYMS AND ABBREVIATIONS

%DW – Dry biomass percentage

DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea

Fm – Maximum fluorescence

Fo – Minimum fluorescence

Fm' – Dissipated maximum fluorescence

Fo' – Dissipated minimum fluorescence

f – Basal fluorescence

Fv – (Fm-Fo)

Fv/Fm – Maximum photosynthetic quantum yield

PBS – Phycobilisomes

PSI – Photosystem I

PSII – Photosystem II

PQ – Plastoquinone

1. Introduction

Cyanobacteria is a group of photosynthesizing prokaryotic microorganisms essential for the functioning of the biosphere. They play an important role in the global oxygen supply, biological fixation of CO_2 and nitrogen, and primary biomass production, being considered one of the most ancientand diverse groups present today (Singh et al., 2005; Kumari & Rai, 2020).

The broad morphological, physiological, and metabolic diversity of this group allows its application in various areas, showing growth and CO_2 fixation rates higher than those of plants, and they may not require large areas of land for production (Wang et al., 2008). Biomass can be consumed as a dietary supplement, as it contains a high concentration of nutrients, including a high protein content (Geada et al., 2021; Mutale-Joan, Sbabou & Hicham, 2023). The lipids and carbohydrates produced by cyanobacteria can also be used in the production of biofuels, such as biodiesel and bioethanol (Priyadarshani & Rath, 2012; Arias et al., 2021).

Cyanobacteria are also promising for the discovery and production of biochemically active compounds (Singh et al., 2005; Wijffels et al., 2013; Hachicha et al., 2022); they can modulate their pigment composition to enhance light absorption, protecting the cell from oxidative damage (Gupta et al., 2013; Sandybayeva et al., 2022). Pigments such as phycocyanin and carotenoids, in addition to phenolic compounds they produce, can be applied in the food and cosmetic industries, for example, as natural dyes (Priyadarshani & Rath, 2012; Gupta et al., 2013; Sandybayeva et al., 2022). The antioxidant capacity of these compounds are related to specific health benefits in the cardiovascular system, Alzheimer's disease, and certain types of cancer, with hepatoprotective and anti-inflammatory effects applicable in the pharmaceutical industry (Goiris et al., 2012; Perera et al., 2023).

The cyanobacterium *Rhabdoderma lineare* stands out as a potential species for biotechnological applications. Initially described in 1900 by Schmidle & Lauterborn, it is considered a cosmopolitan organism, mainly found in oligotrophic and mesotrophic lakes in temperate regions (Azevedo et al., 2003; Sant'anna et al., 2004). The first biochemical characterization of this species was conducted by Valdez et al.(2022). Its composition when exponentially growing under healthy conditions showed a potential commercial application, as its dry

biomass contained approximately 86% total proteins. This protein content is higher than that of the main species of microalgae (*Chlorella* sp.) and cyanobacteria (*Spirulina* sp.) currently marketed as dietary supplements. Furthermore, according to Valdez et al. (2025 – under review) the species shows high growth rates and an absence of toxins. However, these promising results were obtained under optimal temperature and light conditions (25°C; 250 μmol photons m^2 s^{-1}) in bench-scale reactors, and such conditions may not be guaranteed in large-scale cultivation. Artificial ponds, raceway reactors, tubular photobioreactors, and biofilms are among the main industrial methods for producing microalgae and cyanobacteria, most of which are exposed to open or semi-open spaces, subject to variations in sunlight and temperature. According to Novoveská et al. (2023), the above-mentioned growing methods for scaled production offer little or no temperature control.

Cyanobacteria can withstand high light intensities without suffering photoinhibition, unlike plants and eukaryotic microalgae. With higher PSI content relative to PSII in comparison to the eucaryotic microalgae, the cyanobacteria share electrons with respiratory pathways, and have a photosynthetic apparatus different from eukatyotic microalgae. In cyanobacteria, the cyclic electron transport in the thylakoid membranes prevents complete reduction of PSII (Campbell et al., 1998). This imposes difficulties in the investigation of photosynthesis in cyanobacteria by the straightforward method of pulse amplitude modulated chlorophyll a fluorescence (PAM). This is because the electron sharing between the photosynthetic and respiratory systems prevents the complete oxidation of PSII. Since photosynthesis is the primary process for producing biomaterials and biomass, understanding how it works is essential for improving cultivation systems, whether in the laboratory or on a larger scale.

The pulse amplitude modulated chlorophyll a fluorescence (PAM) is a non-destructive technique that has been used for decades to estimate various photosynthetic parameters (Krause & Weiss, 1984; Shreiber et al., 1986; Govindjee, 1995). As described in Campbell et al. (1998), the PSII photosystem is a protein complex loaded with pigments that acts as the apparatus for energy absorption, which is either directed to the photosynthetic pathways (photochemistry – qP) or dissipated through fluorescence (qN) and/or heat emissions (NPQ). In the PhytoPAM used in the present research, a modulated

light pulse fluorimeter emits short light pulses that prevent heat loss in the samples and measures the fluorescence they emit. Knowing the incident energy and the fluorescence emitted, it is possible to calculate the percentage of energy directed to photosynthesis, or the system's efficiency. The maximum photosynthetic quantum yield (Fv/Fm) is a widely used parameter to determine the physiological state of the photosynthetic organism, as it represents PSII potential quantum yield (Govindjee et al, 1968; Kitajima & Butler, 1975). Its determination is automatically performed by the PhytoPAM II fluorimeter after measuring the minimum (Fo) and maximum (Fm) fluorescence values.

In eukaryotic cells, such as microalgae and plants, PSII quantum yield can be determined by acclimating the sample in the darkness (15 to 20 min), where the absence of incident light energy halts the electron flow, causing the plastoquinone (PQ) pool, the first electron acceptor, and the PSII reaction centers (Qa) to be completely oxidized. Under these conditions, the reaction centers are "open" or ready to receive energy (electrons), and therefore, incident energy is preferentially directed to photosynthesis, resulting in minimal fluorescence, Fo. A saturating light pulse is then applied, which causes the Qa reaction centers to become fully reduced or "closed," and in this case, incident energy cannot be diverted to the photosynthetic pathways and is dissipated as high fluorescence values (Fm).

Under optimal conditions, photosynthesizing eukaryotic organisms typically show fluorescence yields between 0.7 and 0.8 (Ogawa & Sonoike, 2016); however, in cyanobacteria, the yields usually range between 0.2 and 0.5 (Raateoja et al., 2004; Suggett et al., 2009). This does not mean they are photosynthetically less efficient than the other photosynthetic organisms, but rather that the reaction centers are not totally oxidized and avid to receive electrons. The commonly used PAM fluorimeters measure and calculate fluorescence based on characteristics of terrestrial plants and green microalgae, while cyanobacteria have distinct characteristics that prevent accurate measurements by conventional methods (Ogawa, Misumi & Sonoike, 2017; Stirbet et al., 2019). Ogawa & Sonoike (2016) present a protocol that circumvents these issues and allows for estimating Fv/Fm in cyanobacteria that are closer to reality, like those of green algae and plants. However, to date, this protocol has only been tested on the cyanobacterium *Synechocystis* sp. In the present

research we intend to validate the same protocol in *R. lineare*, thus being able to evaluate the effects of temperature in its physiology, including the photosynthetic response.

Several studies indicate that both daily and seasonal temperature changes influence growth, photosynthesis efficiency, and the content and composition of proteins, carbohydrates, lipids, and pigments in microalgae and cyanobacteria (Davis et al., 2009; Hongsthong et al., 2009; Lürling et al., 2013; Makey et al., 2013; Ceopi, 2019; Carneiro et al., 2020; Masojídek et al., 2021; Kholssi, Lougraimzi & Moreno-Garrido, 2023). Considering that large scale cultures offer little to no control of temperature (Novoveská et al., 2023) and that temperature variation can either promote or inhibit the production of compounds of interest, this leads to variability in the microorganism biochemical composition and in the production throughout the year. To understand the effects of temperature in *R. lineare* and ensure the success of its biotechnological applications, information regarding its physiological responses to different temperatures and how they affect growth, biochemical composition, and photosynthetic physiology is necessary.

This study is divided into two chapters. Chapter 1 consists of evaluating and validating the protocol proposed by Ogawa & Sonoike (2016) for determining the maximum photosynthetic quantum yield in cyanobacteria. Validation was done using the cyanobacteria *Synechocystis* sp., the species with which the protocol was developed. Chapter 2 evaluated the physiological changes in *R. lineare* cultures exposed to different temperatures, assessing growth, maximum photosynthetic quantum yield and biochemical profiles. Therefore, the present study is a contribution to the biotechnology of cyanobacteria, helping in the selection of suitable large-scale production systems for the species

2. Objectives

2.1. General Objective

To standardize the protocol for determining the maximum photosynthetic yield in the cyanobacteria *Rhabdoderma lineare* and study physiological responses induced by culturing them at different temperatures.

2.2. Specific Objectives

• To grow *R. lineare* in batch cultures at laboratory scale in controlled environmental conditions;

• To standardize the PAM technique protocol for determining the maximum photosynthetic quantum yield in *R. lineare* based on an existing protocol in the literature.

• To determine the growth rate and the yield of dry biomass at the end of the cultures (last day of the exponential phase) for the different culture temperatures;

• To determine the biomolecules (lipids, proteins, carbohydrates, and total pigments) in the dry biomass for the different culture temperatures.

3. Materials and Methods

3.1. Cultures

The *Rhabdoderma lineare* strain (3083)(Nome da coleçao) was kindly provided by Prof. Dr. Célia Leite Sant'Anna (Institute of Environmental Research, SP, Brazil). It was cultivated in houx air-lift type photobioreactors, containing 800 mL of BG-11 medium (Rippka, 1988), under three different temperatures (25, 30, and 35 °C), photoperiod of 12:12 h light:dark cycle, initial pH adjusted to pH 7 and light intensity of 250 μmol photons m^2 s^{-1}. The cultures at 25 °C are considered as controls. All cultures were maintained under constant aeration by bubbling filtered atmospheric air that passed through a membrane with 0.22 μm pore size. For the temperature experiment, all cultures were maintained in a thermostatic water bath.

Growth monitoring was carried out by daily sampling for *in vivo* chlorophyll a determination using a fluorimeter (Turner Designs, USA). At the end of the exponential fase, this data was used to calculate growth rate, final dry biomass yield was determined by lyophilization (Solab – SL-404, Brasil) and weighing (Mettler Toledo with 10^{-6} g precision) of samples, and maximum quantum yield of photosynthesis (Fv/Fm) was determined using the PAM technique with a Phyto

PAM-II equipment (Walz, Germany) on the last day of the exponential phase using the modulated amplitude fluorescence (PAM) technique.

3.2. Photosynthetic Analysis

The photosynthesis protocol was standardized with exponentially growing cells kept at the control conditions. Considering the challenges of determining photosynthesis parameters in cyanobacteria using the PAM technique, the protocol described by Ogawa & Sonoike (2016) was used as a basis for Fv/Fm determination in the present research.

The method involves acclimating the cells to darkness (15 min) to obtain the dark dissipated maximum and minimum fluorescence (Fo´, Fm´), then the cells are acclimated to a weak blue light (5 min, 480 nm, 60-70 μmol photons m^2 s^{-1}). This preferentially excites PSI and generates greater oxidation of PSII reaction centers, allowing for the determination of a more accurate minimum fluorescence (Fo). Finally, the cells are acclimated to white light for 5 min in an intensity similar to that used during cultivation (~250 μmol photons m^2 s^{-1}), but exposed to DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) at a final concentration of 20 μM.

DCMU inhibits photosynthesis by binding to the Qb plastoquinone site of PSII, preventing electron flow from PSII to plastoquinone. This disruption specifically affects PSII without impacting PSI or other photosynthetic processes. As a result, DCMU blocks the linear electron transport chain in photosynthesis. With the PSII oxidized because of the use of the DCMU, a saturating light pulse (SAT-PULSE) is applied and the maximum fluorescence (Fm) is obtained. Once the four measurements (Fo´, Fm´, Fo, and Fm) are obtained, equation [2], developed by Pfündel et al. (2013), is used to calculate the basal fluorescence (f) arising from Photosystem I (PSI) and the Phicobilisome (PBS). Finally, the maximum quantum yield of photosynthesis is calculated using equation [3]. This results in corrected yields closer to the actual values obtained for microalgae and plants.

As presented in Ogawa & Sonoike (2016) red light was used for Fv/Fm quantification and the cultures had a mean density of 0.2 A.U., measured by

absorbance in 750 nm using a spectrophotometer (BELPHOTONICS – UV-M51, UV/VIS, Italy).

$$[2] \qquad f = \frac{Fo'Fm - FoFm' - \sqrt{(Fm - Fo)(Fm' - Fo')(Fo - Fo')(Fm - Fm')}}{(Fm - Fm') - (Fo - Fo')}$$

$$[3] \qquad F_v/F_m = (F_m - F_o)/(Fm - f)$$

3.3. Dry biomass and biomolecules

Dry biomass was obtained in the last exponential day (day 4). The entire culture was centrifuged at 4000 rpm - Thermo Scientific, Sorvall Legend XTR, EUA), the supernatant was discarded, and the pellet was freeze-dried (Solab – SL-404, Brasil). From this dry biomass, aliquots were weighed using a microanalytical balance (Mettler Toledo with 10^{-6} g precision) and then used for total proteins and carbohydrates determination. Total lipids were determined by gravimetry according to Parrish (1999).

Total proteins were extracted following the method of Slocombe et al. (2013) and determined by spectrophotometry according to Lowry (1951), which is based on a calibration curve made with bovine serum albumin. Total carbohydrates were quantified by spectrophotometry according to Albalasmeh et al. (2013), using glucose as the standard. The spectrophotometer used for all determinations was BELPHOTONICS – UV-M51, UV/VIS (Italy).

The pigments chlorophyll a (Chl a) and total carotenoids were determined according to Wellburn (1994), using dimethyl sulfoxide (DMSO) as the extraction solvent. The phycocyanin content was determined following the spectrophotometric protocol described in Yéprémian et al. (2016). For this, the samples were centrifuged (Thermo Scientific, Sorvall Legend XTR, EUA) , and the pellet was digested with phosphate-buffered saline solution (NaCl 0.15 mol L^{-1}, KCl 27 mmol L^{-1}, Na_2HPO_4 80 mmol L^{-1}, KH_2PO_4 20 mmol L^{-1}, Na_2EDTA 10 mmol L^{-1}) for 16 hours. After digestion of the sample, absorbance was measured

at 620, 650 and 750nm, values measured at 750nm were subtracted from those measured at 620 and 650nm to eliminate residue from measure and phycocyanin content was calculated according to the equation proposed by Tandeau de Marsac & Houmard (1998).

$$\text{Phycocyanin}\left(\text{mg L}^{-1}\right)=\frac{\left(\text{Abs } 620-0.7\times\text{Abs } 650\right)\times\text{Ve}}{7.38\times\text{Vs}\times\text{I}}$$

where
Ve = volume of buffer extract (mL)
Vs = volume of water sample (litre)
I = path length of cuvette (cm)

3.4. Temperature

The cultures were exposed to different temperatures in thermostatic water baths. Three experimental replicates of the cultures were kept partially immersed in the water bath. To maintain the temperatures at 25 ± 1ºC, 30 ± 1ºC, and 35 ± 1ºC, aquarium heaters with thermostats were used.

3.5. Statistical Analysis

The results were analyzed using ANOVA and Tukey’s test at 95% confidence level with the help of Minitab 17 (v17.1.0). Graphs were plotted using IgorPro software.

4. Results and Discussion

4.1. Chapter 1: Standardization of the Protocol for Obtaining Maximum Photosynthetic Yield in Cyanobacteria

4.1.1. Characteristics of Fluorescence Emission in Cyanobacteria

As prokaryotic organisms, cyanobacteria do not have mitochondria nor chloroplasts, meaning that the electron transport pathways for photosynthesis

and cellular respiration share the thylakoid membranes. The thylakoid membranes are dispersed within the cells and various molecules, such as the PQ pool, are also involved in the photosynthesis and respiration in cyanobacteria (Campbell et al., 1998). When cells are acclimated to darkness, the PQ pool is not fully oxidized as it continues to be reduced by the respiratory pathways (Peschek & Schmetterer, 1982; Campbell & Öquist, 1996). Consequently, incomplete oxidation of the quinone A (Qa) reaction centers occurs, causing a transition from state 1 to state 2, moving the phycobilisome (PBS) from PSII to PSI. In cyanobacteria, the phycobilisome forms the antenna system meaning that energy will be preferentially allocated to PSI (Ogawa, Misumi & Sonoike, 2017). Overall, this process leads to non-photochemical dissipation, causing Fo' and Fm' values that do not accurately reflect photosynthetic reality when comparing to eucaryotic microalgae and plants.

Cyanobacteria have crucial differences in their pigment composition compared to eukaryotic microalgae. In eukaryotic cells, the PSI/PSII ratio is typically close to 1, with a similar allocation of chlorophylls in both photosystems. However, in cyanobacteria, this ratio can range from 2 to 10 (Stirbet et al., 2019), with a considerably higher percentage of chlorophyll allocated in PSI. This factor, combined with the presence of phycocyanin in the PBS, leads to the emission of a basal fluorescence (f) that interferes with the measured values of Fo and Fm (Ogawa, Misumi & Sonoike, 2017). Therefore, due to these distinctive characteristics, the obtained Fv/Fm in cyanobacteria is usually in the range of 0.2 to 0.5 and does not represent the actual potential functioning yield of PSII. To overcome these issues, Ogawa & Sonoike (2016) proposed a protocol that results in an Fv/Fm in cyanobacteria comparable to that of other microalgae and plants.

4.1.2. Protocol for Obtaining Fv/Fm in Cyanobacteria

The protocol proposed by Ogawa and Sonoike (2016) was developed using *Synechocystis* sp. 6803 as organism test. It uses measuring light in the 650 nm range since this wavelength preferentially excites PSII. The procedure begins by 10-minute dark aclimmation of the cells and the application of a saturating pulse (SAT-PULSE) to obtain the minimum (Fo') and maximum (Fm’) dark dissipated fluorescence values. Subsequently, the cells are acclimated to weak

blue light (60–70 μmol photons m² s^{-1}, 480 nm) for 5 minutes. The chosen wavelength preferentially excites PSI, and its low intensity is designed to minimize the excitation of pigments in the PBS (Schreiber et al., 1995). This leads to a progressive oxidation of the PQ pool, which helps oxidation of the Qa centers of PSII, resulting in a transition from state 2 back to state 1 (Ogawa, Misumi & Sonoike, 2017). This approach prevents losses due to non-photochemical dissipation and yields Fo values closer to reality. However, the Fm values obtained under these conditions, while higher than Fm', still show a small but significant degree of dissipation, suggesting that light may also be causing slight excitation of PSII.

According to the protocol, Fm is obtained by first adapting the sample to white light (5 minutes) in the presence of DCMU, followed by the application of the SAT-PULSE. DCMU is a compound that blocks the Qa reaction centers, preventing photochemical energy dissipation (Campbell & Öquist, 1996). Consequently, the light energy from the saturating pulse is dissipated as fluorescence, revealing real Fm values. However, the Fv/Fm values calculated using Fo and Fm remain in the range of 0.2–0.5 due to the interference of basal fluorescence (f).

Oxborough & Baker (1997) developed equation [1] to calculate Fo' in eukaryotic cells, using Fo, Fm, and Fm', considering that Fo and Fm are dissipated into Fo' and Fm' in similar proportion, respectively.

$$[1] \qquad F_o' = F_o/(F_v/F_m + F_o/F_m')$$

The study conducted by Pfündel et al. (2013) identified that the measured Fo' values were often higher than the calculated values, and they attributed this difference to the basal fluorescence (f) emitted by PSI. The authors incorporated the basal fluorescence to equation [1], leading to the development of equation [2], which allows the calculation of f using Fo', Fm', Fo, and Fm.

$$[2] \qquad f = \frac{Fo'Fm - FoFm' - \sqrt{(Fm - Fo)(Fm' - Fo')(Fo - Fo')(Fm - Fm')}}{(Fm - Fm') - (Fo - Fo')}$$

Ogawa & Sonoike (2016) applied equation [2] to calculate the basal fluorescence from the PBS and PSI using the values of Fo' and Fm' obtained from dissipated fluoresence after dark acclimation, Fo obtained during acclimation under weak blue light, and Fm obtained during acclimation to light in the presence of DCMU. Finally, the value of ***f*** obtained was subtracted from Fo and Fm, leading to equation [3], which allowed the calculation of the maximum photosynthetic yield in *Synechocystis* sp. 6803, with a value around 0.8 under ideal growth conditions.

$$[3] \qquad F_v/F_m = (F_m - F_o)/(Fm - f)$$

4.1.3. Standardization of the Protocol and Application in Different Species

The protocol described above has not been widely reported in the literature, and its implementation by Ogawa & Sonoike (2016) was carried out using a different PAM fluorimeter than the one available for the present study. Therefore, to use it with *R. lineare* in this study, we have validated it in our conditions and PhytoPAM II unit. We used a strain of *Synechocystis* sp. as control, and tested it on *R. lineare* and the filamentous cyanobacterium *Nostoc* sp. The cultures were grown under controled light and temperature conditions, and measurements were performed in exponential growth phase of the strains (day 4 of the culture).

The measurements made in *Nostoc* sp. were limited by the settling of filaments on the bottom of the cuvette, during the acclimations to the blue and white lights,. The protocol assumes samples with an optical density equal to or less than 0.2 at 750 nm; however, the accumulation of filaments at the bottom of the cuvette resulted in a high concentration of cells near the detector, leading to inconsistent measurements. This represents a limitation for the application of the protocol in PAM II fluorimeters, which, unlike the PAM I, do not have an integrated mixing device. On the other hand, *Synechocystis* sp. and *R. lineare* are unicellular coccoid species that are evenly distributed in the sample and did not exhibit this

problem. The results obtained for each culture are listed in Table 1. The values obtained for *Nostoc* sp. were excluded, as they did not meet the requirements outlined by the protocol.

***Table 1*. Results obtained in the validation of the Ogawa & Sonoike (2016) protocol with *Synechocystis* sp. and *Rhabdoderma lineare*. Fo' and Fm values result from cell acclimation to dark conditions, Fo from adaptation to weak blue light, Fm from white light with DCMU in the sample, f is the value calculated based on equation [2], and Fv/(Fm-f) is based on equation [3].**

Species	Fo´	Fm´	Fo	Fm	f	Fv/(Fm-f)	Mean (SD)
Synechocystis sp. 6803 *(Ogawa & Sonoike, 2016)*	91	159	94	194	58	0,73	-
Synechocystis sp.	205	241	216	439	181	0,86	0,83 (0,03)
Synechocystis sp.	191	227	211	454	157	0,81	
R. lineare	358	419	399	882	296	0,82	0,81 (0,00)
R. lineare	356	428	400	907	284	0,81	

All analyzed samples showed maximum photosynthetic quantum yield consistent with the literature, with values close to 0.8 under healthy conditions. Compared to the control, a difference between the fluorescence values obtained in this study and those in Ogawa & Sonoike (2016) were obtained. This can be attributed to the use of different equipment models. However, the application of equations [1] and [2] resulted in similar photosynthetic yields, validating the functionality and applicability of this technique across different equipment and species. In Chapter 2, this method will be used to assess the effects that different temperatures had on the photosynthetic physiology of *R. lineare*.

4.2. Chapter 2: Biochemical and Physiological Effects Induced by Different Cultivation Temperatures in *Rhabdoderma lineare*

Temperature is one of the key variables governing the cultivation of microalgae and is one of the major limitations for outdoor cultivation (Masojídek et al., 2021). With the exception of rare thermophilic species, most algal cultures do not survive in temperatures above 40 °C, and the main large-scale production methods have little or no temperature control (Novoveská et al., 2023), as this

would lead to increased costs. This chapter will present the results and discuss the physiological responses of *R. lineare* to different cultivation temperatures.

4.2.1. Growth

Figure 1a shows the growth curves of *R. lineare* under three treatments (25, 30, and 35 °C), obtained through *in vivo* chlorophyll a fluorescence as a function of experimental time (days). From these curves, the growth rate was calculated considering days 1 to 4 (Figure 1b). No statistically significant differences between the treatments 25 °C (0.28 (0.03) d^{-1}) and 30 °C (0.33 (0.06) d^{-1}). Nevertheless, at 35 °C with a growth rate of 0.10 (0.02) d^{-1}, a significant decrease was obtained ($p<0.05$).

These results are consistent with several studies in the literature, which indicate a decrease in the growth rate of cyanobacteria at temperatures above 35 °C (Lürling et al., 2013; Amin, Jaiswal & Kannaujiya, 2024). Lürling et al. (2013) analyzed the growth of 8 species of cyanobacteria at 6 temperatures in the range of 20 – 35 °C, 7 of which reached their highest growth rates between 25 and 32.5 °C, showing a decrease at 35 °C. Amin, Jaiswal & Kannaujiya (2024) compared the effects of temperature on rice-field cyanobacterium *Anabaena* sp. VKB01 and hot-spring cyanobacterium *Nostoc* sp. VKB02, growth rates decreased at 35 and 40°C respectively, attributed mainly to a reduction in cell viability, photosynthetic activity and the occurrence of cell bleaching.

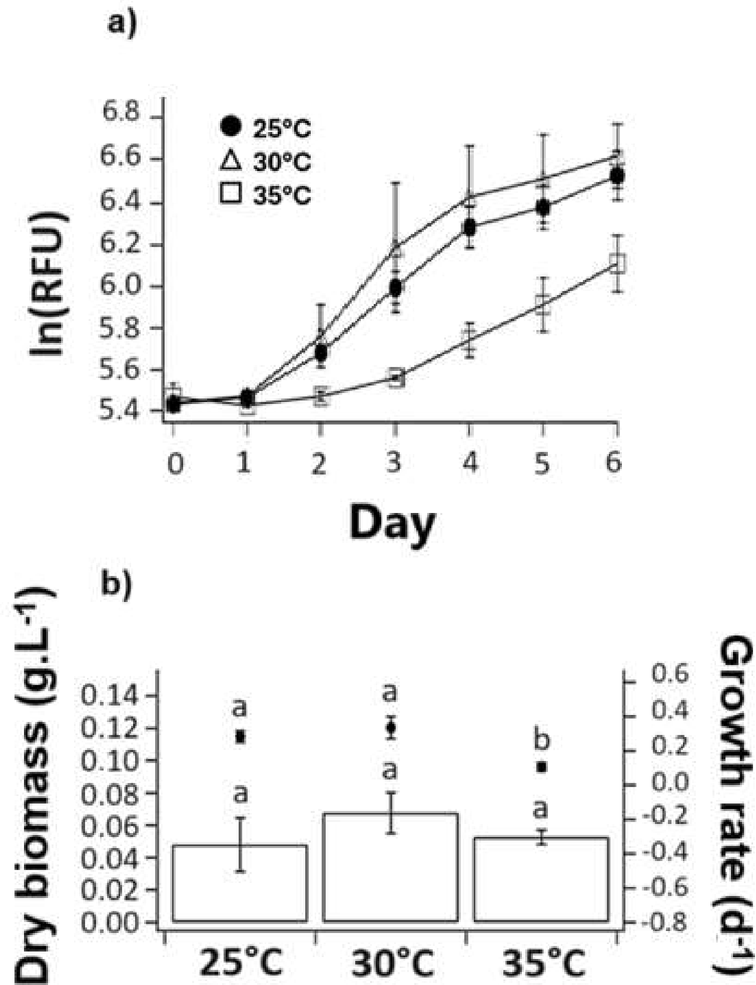

***Figure 1*. a) Growth curves as a function of experimental time (days). Data points are the mean values of in vivo chlorophyll a fluorescence; b) Dry biomass yield (g.L^{-1}) in the last culture day (bars) and growth rate (d-1) for the cultivation temperatures (points). All values are the mean and bars the standard deviation from the mean (n=3).**

The final dry biomass yield (Figure 1b) showed no significant differences among the treatments, meaning that even though at the highest temperature the lowest growth rate was obtained, its biomass did not decrease. This can be related to differences in chlorophyll a content at each temperature. The similarity in biomass production at different temperatures independently of growth rates can be due to different biomolecules composition in the cells. Temperature can promote or inhibit the accumulation of biomolecules such as lipids and carbohydrates, as has already been shown in the literature (Cepoi, 2019; Wang et al., 2024), leading to changes in cell diameter and volume (Barten et al., 2022).

Sheng et al. (2011) exposed *Synechocystis* sp to several temperatures in an experiment that lasted 46 days and, comparing to controls, they observed a decrease in growth rate and dry biomass yield at both lower (22 and 18 °C) and higher (44 °C) temperatures, but obtained little biomass variation when the cells were grown in the 30 – 33 °C range. On the other hand, Cepoi (2019), after increasing the temperature from 30 to 40 °C in *Spirulina platensis* cultures, found no significant differences in dry biomass content, despite several variations in the biochemical profile of the treatments.

Our results showed that *R. lineare* was able to grow at all three temperatures, with a better cultivation temperature range between 25 – 30 °C. The lower growth rate observed at 35 ºC can be related to the lower photosynthetic capacity, detected by the maximum quantum yield of photosynthesis as will be discussed below. With potentially lower photosynthetic capacity at higher temperatures, lower growth rate can be expected since less biomaterial and less CO_2 will be fixed by the temperature stressed cells.

4.2.2. Maximum Photosynthetic Yield

The maximum photosynthetic quantum yield (Fv/Fm) is an important parameter for assessing the state of photosynthesis, and consequently the health status, of the microalgae cells during cultivation. It represents the maximum potential efficiency of PSII and, therefore, of the overall system. Figure 2 shows the maximum quantum yield obtained for *R. lineare* at each temperature tested. A progressive decrease in the maximum photosynthetic quantum yield as the temperature increased was observed. Similar to the present results, Barten et al. (2022) and Wang et al. 2024 showed a decrease in Fv/Fm in *Picochlorum* sp. and *Vischeria* sp. respectively, when culture temperature surpassed 30-31°C. Several studies also show a decrease in Fv/Fm values for cyanobacteria at higher temperatures (eg. Amin, Jaiswal & Kannaujiya, 2024), nevertheless, these studies do not take into consideration electron sharing with respiratory pathways and basal fluorescence, so its results do not represent the actual state of the photosynthetic system and will not be used as comparison to this study.

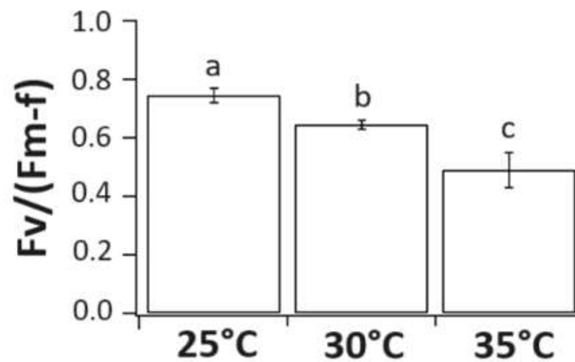

Figure 2. **Maximum photosynthetic quantum yield obtained in *R. lineare* cultures maintained at temperatures of 25, 30, and 35 °C. Values are the mean and bars the standard deviation from the mean (n=3).**

According to Matur, Agrawal & Jajoo (2014), PSII in the photosynthetic apparatus is one of the most temperature-sensitive components. As the temperature increases, the fluidity of the thylakoid membrane also increases, which can lose PSII present in the thylakoid membrane and dislodge it. Furthermore, the ability to oxidize water molecules is reduced due to the dissociation of cofactors like Ca^{2+} and Mn^{2+} whose chemical bonds can be affected by the increased temperature. Additionally, the plastoquinone (PQ) pool can also be affected due to an increased formation of reactive oxygen species (ROS) under these conditions. This leads to changes in the transition state, an increase in Fo, and a decrease in Fm and Fv/Fm (Matur, Agrawal & Jajoo, 2014). In the present research, the average fluorescence values obtained during the analysis (Tabel 2) showed an increase in Fo at 35 °C compared to the control (25 °C) and a decrease in Fm and Fv/Fm, nevertheless they were not significantly different under the different temperatures(ANOVA $p<0.05$). This decrease in photosynthetic yield may also be viewed as a strategy to combat ROS formation, which is accelerated with increasing temperature, and is often associated with changes in pigment content (Barten et al, 2022).

The use of the protocol analyzed and validated in Chapter 1 allowed the observation of real changes in the Fv/Fm values across different treatments, defining 25 °C as the optimal temperature for the functioning of the photosynthetic apparatus in *R. lineare*.

Table 2. Average fluorescence values (A.U.) and maximum photosynthetic yield obtained from R. lineare culture samples subjected to temperatures of 25, 30, and 35 ºC using the protocol of Ogawa & Sonoike (2016). Values are the mean and the standard deviation from the mean (n=3) is presented in brackets.

Culture Temperature	Fo´	Fm´	Fo	Fm	f	Fv/(Fm-f)
25 °C	231(42)[a]	264(61)[a]	274(41)[a]	547(41)[a]	181(23)[a]	0,74(0,025)[a]
30 °C	261(35)[a]	281(38)[a]	334(47)[a]	565(10)[a]	207(28)[a]	0,64(0,015)[b]
35 °C	197(28)[a]	213(33)[a]	288(13)[a]	432(15)[a]	135(39)[a]	0,49(0,06)[c]

4.2.3. Biochemical composition

The increase of temperature has been associated with changes in the biochemical profile of cyanobacteria, such as a decrease in lipid content, accumulation of carbohydrates, and changes in total proteins content (Sheng et al., 2011; Cepoi, 2019; Carneiro et al., 2020; Wang et al., 2024; Amin, Jaiswal & Kannaujiya, 2024). However, no consistent pattern to predict the effects of temperature on biochemical composition has been proposed, as it can vary among species (Nalley et al., 2018; Teoh et al., 2010) and even within species (Sayegh & Montagnes, 2011). Therefore, understanding the specific behavior of a strain under different temperatures is crucial to predict outcomes under industrial production conditions.

Figure 3 shows the total concentration of proteins, carbohydrates, and lipids as a percentage of dry biomass (%DW) for *R. lineare* under the temperatures 25 °C, 30 °C, and 35 °C that were tested in this study.

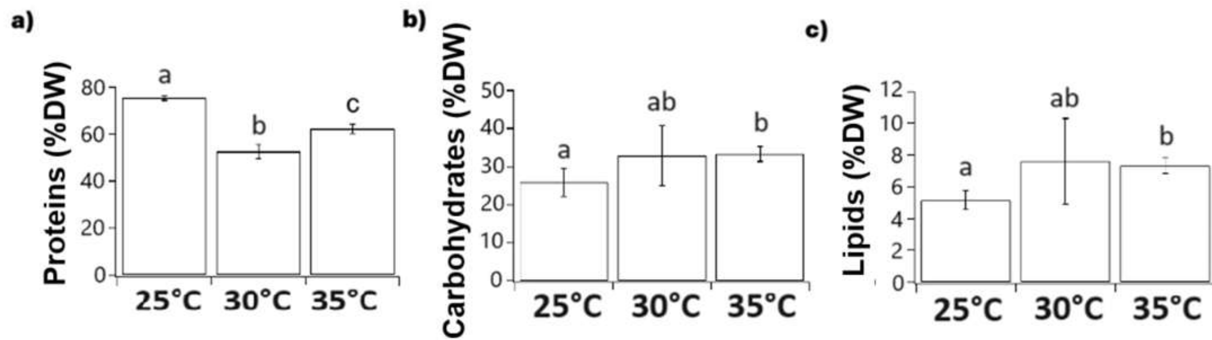

***Figure 3.* Percent biomolecules in the dry biomass (%DW) in *R. lineare* cultured at different temperatures, 25, 30, and 35 °C. Total proteins concentration (a), carbohydrates (b), and lipids (c). Values are the mean and bars the standard deviation from the mean (n=3).**

In the present research at the control condition (25 °C), the contents of total proteins, carbohydrates, and lipids are in accordance with literature data on the composition of *R.lineare*. The proportion of biomolecules they usually present is higher total proteins, followed by carbohydrates and the least biomaterial is lipid. In terms of absolute amount of the biomaterials comparing to other cyanobacteria, *R. lineare*, with ~ 75% DW of total proteins and 25% DW total carbohydrates contents are equal to or greater than the current organisms commercialized as dietary supplements, such as the cyanobacteria *Arthrospira platensis* (46-71% DW) and the Chlorophyta *Chlorella vulgaris* (50-58% DW) (Koyande et al., 2019). Similar to the literature (Amin, Jaiswal & Kannaujiya, 2024; Sayegh & Montagnes, 2011), with no consistent pattern, at the two higher temperatures tested, 30 °C and 35 °C, comparing to the control, lower total proteins were obtained, whereas carbohydrates and lipids increased. When compared to other species of cyanobacteria, *R. lineare* remains one of the species with the highest protein production even under thermal stress (Geada et al., 2021) since in the present study, its protein content varied within the range 52% DW to 75% DW. This result is promising, as in an industrial production scenario for the species, since temperature fluctuations would not significantly affect the production of its primary biomolecule of interest.

The higher total proteins in the control is a confirmation that the cells were in better growth conditions than at higher temperatures. Proteins are not reserve materials, as are carbohydrates and lipids, which increased under stressing

conditions. *R. lineare* is known for its high protein content. Valdez et al (2025 – under review) reported 86% DW of proteins in *R. lineare* under optimalconditions. To deepen our knowledge on the proteins of *R. lineare*, future studies should focus on the aminoacid composition and the proteins that are being synthesized.

Considering carbohydrates and lipids, a similar pattern can be observed, with a progressive accumulation as temperature increased, showing significant differences between 25°C and 35°C. However, the observed increase in carbohydrates was 8%, while lipids increased by 2%, indicating a preference for carbohydrate accumulation at high temperatures in this species. This preference has also been reported for *Arthrospira platensis*, and *Synechococcus sp. PCC 6741* (Cepoi, 2019; Panyakampol et al., 2014). In the present research, the carbohydrate content remained between 25% and 33% DW for all three tested temperatures. These values are higher or similar to the content of carbohydrates found in other currently marketed cyanobacteria as *Arthrospira platensis* (8-14% DW), and *Aphanizomenon flos-aquae* (20-30% DW), and the Chlorophyta *Hematococcus pluvialis* (15-40% DW) and *Chlorella vulgaris* (12-17% DW), (Koyande et al., 2019).

The total lipids content in *R. lineare* remained between 5% and 7% DW (this study). These values are common in cyanobacteria (Baracho & Lombardi, 2023; Morales et al. 2021). In fact, cyanobacteria are known to produce low amounts of lipids. Baracho & Lombardi (2023) investigated the biochemical composition of 20 cyanobacteria strains and showed that lipids ranged from 2 to 15%DW for all species with few of them surpassing 10%DW. Under non stressing conditions, as those reported in Baracho & Lombardi, eucaryotic species present around 5 to 20% lipids, as reported for *Chlorolobion lunulatum* in Faria (2023). Lombardi and Wangersky (1991) showed that a diatom, *Chaetoceros gracilis* presented half the amount of intracellular lipids under healthy conditions in comparison to stressed cells. The accumulation of lipids under healthy conditions is, in fact, uncommon in eukaryotic microalgae and cyanobacteria, with few exceptions. For example, some oleaginous species as the green algae *Botryococcus braunii* that accumulated up to 60% lipids under specific stressing conditions (Morales, Aflalo & Bernard, 2021). However, the increase in lipids under stressing situations has been reported to affect the fatty acids composition. Literature has shown that temperature fluctuations result in the variation of

saturated, unsaturated, and polyunsaturated fatty acid contents, which could affect the nutritional value of the biomass (Zao, Han & Cao, 2020; Aussant, Guihéneuf & Stengel, 2018). Therefore, future investigations could focus on the lipid composition of *R. lineare* to assess the presence and abundance of these compounds at different cultivation temperatures.

4.2.4. Pigments

Figure 4 shows the contents of chlorophyll a (Chl a), total carotenoids, and phycocyanin in the dry biomass of *R. lineare* in the different temperatures.

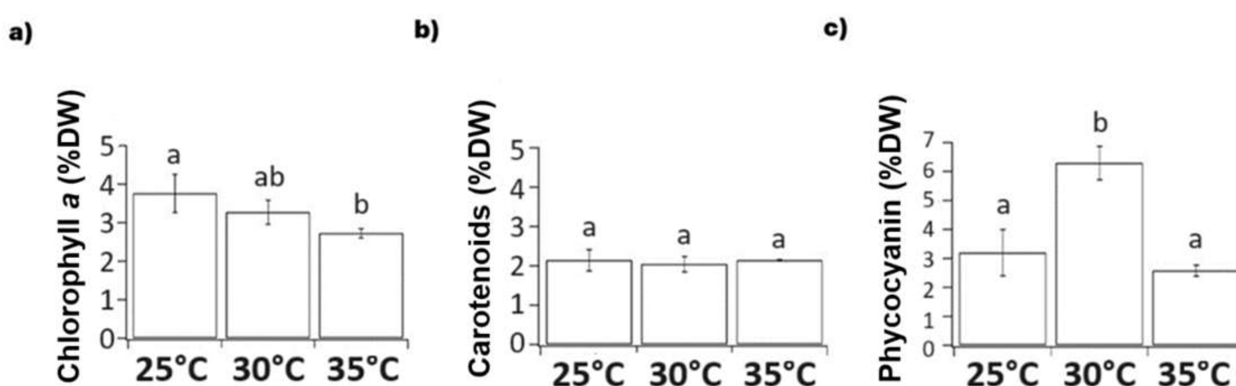

Figure 4. **Concentration of pigments as percent of dry biomass obtained in *R. lineare* cultures at different temperatures, 25, 30, and 35 °C. Chlorophyll a (a), total carotenoids (b), and phycocyanin (c). Values are the mean and bars the standard deviation from the mean (n=3).**

The content of chlorophyll a in the control (3,7%) is in accordance with literature results. Jaiswal et al (2018), reported a chlorophyll a range of 0,9 to 2,9 for *Westelliopsis*, *Anabaena*, *Nostoc, Aulosira* e *Tolypohrix* on the first seven days of culture. However, temperature increase was able to decrease the content of Chl a in *R. lineare* (Figure 4a). The decrease observed at 35 °C may be associated with a reduction in the biosynthesis and/or an acceleration of the degradation of the pigment under thermal stress conditions. This effect has been reported in the literature (Kumar et al., 2011; Matur, Agrawal & Jajoo, 2014; Wang et al., 2024). Wang et al. (2024) showed that cultures of microalgae *Vischeria sp.* exhibited an accelerated decrease in Chl a concentration above 31 °C. Kumar et

al. (2011) showed that cultures of *Spirulina platensis* under different temperatures varying from 20 to 40 °C had a gradual increase in chlorophyll a concentration up to 35 °C, followed by a significant decrease above this temperature.

The total carotenoids content in *R. lineare*, which was around 2% independent of the temperature (Figure 4b), is not in accordance with literature, which suggests an increase in total carotenoids under increasing temperature. This is due to their antioxidant properties and their role in energy dissipation as temperature is increased (Kumar, Kulshreshtha & Singh, 2011; Amin, Jaiswal & Kannaujiya, 2024). In cyanobacteria, carotenoids are typically associated with the orange carotenoid protein (OCP), which exists in a soluble state and interacts with the phycobilisomes (PBS) when there is an excess of excitation energy (light). Their function is to dissipate the excess energy as heat (Stirbet et al, 2019). We hypothesize that the consistent total carotenoid concentration in the biomass is linked to the optimal light intensity used for cultivating *R. lineare*, with only the temperature being varied.

The content of phycocyanin varied among the temperatures tested, with the highest value at 30 °C. It was more than twice the values at 25 ºC and 35 ºC (Figure 4c). Literature data show that the content of phycocyanin in cyanobacteria vary significantly among species. Baracho & Lombardi (2023) reported production ranging from ~1 to 62 µg.mL^{-1} in 20 different species, Basheva et al. (2018) tested 18 strains from 10 different genera and obtained values from ~10 to 200 µg.mL^{-1} of phycocyanin, while our results for *R. lineare* ranged from 0,9 to 4,5 µg.mL^{-1} under the different temperatures tested. In *Arthrospira platensis* phycocyanin content has been reported to be over 15%DW depending on the growth conditions, being currently the most used species for producing this pigment (Eriksen, 2016). This is a pigment with high commercial interest, since it is a natural blue colorant that aggregates antioxidant potential and diverse nutraceutical properties and bioactivities (Fabre et al 2022; Basheva et al, 2018; Eriksen, 2016).

Phycocyanin, a phycobilin, is the main pigment associated with the PBS, being essential in photosynthesis, for light energy capture by the antenna system and its redirection to the photosystems (Stirbet et al, 2019). Phycobilins are capable of processing and neutralizing free radicals whose concentrations increase under thermal stress conditions (Cepoi, 2019). This may explain the

increase in phycocyanin observed at 30 °C, which would have acted as a defense mechanism against oxidative stress. The decrease observed at 35 °C may be associated with the reduction in photosynthetic activity or difficulties in pigment synthesis under thermal stress. Even though *R. lineare* is not a producer of phycocyanin as compared to other cyanobacteria, we may suggest that increasing the growth temperature can be tool for manipulation of Cyanobacteira inducing the increase of the blue pigment.

5.Conclusions

In chapter 1 we showed that the protocol of Ogawa & Sonoike (2016) was valid for *R. lineare*, resulting in photosynthetic quantum yields similar to eucaryotic photosynthetic microalgae. This confirmed the functionality and applicability of the technique across different equipment and species.

In chapter 2 we showed that temperature was an environmental parameter effective in interfering on both the biomass composition and on the physiological variables investigated, but not in total carotenoids and biomass yield. Related to the total biomolecules content, *Rhabdoderma lineare* can be considered a promising organism for biotechnological applications due to its high protein content, e.g. 70 to 80%. Even though increasing temperature decreased proteins, this biomolecule still remained higher than other cyanobacteria currently commercialized. Carbohydrates and lipids increased slightly with temperature, with carbohydrates surpassing other cyanobacterial species. However, the physiological variables as growth rate, photosynthetic yield, and chlorophyll *a* decreased with increasing temperature. Notably, phycocyanin content significantly increased at 30 °C, therefore we suggest that moderate temperature increase can be tested as a tool for manipulating other cyanobacteria for increasing the blue pigment.

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