Universidade de Aveiro
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VERA RITA FERREIRA CARDOSO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica do Prof. Doutor João Serôdio, Professor Auxiliar c/ Agregação do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Silja Frankenbach, Investigadora Júnior, CESAM & Departamento de Biologia da Universidade de Aveiro.

À família e amigos, presentes em todos os momentos.

o júri

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palavras-chave

Arthrospira platensis , Spirulina, meio de cultura, condições de crescimento, salinidade, temperatura, fotofisiologia, identificação genética.

resumo

Arthrospira platensis é uma cianobactéria helicoidal e filamentosa, popularmente conhecida como *Spirulina*, naturalmente presente em lagos altamente alcalinos. Com alto valor nutricional, é aplicada em suplementos para alimentação humana e animal, sendo valorizada pelo elevado teor em proteína (cerca de 60%), propriedades antioxidantes e anti-inflamatórias e presença de pigmentos como ficocianina e *β*-carotenos, bem como vitamina B12 e ómega-3. Devido ao crescente interesse e popularização pela indústria do bem-estar, é industrialmente produzida numa escala cada vez maior. A testagem das condições de cultivo utilizadas pela indústria, bem como o seu aprimoramento, com aplicabilidade prática é, portanto, de grande relevância. No presente estudo, a evolução do crescimento e fotofisiologia de duas estirpes de *A. platensis* (Chad e UTEX LB2340) é testada com diferentes salinidades e temperaturas, tendo como objetivo principal o melhoramento das condições de cultivo. Tendo como base as condições de crescimento padrão ("*Spirulina* medium", 35ºC, salinidade 15 e elevada intensidade luminosa), uma das estirpes (Chad) foi submetida a um teste de salinidades de 15 (controlo), 25 e 35, bem como a meio de cultura industrial. Ambas as estirpes foram sujeitas a um teste agudo de temperatura, num espetro de 5 a 65ºC, de forma a avaliar a sua resposta fotossintética. Foram determinadas curvas de crescimento através de densidade ótica e avaliação do teor de clorofila, bem como avaliação de respostas fotofisiológicas recorrendo a fluorometria de pulso modelado. Os resultados demonstram que a espécie em estudo prefere meio laboratorial, no entanto, demonstra potencial para ser produzida em água salgada, uma vez que evidencia eficiência fotossintética quando sujeita a salinidades de 25 e 35. O meio industrial mostrou ser o menos promissor em termos de resposta fotofisiológica e quantidade de pigmento. Em relação ao teste de temperatura, ambas as estirpes de *A. platensis*, demonstraram uma clara preferência por valores acima dos 35°C, estabelecidos como ótimos pela literatura, atingindo o máximo de eficácia fotossintética (determinada pelo parâmetro "taxa relativa de transporte de eletrões"), aos 50 e 55ºC, respetivamente para a estirpe UTEX2340 e "Chad". Foi ainda realizado um teste baseado na metodologia de PCR, para identificação das duas estirpes, cujos resultados são preliminares.

keywords

Arthrospira platensis , Spirulina, culture medium, growth conditions, salinity, temperature, photophysiology, genetical identification.

abstract

Arthrospira platensis is a spiral and filamentous cyanobacteria, popularly known as *Spirulina*, naturally present in highly alkaline lakes. With high nutritional value, it is applied in supplements for human and animal food, being valued for its high protein content (about 60%), antioxidant and anti-inflammatory properties and the presence of pigments such as phycocyanin and *β*-carotenes, as well as vitamin B12 and omega 3. Due to the growing interest and popularization by the wellness industry, it is industrially produced on an ever-increasing scale. The testing of the cultivation conditions used by the industry, as well as their improvement, with practical applicability is, therefore, of great relevance. In the present study, the evolution of growth and photophysiology of two strains of *A. platensis* (Chad and UTEX LB2340) is tested with different salinities and temperatures, with the main goal of improving its growth conditions. Based on standard growth conditions ("*Spirulina* medium", 35°C, salinity 15 and high light intensity), one of the strains (Chad) was subjected to a test of salinities of 15 (control), 25 and 35, as well as industrial culture medium. Both strains were subjected to an acute temperature test, in a range of 5 to 65°C, in order to assess their photosynthetic response. Growth curves were determined through optical density and assessment of chlorophyll α content, as well as assessment of photophysiological responses using modeled pulse fluorometry. The results demonstrate that the species under study prefer laboratory growth conditions, however, there is potential to produce in salt water, since it shows photosynthetic efficiency when subjected to salinities of 25 and 35. The industrial environment proved to be the least promising in terms of photophysiological response and amount of pigment. Regarding the temperature test, both strains of *A. platensis* showed a clear preference for values above 35°C, established as optimal by the literature, reaching the maximum photosynthetic efficiency (determined by the parameter "relative electron transport rate"), at 50 and 55°C, respectively for strain UTEX2340 and "Chad". A test based on the PCR methodology was also carried out to identify the two strains, the results of which are preliminary.

INDEX

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

SM – Spirulina medium, Modified

JM – Jourdan's medium (industrial)

RLC – Rapid Light Curves

E – Spectrally averaged irradiance of Photosynthetically Active Radiation (400 to 700 nm) (µmol m^{-2} s^{-1})

Fo, Fm – Minimum and maximum fluorescence of a dark-adapted sample

Fs, Fm' – Steady state and maximum fluorescence of a light-adapted sample

PSII – Photosystem II

ETR –PSII relative electron transport rate

rETR – relative PSII electron transport rate

ETR_m –Maximum ETR in an ETR vs *E* curve

α – Initial slope of the ETR versus E curve

E_k – light saturation parameter of an ETR vs *E* curve

E_{opt} – optimal irradiance

INTRODUCTION

Framework

Oceans cover the largest part of our planet, up to 70% of the surface, but most of them are still underexplored (Gerretsen, 2022). Despite this, oceans should not be underestimated, as they provide precious resources to maintain life as we know it in all ecosystems, even the terrestrial ones. It is estimated that at least half of Earth's oxygen is produced by marine organisms, being it plants, algae, or some photosynthetic bacteria, each of them playing a particular role in their particular ecosystems (NOAA, 2022).

During the formation of the Earth, about 4.5 billion years ago, the atmosphere was mainly composed by carbon and nitrogen compounds, such as CO_2, CH_4, N_2, NH_3, and H_2O vapor caused by intense volcanic activity, with little or no oxygen at all (Windley, 2021). Because of this, the earliest living forms were anaerobic (and chemoautotrophs), meaning they used chemicals, other than oxygen and light, to produce energy. However, today's anaerobic organisms do not represent such a big proportion of existing living organisms, as in the past, due to a big change in the atmosphere throughout the decades, that made aerobic organisms appear and expand: the Great Oxidation Event. This happened by the end of Archean, the beginning of Proterozoic Eon, about 2.5 to 3 billion years ago, when the first oxygenic photosynthesizers appeared, the cyanobacteria (prokaryotes) (Schirrmeister et al., 2015). There is evidence that group of primary producers brought photosynthesis to the world, although this is still a debated topic (Blankenship, 2010). They started to use light to convert chemical compounds into organic matter, and for this, captured carbon dioxide from the highly carbon concentrated atmosphere, H_2O and sunlight as source of energy. Through the process they formed oxygen as a by-product, that was released to the environment. This caused an increase in atmospheric and oceanic oxygen levels like never before. Great oxidation of iron in the oceans and its deposition occurred, and the biggest iron banded formations deposits happened during this period (hence the name of the history event).

Little is known about the origin of cyanobacteria, but its evolutionary importance can't be questioned. These were the first organisms with photosynthetic ability, and the only where oxygenic photosynthesis evolved by itself. The other we know today, like algae or plants have evolved through endosymbiosis with cyanobacteria, forming chloroplasts (Schirrmeister et al., 2015). These little beings were responsible for the greatest change in the atmosphere and oceans in the history of our planet, which changed the Earth from then on. Still, it is very challenging to study the origin of these organisms because the existing fossils, known as

Stromatolites and MISS (microbially induced sedimentary structures), associated with cyanobacteria activity, have been found to also hold the record of anaerobic phototrophic activity. Also, discrepancies between fossils and molecular phylogenies remain, so this subject is studied and discussed to the day (Demoulin et al., 2019).

Cyanobacteria are not strict to one habitat, instead, species of this group can be found in marine, inland waters, and soil, in a range of temperatures from arctic to equatorial regions. These prokaryotes are divided in five group, being section I the simplest, and V, the most complex, based on their characteristics: uni or multicellularity, filamentous and the ones able to differentiate, forming hormogonia, akinetes and heterocyst. This Phylum includes around 2000 species in 150 genera (Vincent, 2009) and is a great contributor to global nitrogen and carbon fixation, moreover there are several species of interest to biotechnology, food, health and biofuels (Shih et al., 2013). Some of the most famous cyanobacteria are the genera *Anabaena, Oscillatoria, Nostoc* and *Spirulina*. The latest, is the focus of this dissertation.

The genus *Arthrospira*, commonly known as *Spirulina*, is a filamentous, helicoidal cyanobacteria, naturally occurring in high salt alkaline lakes in tropical and subtropical areas (Dillon et al., 1995). It has been used for centuries, although its study and mass production only begun in the last 50 years. It is considered a superfood, an important source of nutrition for humans and animal feeds (T. Kim et al., 2018), due to its high levels of protein (60-70%), presence of essential amino acids, vitamin B12, *β*-carotenes, phycocyanin, iron, phenolic acids and *γ*-linolenic acid (Dillon et al., 1995). Also, the absence of cellulose cellular walls (that can be found in algae and plants) makes it more digestible. It is also known for its antioxidant and anti-inflammatory properties (Gershwin & Belay, 2007).

Because of its benefits, it has been included in several plans to protect for malnutrition and as a dietary supplement in space missions (ESA, 2016 ; Karkos et al., 2011). A report in combating malnutrition, from the Swiss Agency for Development and Cooperation, in Bangladesh and India, claims that “One gram of (dried) *Spirulina* per day can quickly and permanently reduce infant malnutrition, even in an advanced stage” (Heierli & Weid, 2007). Also, a study performed in children in Zambia says that “The results from this study indicate that a combination of *Spirulina* and mealie meal is effective in improving the linear growth in height for undernourished children” (Masuda et al., 2014).

Because of these excellent properties, *Spirulina* became more and more appealing to wellness market and animal feed industry, which has been growing throughout recent years. As people become more conscious of its nutritional benefits, and a global trend of wellness market grows, the sales of *Spirulina* and similar supplements has been continuously growing (Meticulous Research, 2021).

Considering the above, it is of obvious interest to look at the production requirements of *Spirulina* and ask ourselves if they can be improved. To do this, growth conditions and specific characteristics must be studied: optimum temperature, medium, salinity or pH. Can this be improved? Can some industries benefit from adapting the growth media, and even use seawater? Is it possible to lower the energy costs of production by using different temperatures? Is the quality of spirulina we are consuming really the best?

In the present study, different growth media, salinities and temperatures were tested in order to evaluate their influence in growth and photosynthetic activity of *Spirulina*. To do this, several experiments were carried out, which will be described later in this monography. But first, let us look more closely to this peculiar organism.

Classification and genetical identification

Arthrospira is a prokaryote, classified as follows: kingdom Eubacteria, subkingdom Negibacteria, phylum Cyanobacteria, class Cyanophycea, subclass Oscillatoriophycidae, order Oscillatoriales, family Microleacear and genus *Arthrospira* (Algabase, 2019). The three species of produced edible *Arthrospira* are *A. platensis*, *A. maxima* and *A. fusiformis*, being *A. platensis* the most common (*Herbal Drugs*, 1997)

There is a great confusion whether to name it *Spirulina* or *Arthrospira*, due to several changes on its classification throughout the years. In fact, these are two different genera, both belonging to the subclass Oscillatoriophycidae, as they produce helical trichomes. The genus *Spirulina* was the first to be identified, by Turpin in 1827. Later in 1852, Stizenberger introduced *Arthrospira*, distinguishing it from *Spirulina* by the presence of cross walls and larger trichomes. However, in 1917 using more advanced staining techniques, Gardner found that some species classified as *Spirulina* also had cross walls like *Arthrospira*, and so, these two genera were combined as a single genus, *Spirulina,* with two sections: *Arthrospira* and *Euspirulina* by Gaitler in 1932 (Muhling, 2000).

In the 1970s, however, it was found that there were substantial differences in the DNA composition between two different strains of *Spirulina* (Herdman et al., 1979 as cited in Muhling, 2000). This led to the conclusion that the taxonomy of this group had to be revised again. Further investigations concluded that the ultrastructure of both genera was indeed different: *Arthrospira* had a single row of pores around the trichome while *Spirulina* had several (Guglielmi & Cohen-Bazire, 1982 as cited in Muhling, 2000). This led to again to the splitting in two different genera in the 1980s. However, at this time, the industrial production of this organism had already started and was being commercialized with the name *Spirulina*, so it was established

that for scientific purposes the name *Arthrospira* should be used, while for commercial purposes the name *Spirulina* could be kept (Tomaselli et al 1996 as cited in Muhling, 2000). However, in the scientific community it is common to find the nomination like this: *Spirulina (Arthrospira) platensis*, so researchers can understand what is being referred to. But there is still confusion.

In the past, species classification was organized based on their morphological features, but this is often not enough to identify species and strains morphologically very similar, like *A. platensis.* Because of this, there was a need to go further and understand its genetical characteristics (Kadri, 2019).

Polymerase chain reaction (PCR) is a method developed by Kary Mullis, in 1983, for replicating (amplifying) segments of DNA, for genetic identification of species. This method uses the enzyme DNA polymerase to generate multiple copies of DNA fragments, by breaking the double strand and bonding, repeatedly (Kadri, 2019)..

Nowadays, there are PCR kits with ready-to-use reaction mixtures that save laboratory work time. PCR is carried out in tubes with a mixture of DNA isolate, Taq polymerase, primer pair, and water. The reaction happens in thermocyclers, equipments that allow for a very quick and precise variation of temperature, and has three phases: amplification, hybridization (or annealing), and elongation. The first phase happens at a high temperature (usually 94 °C) to separate the two strands of DNA (as hydrogen bonds break at this temperature). The DNA is now denaturated into single strands. In the second phase, annealing, at a lower temperature (between 40 and 70 °C), primers bond to the corresponding regions of the single strands of DNA. Finally, in the elongation phase (72 °C) it occurs the binding of a complementary strand, catalyzed by Taq polymerase. These three phases are repeated for several cycles, until there is an analyzable number of copies. The number of cycles is chosen considering the of the sequence (Kadri, 2019).

Primers must be used in complementary pairs, one bonding the fragment in the direction 5'-3' and the other in 3'-5'. They are synthetized chemically and can be designed by specialized companies, to the researchers needs. The chosen primers should have published evidence, so the results correspond to a species/ strain etc. (Kadri, 2019). The PCR product is normally analyzed in agarose gel electrophoresis, and the DNA detected by ethidium bromide staining, which turns it visible under UV transillumination. The identification then compares the gene sequence with existing database, to hopefully find correspondence. This is done by comparing nucleotide sequence to a database of nucleotide published sequences, like NCBI (Berkley library, September 2022).

History of human consumption

Human consumption of *Arthrospira* goes back to the Aztec civilization, who collected it from lakes using specific fine nets, being called *tecuitlatl.* This was discovered when the Spanish conquered the present day Mexico, in the sixteenth century (Vonshak, 1997). Also, the Kanembu tribe, living in Chad and Niger, did the same for many years, preparing a "cake" of *Arthrospira*, tomato, and peppers, called *dihé* that used to be sold in local markets. They believed this algae could treat some diseases and improve people's health (Habib et al., 2008). However, it wasn't until the 1960s, during a French-Belgian expedition to Chad, Africa, that a researcher, Jean Léonard, noticed that this "blue-green algae" was being used by locals for centuries and that this could be an interesting organism to produce (Léonard, 1966 as cited in Habib et al., 2008). In this decade, the interest in *Arthrospira* rose and the first research carried out on its growth requirements and physiology started. The doctoral thesis of the French researcher Claude Zarrouk,, in association with the *Institut Français do Petrole*, focused on studying this "blue-green algae" in evaporating ponds in a lake near Mexico City, and established what would later be the basics of every *Arthrospira* medium used nowadays (Vonshak, 1997). This first researches showed the high amount and quality of protein of this organism, information that drew attention to this cyanobacteria and started its industrial production in the 1970s (Habib et al., 2008).

Global production and market

For statistical purposes, *Arthrospira* (*Spirulina*) is often included in the microalgae spectra due to its similar applications. *Chlorella* was the first microalgae produced in large scale, in Japan in the 1960s, followed by *Arthrospira* in Mexico in the 1970s. These two are the most produced species worldwide (Habib et al., 2008). Nowadays, Asia and USA dominate the world production of microalgae and *Arthrospira*. In 2021, the U.S.A. dominated the *Arthrospira* market, however it is estimated that Asia will grow faster in the next years and overtake this due government initiatives to promote its use and investment in research (Meticulous Research, 2021).

Despite most *Arthrospira* biomass being currently produced in Asia and U.S.A., there are also producers in Europe (Fig. 1), spread over 23 countries: 222 companies producing it, France leading, with 147 companies. The facilities of this companies are mainly located inland. Although photobioreactors are the most common system used for microalgae growth (71%), for *Spirulina* the preferred method is producing in open ponds (83% of the companies) (Araújo et al., 2021), the reason being this method provides a higher biomass volumes for this species, and also lower

initial investment, operational and energy costs (Narala et al., 2016), although this has a downside of higher contamination risks (Mayers et al., 2016).

It is often difficult to estimate the production statistics of microalgae in Europe, due to lack and fragmented information, even from FAO and Eurostat. Still, the estimated production is currently around 142 tons (dry weight) per year (Araújo et al., 2021). In Portugal, the last report from DGRM/INE has no information about the amount of microalgae produced, even though there are several companies in the country (INE, 2021).

The bar that settles the market value for microalgae and *Arthrospira* is related to its bioactive compounds: pigments, antioxidants, and unsaturated fatty acids, like omega 3. The market interest in *Arthrospira* has been rising, as this organism proved to be rich in proteins and antioxidants, and produces an important phycobiliprotein, phycocyanin. This blue pigment has several uses, such as cosmetics applications, health and dietary supplements (Mobin & Alam, 2017). Worldwide, it is estimated that the market will rise 18,1% from 2021 to 2028. This trend is related to the growing health and wellness concerns, vegetarian and vegan diets, natural dyeing pigments and inclusion in aquaculture and domestic animal feeds. *Arthrospira* is sold mostly in powder, tablets, capsules, flakes, phycocyanin extracts and frozen biomass. (Meticulous Research, 2021).

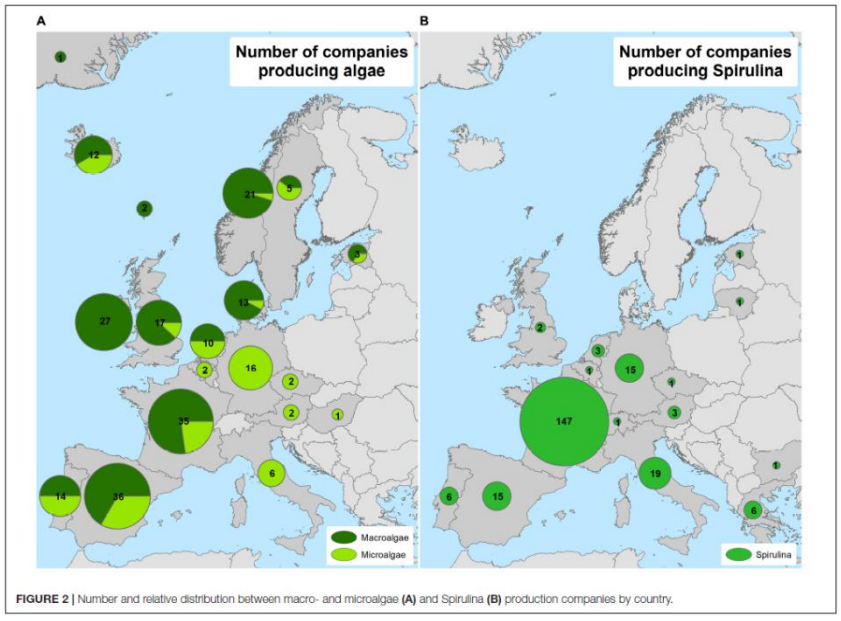

Figure 1 Number and relative distribution between macro and microalgae (A) and Spirulina (B) production companies by country in Europe (image from Araújo et. al., 2021)

Legal framework

Microalgae and *Spirulina* weren't always used worldwide for human consumption, actually this market is quite recent, when compared to other food sources. The European Commission has a specific term for this: "Novel Food", this is "defined as food that had not been consumed to a significant degree by humans in the EU before 15 May 1997, when the first Regulation on novel food came into force.*",* Reg. no 258/97/EC. There is an online catalogue of all the "Novel Foods" EU recognizes, that is updated as more (verified) information comes from state members (European Commission, 2022).

With no surprises, *Arthrospira* and several microalgae lie on this scope, and, according to this regulation, must be packed and labeled properly. The producing companies must ensure that their product is safe from any toxins or harmful components for consumers, therefore certifications and analysis have to be done, before the product enters the market. But here, it gets more complicated, because these are no longer EU laws, but national, and countries have the right to ban certain supplements if they want to. This creates unequal competitiveness among states (Dir. 2002/46/EC). This absence of cohesion between countries, results in lack of legal guidance and legislative ambiguity. Some countries start selling supplements before EU has a position on it, and others forbid it completely (Reg. 1169/2011/EC)

Narrowing it down to Portugal, here *Arthrospira* and microalgae production are under the supervision of DGAV- *Direção Geral de Agricultura e Veterinária*, but it was a long way for producers to finally start producing, even though it had already entered the country market as supplement form. This government department had little knowledge on the production process in the past, so it took a while until it was legal. This process was started by Georges Porta, a French producer in Monchique that brought the producing knowledge to Portugal and presented the subject to DGAV. It took three years, between bureaucracy and building facilities, for the first harvest to happen, in 2015. After this first step, more companies followed. This information was transmitted to the author by meeting this producer in person and visiting his company. Apart from getting a license approved by DGAV, the producing legal requirements must follow these food industry regulations: HACCP- Hazard Analysis and Critical Control Point and ISO 22000. They should also respect the EU norms for heavy metals content: Commission decision 2022/1244/EC. These are the mandatory requirements, but on top of this, the producer can choose to increase the interest of its product by adding certain certifications like Organic Certification, ISO9001, GMP, Global GAP, and so on.

Cyanobacteria growth and photosynthesis

Cyanobacteria, like microalgae are organisms that use H_2O and CO_2, and inorganic salts as source of energy. As any other organisms, they require certain conditions to grow, as temperature, CO_2, pH, availability of nutrients and certain light conditions, as this is the driving force of photosynthesis, and consequently, growth. *A. platensis* relies on a nitrogen source as well as phosphate and carbonate, an alkaline medium (above 8), high levels of light and an optimum growth temperature of ± 35°C (Richmond, 1988; Sánchez-Bayo et al., 2020)

Photosynthesis is a process in which, in short, cyanobacteria, algae, green plants and other photosynthetic organisms convert light energy and water into organic compounds and oxygen. It is a rather complex process, but this information is enough to understand its importance to maintain life on Earth, as these photoautotrophic organisms are responsible for the primary production of organic matter for most living beings (Lambers, H., et al. 2022).

However, natural sun light has variations of intensity throughout the day that can cause damages and obstacles to this process, so these organisms had to find a way to overcome this, by creating regulating mechanisms. As the photosynthetic process uses light, there is a risk of damaging the photosynthetic apparatus with excessive light absorption. So, this process has to balance light absorption, while minimizing damages to the organism.

Cyanobacteria photosynthesis is directly related to chlorophyll *a*, as this green pigment plays a central role in the process: participates in photon harvesting and helps to regulate the excitation energy distribution (Schreiber et al., 1986). This molecule is key to fluorescence measurements, as this pigment-proteins complexes, that exist in Photosystems I and II (PSI and PSII, respectively) and light harvesting complexes (LHCs), are responsible for light absorption, or, in case of excessive energy, re-emitting as heat or re-emitting as light (fluorescence). It is assumed that the fluorescence signal comes mostly from PSII and gives valuable information about the other two processes: quantum efficiency of the photochemistry and heat dissipation (Murchie & Lawson, 2013). Because of this, determination of chlorophyll *a* concentration is investigated in the present study, as assessment of culture wellbeing, as well chlorophyll *a* fluorescence.

Photosynthetic activity can be analyzed by producing rapid light-response curves, RLC using Pulse Amplitude Modulated Fluorometry (PAM), this method is widely used due to its ability to minimize disturbance of the samples (Schreiber et al., 1986 ; Coelho, 2006). These RLCs measures the relationship between photosynthetic electron transport rate through PSII and the incident light - photon irradiance (*E*), (ETR vs *E* curves). This is estimated by the fluorescence index $\Delta F/F'_m$ and the quantum yield of the photosynthesis (Genty et al., 1989), and will be used

in this study. The light used for measurements was in the red zone of the spectra, as cyanobacteria have shown to use this type of frequency (Averina et al., 2020). Chlorophyll *a* has two peaks of absorption, at blue and red frequencies, while phycocyanin has a single peak, at red (between 500-700 nm) (Zhang et al., 2017), so it fits into the selected measuring wavelengths.

Aim of this study

The aim of this study was to investigate the growth conditions of *Arthrospira platensis*, namely temperature and medium composition, that allow for improving the growth of this culture, with the hope to contribute to the producing industry, as well as contributing with more fundamental scientific knowledge to the topic.

MATERIALS AND METHODS

Experimental design

The aim of this study is to test and improve the growth conditions of *A. platensis* cultures. For this, the literature established ones were always used as control, being the medium: *Spirulina Medium, Modified,* referred to as "SM" in this monography and ± 35°C of growth temperature (Richmond, 1988). The setup and growth conditions will be specified in more detail later.

To compel this objective, photo physiologic responses and growth rates were tested with different growth medium, salinities, and temperatures. Two strains of *A. platensis* were used: industrial (kindly provided by *Tomar Natural* company, Portugal) and laboratory obtained (courtesy of Dra. Joana Pereira) (1 and 2, respectively), and are mentioned in the next section.

The first phase of the experiment was to test photosynthetic activity and growth rates of the species with different growth media, salinities, and industrial medium. Only strain 1 was used in this phase due to acclimation difficulties with strain 2 cultures. The treatments in detail were: SM; SM+10 units of salinity; SM + 20 units of salinity and industrial, Jourdan's medium (JM). One flask for each treatment was used, and experiments performed in triplicate. It was not possible to fit more replicates of flask treatments in the setup due to lack of space. Culture growth was measured by monitoring optical density, dry weight, and chlorophyll *a* concentration. Photosynthetic activity was measured by performing light-response curves of the relative electron transport rate of PSII (Rapid Light Curves, RLC).

After obtaining the results for the first phase and determining what was the best medium for growth and photosynthetic activity, the second phase of the work begun. In this stage, different temperature tolerances were tested, using in the medium considered the best. The two strains were available at this point, so the experiments were done with both, three flasks of culture for of each strain. The growth of the cultures was measured by following optical density, dry weight, and chlorophyll *a* concentration, at the temperature of ±35 °C. The different temperatures were induced when performing the RLC and not during the growth of the cultures, because the laboratory setup did not allow to have different temperatures. The temperatures tested were: 5, 20, 35, 50, 65 °C. These temperatures were selected in an exploratory way, having ± 35 °C as control, and then, some degrees above and some degrees below. In the diagram below (Fig. 2) the experimental design is illustrated, for better understanding. The methods for each phase will be described in detail in the next sections.

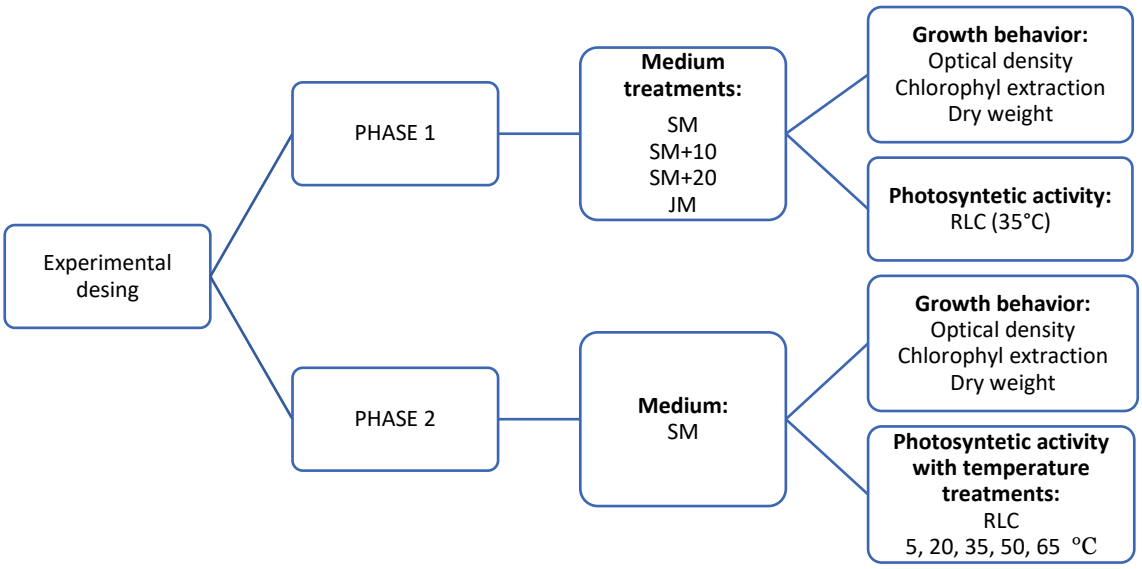

Figure 2 Planning of the experiments performed in this study.

Setup and growth conditions

A. platensis is a cyanobacteria that, as all organisms, needs certain conditions to grow. According to early studies, and industry used, the optimum growth conditions of this species was determined to be ± 35 °C medium temperature, as mentioned before, and high levels of light (Richmond, 1988), this has been settled and used in further research. Besides the information available from the literature, these growth conditions information were kindly provided by *Tomar Natural,* a company specialized in producing industrial *A. platensis* for human consumption, in a green and artisanal way.

As in our laboratory we didn't have any setup with these conditions, a new one was prepared. For that, it was used an 80 liter glass aquarium filled with water, to use as a "water bath" in order to keep the temperature of the cultures constant. Two thermostats selected to 35 °C (Tetratec HT50 230 V - 50 Hz - 50 W and Protemp S100 230V - 50 Hz - 100 W), a light source consisting in 4 fluorescent lamps (Philips Tornado ESaver 42W - 220-240 V – 50-60 Hz), making up a light intensity of ± 240 µmol quanta m^{-2} s^{-}1, with 12:12 photoperiod. These light conditions were selected based on the average levels used in *Tomar Natural* company, and were measured by the author in their facilities with a quantum sensor equipped with a semi-spherical sensor (MQ- 200, Apogee Instruments, Logan, USA).

To help to increase the light incident on the cultures, a hood made of aluminum foil was placed on top of the lamps. Also, a water pump (NewJet 1700 - 230 V - 50 Hz - 33 W) was placed inside the aquarium to provide water movement, keeping the water homogenized and the temperature stable (Fig. 3 A). A glass and mercury thermometer was used to measure the water temperature (Fig. 3 C). For aeration of the cultures, an air pump (HiBlow SPP-40GJ-L 240 V - 50 Hz - 44 W) was placed beside the aquarium, connected to a plastic tube with several silicon tubes as an air output, each silicon tube was connected to a syringe filter (PES 0,20 µm) to filter the air, avoiding particles and contamination going into to the cultures (Fig. 3 B). To minimize evaporation losses, a glass panel was used to cover the setup (Fig. 3 C).

The cultures were prepared in 1 liter glass flat bottles (Fig. 3 D), covered with parafilm, fastened with a rubber band, through which a hole was made for the aeration tube, to minimize evaporation (Fig. 3B).

Figure 3 Setup assembly: A - glass aquarium where, a) circulation pump, b) and c) thermostats, d) Light bulbs; B - aeration tubes and flasks with parafilm and rubber bands; C - covering glass panel and e) mercury thermometer; D - 1L glass bottles of cultures.

Culture medium

One of the aims of this thesis is to test physiological responses with different culture mediums. For this purpose, the previously mentioned "*Spirulina* medium", that will be referred to as "SM" further in this monography, was used, as well other medium (Industrial) and salinity changes.

SM has a salinity of 15 and, to test different salinity tolerances, the salinity was adjusted to 25 and 35, by increasing the amount of sodium bicarbonate in SM. The decision to test with different salinities was made thinking of its possible benefits for *A. platensis* producers, as it could be an advantage for companies settled near the sea to use seawater. This could reduce aquifer consumption, as well as costs in freshwater intake. On the downside, the rising of salt content in the product could be an obstacle for nutritional applications, and equipment wearing.

A. platensis culture medium was prepared according to Aiba and Ogawa (1977) and Schlösser (1994). The medium name is "*Spirulina* Medium, Modified", and can be found in Appendix A of *Recipes for Freshwater and Seawater Media: Academic Press* (Andersen, et. al., 2005). Its composition is listed on tables 1, 2 and 3 below.

Industrial medium (table 4) was also tested and prepared according to Jean Paul Jordan's manual, *Manuel de Culture Artisanale de Spiruline* (J. P. Jourdan, 2018). This medium will be referred to as "JM" in this monography. J. P Jourdan was a french pioneer in *Arthrospira* study and production, who developed a growth media (based on Zarrouk's work) that is used in industry (*Fédération des Spiruliniers de France*, 2022). This manual is not yet published by an editor, but its information is accessed and used widely by *Arthrospira* producers. It was obtained by attending to the *Curso de cultivadores de eSpirulina profesionales- online* in March 2021, a training course taught by Juan Sole, the CEO of *OrganaeSpirulina*, a spanish *Arthrospira* company focused on breaking knowledge barriers regarding the production of this cyanobacteria, sometimes practiced by the industry, and spreading the concept of producing sustainable *Arthrospira*, also with a research component.

The composition of the mediums is listed below. The solid components were weighted in a precision balance (Radwag AS220/C/2), with balance boats and stainless steel spatulas, and the liquid ones measured with micropipettes and measuring cylinders, according to the needs.

Table 1 Composition of *Spirulina* Medium, Modified (Aiba and Ogawa 1977, Schlösser 1994, as described in Andersen et al., 2005)

Composition of SM

Component	Concentration (g L^{-1} dH_2O)
$NaHCO_3$	13,61
Na_2CO_3	4,03
K_2HPO_4	0,50
$NaNO_3$	2,50
K_2SO_4	1,00
NaCl	1,00
$MgSO_4 \bullet 7H_2O$	0,20
$CaCl_2 \bullet 2H_2O$	0,04
$FeSO_4 \bullet 7H_2O$	0,01
Na_2 EDTA $\bullet$ $2H_2O$	0,08
Trace metals solution (see below)	1 mL
Vitamin solution (see below)	1 mL

Table 2 Trace metals solution composition in *Spirulina* Medium, Modified (Aiba and Ogawa 1977, Schlösser 1994, as described in Andersen et al., 2005)

Trace metals solution

Component	Stock solution (g L^{-1} dH_2O)	Amount in solution
Na_2 EDTA $\bullet$ $2H_2O$		0,8 g
$FeSO_4 \bullet 7H_2O$		0,7 g
$ZnSO_4 \bullet 7H_2O$	1,00	1 mL
$MnSO_4 \bullet 7\ H_2O$	2,00	1 mL
H_3BO_3	10,00	1 mL
$Co(NO_3)_2 \bullet 6H_2O$	1,00	1 mL
$Na_2\ MoO_4 \bullet 2H_2O$	1,00	1 mL
$CuSO_4 \bullet 5H_2O$	0,0005	1 mL

Table 3 Vitamin solution in *Spirulina* Medium, Modified (*Aiba and Ogawa 1977, Schlösser 1994,* as described in (Andersen et al., 2005)

Vitamin solution	
Component	Stock solution (g L^{-1} dH_2O)
Cyanocobalamin (vitamin B_{12})	5 mg

SM was prepared according to the recipe in Anderson, 2005. After autoclaved (for 20 minutes) and cool, the medium was stored in 1 liter reagent glass bottles in the fridge (4°C). Vitamin B_{12} solution was sterile filtered with 0,20 µm syringe filters (PES 0,20 µm), stored in 1 mL Eppendorf tubes and frozen (-18°C) and was only added to the solution, while inoculating new cultures: 1 mL L^{-1} culture medium.

Table 4 Composition of J. P. Jourdan's Medium

Jean-Paul Jourdan's medium (JM)	
Component	Concentration (g L^{-1} dH_2O)
$NaHCO_3$	8
$(NH_2)_2CO$	0,01
NaCl	5
KNO_3	2
K_2SO_4	1
$(NH_4)H_2PO_4$	0,2
$MgSO_4 \bullet 7H_2O$	0,2
$CaCl_2 \bullet 2\ H_2O$	0,1
$FeSO_4 \bullet 7H_2O$ solution (10g/L)	100 µL
Trace elements solution - same as in "*Spirulina* Medium"	1 mL

Because JM is about large-scale application, there is no information about how to prepare it for laboratory purposes, so for sterilizing it, research about its components had to be made. The final decision was to sterile filter the medium with 0,20 µm syringe filters, because urea degrades with heating, so it is not recommended to autoclave (Merck, 2000). After filtration, the medium was stored in the fridge (4°C). The pH and salinity of SM and JM was analyzed with pH and salinity meters (Accumet AB200 and TMC refractometer, respectively). SM has a salinity of 15 and pH of 9,77 and JM, a salinity of 10 and pH of 10,00.

Species

In this study, there were used two strains of the species *A. platensis.* The first one, referred to as strain 1 in this study (Fig. 4), is commonly known as the Lake Chad strain due to its origin being in this lake, in central Africa. The other one (strain 2), (Fig. 5) is a laboratory obtained strain, UTEX LB2340 (strain 2). Strain1 has a very pronounced floating behavior, (presumably due to the presence of gas vesicles found in some *A. platensis* strains (Kim, et. al. 2007), while strain 2 when not agitated tends to form filament aggregates hard to mix, that make experiments more difficult to perform.

Figure 4 Strain 1: *A. platensis* (Chad strain), optical microscope.

Figure 5 Strain 2: *A. platensis* (UTEX LB2340), optical microscope.

Inoculation of the cultures

The inoculation of new cultures was always prepared in sterile conditions, inside a Laminar Flow Chamber (UV sterile). The flasks and all the equipment were previously sterilized by autoclaving. Some of the "old cultures" were taken from the setup in its exponential phase and were used ca. 15mL of them to prepare the "new ones" in 1 L culture medium. This process was the same for all medium types and species strains (Fig. 6), not only to prepare for measurements, but to maintain the cultures in between experiments.

Figure 6 Inoculation of *A. platensis* cultures: A - culture flasks, medium, pipet, pipet tips and measuring cylinders inside the Laminar Flow Chamber; B - culture flasks after inoculation, for maintenance (from left to right: two flasks of strain 1 in SM and 2 flasks with JM).

Growth curves

As this cyanobacteria is arranged in multicellular filaments, it was not possible to determine its growth rate by cell counting, so it was estimated by optical density measurements, as detailed below.

1) Dry weight and optical density

These measurements are particularly important when there is interest in biomass production. To correlate optical density (OD) with dry weight (DW), a calibration curve for each medium was determined.

In phase 1, optical density of the cultures in each medium was read in triplicate for 12 days at 630 nm, using a spectrophotometer (Thermo Spectronic Genesys 6) and 2mL glass cuvettes, to do a growth curve. Remember that in this phase there were four culture flasks of strain 1 in the setup, one for each medium treatment: SM; SM+10; SM+20 and JM. This data was saved and graphed as OD vs Time.

To generate a calibration curve for dry weight determination, a series of 10 dilutions of concentrated culture were done with medium, each dilution with 10 mL of total volume (eg. 1:10; 2:10; 3:10 etc.). The optical density of these 10 dilutions was measured at 630 nm (Genesys 6). Then, the 10 mL volume of each dilution was filtered through glass microfiber filters, previously weighted, (VWR no. 693 – 1,2 µm), for this purpose a filtration set connected to a vacuum pump was used (AL17 220V – 50 Hz 0,1 kW) (Fig. 7). The 10 filters with the extracts for each strain were dried in a Binder dry oven at ± 60 °C for 48 h. After this period, the filters were weighted (Radwag AS220/C/2), and the dry weight (DW) of each dilution was determined as below. For a better perception, milligrams (mg) were the chosen units.

$$DW\ (mg) = Filter\ (mg) - (Filter\ (mg) + DW\ (mg)) \quad (1).$$

Then, the correlation between the optical density and the dry weight was graphed and a calibration curve was determined. The equation that resulted from this was used to extrapolate the dry weight of the cultures further in this study.

In phase 2, there were 3 flasks of strain 1 culture, as well as 3 flasks of strain 2 culture in the set, both with SM. The optical density measurements were performed in triplicate (technical replicates), with the same method as in phase 1, and a graph of OD vs Time was plotted. To determine the DW calibration curve of both strains, the same method was also used, a DW vs OD graph plotted, and equations used for further analysis.

Figure 7 Filtration set: A- vacuum pump; B- filtration flaks; C- dilutions of culture; D- glass microfiber filters.

2) Chlorophyll *a* extraction

Chlorophyll *a* is the only type of chlorophyll that cyanobacteria have. It was extracted from the cultures when they reached a concentrated state, OD > 0.200 (in our case, at least 6 days old), to improve spectrophotometric measurements of the extracts.

Th extraction method, the acetone extraction method, was based on Lorenzen's work (1967), for both phases of the experiment. Note again that phase 1 has one flask of strain 1 culture per treatment, and phase 2 has three flasks for strain 1 and three for strain 2 in SM, so for phase 1, technical replicates, and phase 2, biological and technical. Although there are more ways to extract pigments, the decision to choose this method was based in the previous experience with similar samples.

The following steps were measured in triplicate for each culture flask. Before the extraction, optical density of the samples to use were measured in the spectrophotometer at 630 nm (to later correlate with optical density of growth stage). Then, 5 mL of the culture were filtered through glass microfiber filters (VWR no. 693 – 1,2 µm), using the same filtering system mentioned above. The filters were then folded and put in 15 mL falcon tubes. 5 mL of 90 % acetone were then added to each of this falcon tubes. This samples were crushed with a glass stick to dissolve the pigments in the solvent as much as possible. The tubes were then stored in the fridge for 24 hours, at 4°C. In the next day, the falcon tubes with the acetone and filters were centrifuged at 4°C, 10 000 g for 15 minutes (Thermo Scientific™ Megafuge 16R, ThermoFisher). Always working on ice, the supernadant was carefully taken out and placed in new falcon tubes. The pellet was later discarded accordingly to the environmental safety measures of the UA. Each sample was then measured, in the spectrophotometer mentioned above, at 665 and 720 nm, according to Ritchie's equation for cyanobacteria (Ritchie, 2006):

$$\text{Chl } a \text{ (µg/mL)} = 12{,}9447\ (A_{665} - A_{720}) \qquad (2).$$

The data was then analyzed. The chlorophyll *a* acontent was compared to the optical density of the samples before extraction, and the dry weight extrapolated through the calibration curves equation, so it could be analyzed whether can a correlation happen between dry weight, optical density and chlorophyl content.

On phase 1, the chlorophyll *a* extraction was done on day 11 of growth and the results are regarding three technical replicates, as there is only one flask per treatment. One phase 2, this experiment was done on day 6, with three technical and biological replicates, as there were 3 flasks of each strain culture.

Photophysiology analysis

In the present study, RLCs were performed using a Multiple Excitation Wavelength Chlorophyll Fluorescence Analyzer - MULTI-COLOR-PAM (Walz, Germany), controlled by the software PamWin-3. A recirculation bath ultrathermostat (Frigiterm -10 6000382, Selecta) with a selected temperature was also used, so the RLCs were measured with constant selected temperature. To perform measurements in the MC-PAM it was used 1250 µL of sample in an optical glass cuvette (Hellma- 6030- 10mm). RLCs were all performed in triplicate, as specified below.

In phase 1, the RLCs of strain 1 with different salinities were generated having in mind that the used SM has a salinity of 15, and the author wanted to test photophysiological responses with higher salinity levels, to evaluate if in the future seawater could be used to grow *A. platensis,* so 35 was also used, as well as a level in between, 25. Industrial medium, which has a salinity of 10 was also tested. The samples were acclimated in the dark for 15min at ± 35°C, and the measurements in MC-PAM were also carried out at this temperature.

In phase 2, RLCs at different temperatures of the cultures of strain 1 and 2 with SM were done. As mentioned before, these cultures were not grown at the tested temperatures, so these measurements represent an acute stress test. The samples were acclimated in the dark for 30 min, at the stress temperature selected in the ultrathermostat. The temperatures chosen to run the tests were selected in an exploratory way. Considering that the growth temperature of the setup was ± 35°C, all the others were tested to analyze the culture resilience to different temperatures, and perhaps find a more suitable growth temperature. The temperatures used were [±°C]: 5, 15, 20, 25, 35, 45, 50, 55, 65.

In the software settings, the "blank" was done with the corresponding medium, before measurements and the measuring light was selected to 625nm (red light best suited for cyanobacteria). A protocol was prepared with 9 steps of light pulses of different irradiances (*E*) every two minutes: 0, 17, 60, 128, 222, 298, 506, 974 and 1634. The used parameters to plot the RLC were F_o and F'_m, to calculate rETR (relative PSII electron transport rate).These curves were plotted and analyzed, as well as the parameters α (initial slope of an ETR vs *E* curve), $rETR_m$ (maximum ETR in an ETR vs *E* curve), E_k (light saturation parameter of an ETR vs *E* curve) and E_{opt} (optimal irradiance). These measurements are used to access the healthiness of the cultures with different treatments.

Strain identification

The two strains used in this study are of the same species, however, as its visible on figures 4 and 5, they morphologically look very different under the microscope. Because of this, it was decided to genetically identify the strains. Although we have this information for strain 2, provided by the laboratory of origin, for 1 we don't, but still the author aimed to identify both, with PCR method. Research was made about the primers to use for the reaction, and the chosen pairs were CYA106F, CYA781R, cyanobacterium specific (Srivastava, et al., 2015) and 16S27F, 23S30R (Taton, et al., 2003). The nucleotide sequences are listed in table 5.

Table 5 Primer sequences

Primer	Sequence (5' → 3')
CYA106F	CGG ACG GGT GAG TAA CGC GTG A
CYA781R	GAC TAC TGG GGT ATC TAA TCC CAT T
16S27F	AGA GTT TGA TCC TGG CTC AG
23S30R	CTT CGC CTC TGT GTG CCT AGG T

1) DNA isolation

The first thing to do is to obtain biomass from the cultures. This is normally accomplished by the pellet resulting from centrifugation of the culture, and for strain 2 this works. However, for strain 1 after several tries to centrifuge it, it did not formed pellets, because of its highly floating behavior. Other method had to be used then, and filtration was chosen. For strain 1, some of the culture, ± 4 mL, was filtered through a glass microfiber filter (VWR no. 693 – 1,2 µm) using a filtration set connected to a vacuum pump (AL17 220V – 50 Hz 0,1 kW). Then, a tiny bit of the product of the filtering was scraped and put in a 1,5 mL Eppendorf tube. To this tube, 200 µL of elution buffer (Batch YL101, NZYTech) was added and mixed, 50µL taken out and put in another tube, and 150µL of elution buffer added, to reduce medium salts so they wouldn't interfere with the reaction.

As for strain 2, It can form pellets, so 1,5 mL of the culture was centrifuged in 1,5mL Eppendorf tubes, for 10 min, 15 000 g (Microcentrifuge 1524, Gyrozen, Korea), the supernatant discarded, the pellet washed with 200 µL elution buffer, and centrifuged again, so the number of medium salts was reduced. The supernatant was taken out again and 200 µL of elution buffer added and mixed.

The two Eppendorf tubes resulting from this (one for each strain), were then placed in a heat block (VWR digital heat block), 100 °C for 5 min.

2) DNA Amplification

The amplification was carried out in a total of 25 µL reaction mixture (Fig. 8), in 200 µL Eppendorf tubes, consisting of 24 µL of Super Master Mix (SMM) and 1 µL of the DNA isolate, one tube for each strain and primer pair, plus one negative (without DNA). The SMM was prepared in 1,5mL Eppendorf tubes and consists in a mixture of 63 µL of PCR grade water, 25 µL of NZYTaq II 2x green Master Mix (NZYTech), 4 µL of the forward primer and 4 µL of the corresponding reverse primer. These primers were previously prepared in a proportion of 1:10 (working stock solution and PCR grade water).

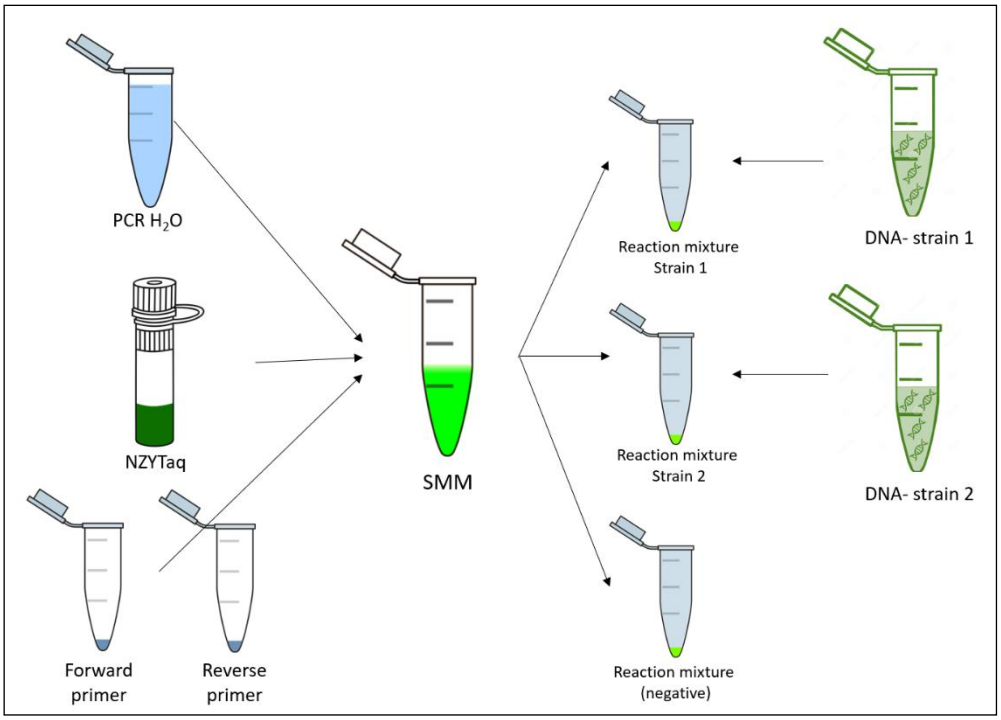

Figure 8 Reaction mixtures composition process.

The reaction mixture tubes were then used for PCR. This was carried out in two thermal cyclers: C1000 Touch Thermal Cycler, for the primer pair 16S27F / 23S30R, and BioRad MyCycler™ for CYA106F / CYA781R. Two were used, to optimize the time of the tasks, and were selected based on complexity of the cycle steps (the older equipment with the primer with least cycles and the most recent with the more complex protocol).

For the primer pair 16S27F / 23S30R the cycles were the following: first denaturation cycle of 10 min at 94°C, followed by 10 amplification cycles of 1 min denaturation at 94°C, 1 min

annealing at 57°C and 2 min elongation at 72°C, this was followed by more 25 amplification cycles of 1 min at 92°C, 1 min 54°C and 2 min 72°C, with a final elongation cycle of 10 min at 72°C. This thermal cycling profile was based on Taton, et. al., 2003, and slightly modified (the 1 min times were originally 45 s, and the first denaturation was 5 min) .

For the primer pair CYA106F / CYA781R the thermal cycling steps were performed as follows: 3 min initial denaturation at 94°C, followed by 35 amplification cycles consisting in 1 min denaturation cycle at 94°C, 1 min annealing at 59,5°C and an elongation of 2 min at 72°C. Final elongation at 72°C for 7 min (Srivastava, et al., 2015).

After obtaining the first results for these cycles, some adjustments of the steps were done, to obtain better results. The last try was carried out in C1000 Touch Thermal Cycler, for both primer pairs and the steps were 7 min denaturation cycle at 94°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 61°C and 2 min elongation at 72°C with a final 72°C elongation.

3) PCR product detection and analysis

After the PCR products were ready, the electrophoresis phase began. An 8 well agarose gel matrix was prepared. For this, 2% agarose gel was prepared: 1,2 g in 60 mL buffer consisting of 20 mL L^{-1} of 40 mM Tris-Acetate and 1 mM EDTA (Fisher Bioreagents). The agarose was then dissolved in the buffer by microwave heating and manual agitation. After it cooled down a little, it was poured in a 60 mL gel casting tray and a “comb” was placed in one end to form 8 wells. When completely cooled and solidified, the “comb” was removed from the gel, and the gel was removed from the tray. The gel was then placed in an electrophoresis tank (Horizon 11.14), filled with electrophoresis buffer, connected to a power supply (EC250-90). The agarose gel was covered by the buffer, to conduct the current. Then, 5 µL of NZY DNA Ladder VI (NZY Tech MB089) was poured in the first well. The PCR products were pipetted, one in each well (5 µL). The power was set to 90 V for 1 hour.

After this time, the gel was removed and immerse in an ethidium bromide solution, 2 drops of 10mg mL^{-1} in a 2 L tray, for 15 min, that works as an intercalating agent with nucleic acids and provides fluorescence, so it is possible so see the results of the electrophoresis. Then, the gel was placed in distilled water for another 15 min The agarose gel was then analyzed a in Molecular Imager (Gel Doc™ XR+ BioRad), connected to Image Lab™ Software.

RESULTS

Phase 1

Growth curves

1) Optical density and dry weight

Figure 9 shows the results for the optical density measurements (three technical replicates) over the measuring time of 12 days for four different medium treatments: original *Spirulina medium*, that had a salinity of 15, and *Spirulina medium* adjusted for the salinities of 25 and 35, as well as industrial medium (Jourdan's medium; JM). It has to be taken in consideration in this phase of the experience the data is regarding strain 1 results and that there is only one culture flask of each treatment. These results show that the evolution of growth is very similar for the various media until day 5, and that from that day on, the growth with SM became noticeably slower than with the other media.

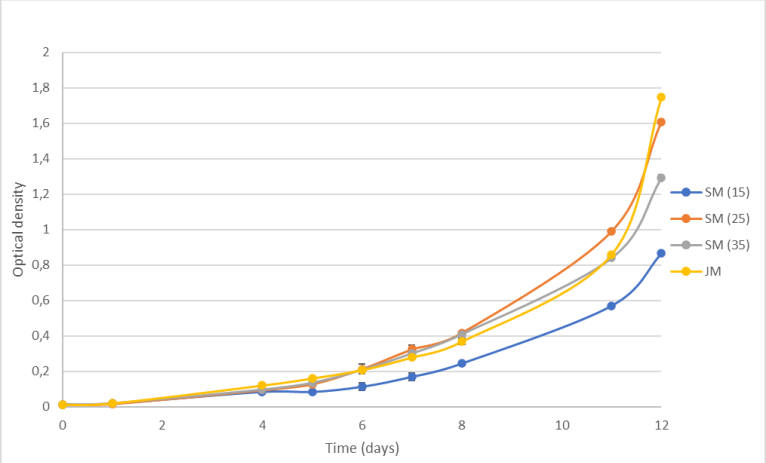

Figure 9 Optical density of strain 1 cultures over 12 days of counting, for four medium treatments (average data).

Figure 10 and Table 6 shows the observed relationship between dry weight and OD. The strong linearity that was found ensures that this relationship can be used as a calibration curve to predict dry weight from OD measurements. for each growth medium. The equations show that the results for the calibration are quite consistent (high R^2). It is visible that the medium with the highest salinity was the one for which DW reached highest values for the same OD. This medium was followed by JM, SM(25), and SM(15), that reached the lowest dry weight for the same OD.

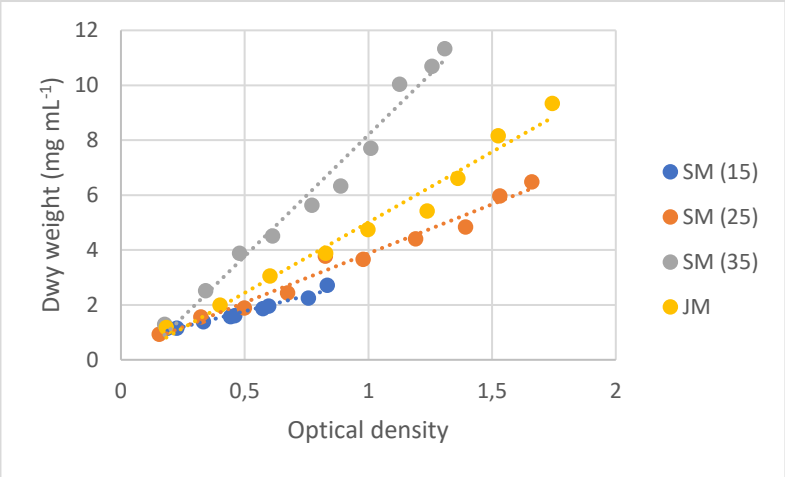

Figure 10 Dry weight calibration curves for the four treatments.

Table 6 DW equations and R^2 for the four treatments.

Medium	Equation	
SM (15)	y = 2,2772x + 0,6281	R^2 = 0,9663
SM (25)	y = 3,5793x + 0,2948	R^2 = 0,9767
SM (35)	y = 2,2772x + 0,6281	R^2 = 0,9663
JM	y = 5,1357x - 0,1311	R^2 = 0,9761

2) Chlorophyll *a* extraction

As previously mentioned, before chlorophyll *a* extraction, the OD of the cultures was measured, to later extrapolate the content of pigment per milligram of DW, based on the equations listed above (on Table 6).

In Figure 11 it can be seen that SM (15) reached the lowest OD of the four media, while SM (25), SM (35) and JM did not differ substantially . Despite this, JM proved to be the medium that yielded the lowest chlorophyll content per mL and per mg of DW, followed by SM (35). SM (25) happened to have the highest amount of chlorophyll per mL (Fig.12), but it was also the most concentrated one in this growth stage (11 days old), as confirmed by Fig. 9. However, per mg of DW, which is a result independent of the growth stage, SM presented the highest value of chlorophyll *a* content (Fig. 13).

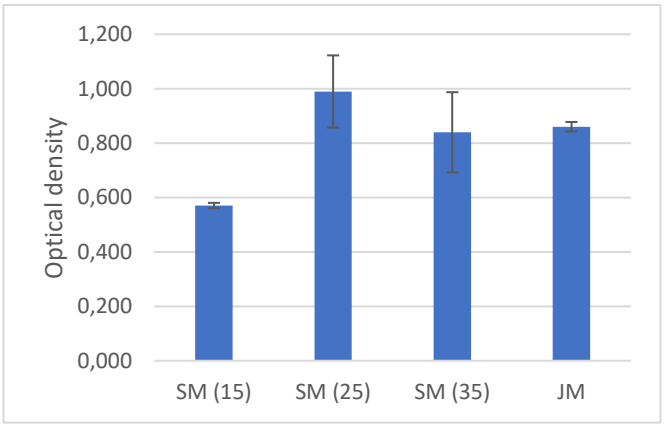

Figure 11 Optical density of the cultures on day 11 of each treatment, before extraction (average data of technical replicates).

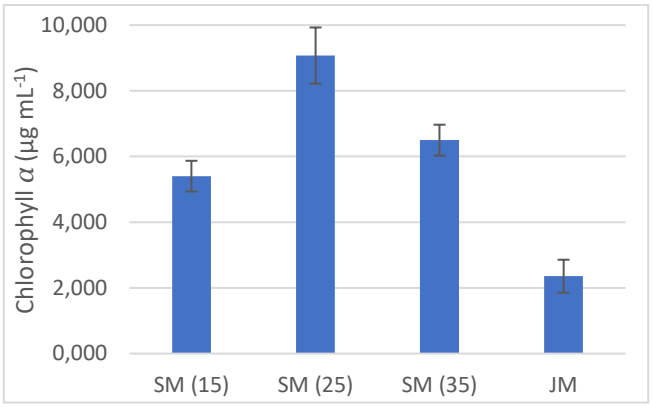

Figure 12 Chlorophyll *a* content per mL of culture, of each treatment.

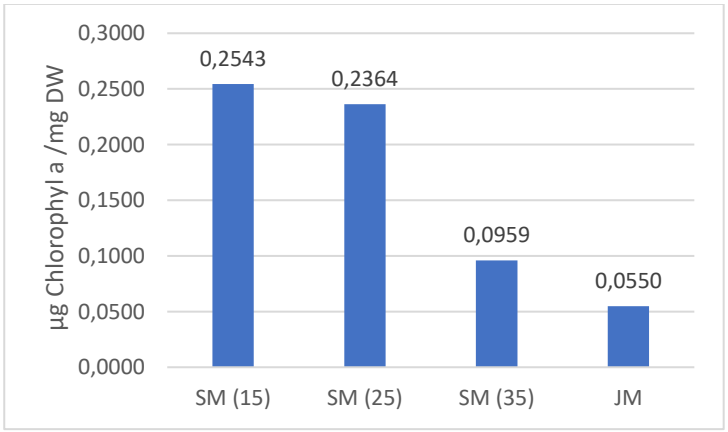

Figure 13 Chlorophyll *a* content per mg of DW as predicted from the relationships in Table 6.

Rapid Light Curves

The light response of rETR of the cultures grown under the various tested growth media, measured at growth temperature (± 35°C) is presented in Fig. 14. It is evident that the medium that yielded the highest values of rETR is the original medium SM, closely followed by SM (25). JM stands out for being the treatment that yielded the lowest photosynthetic efficiency at all irradiance levels.

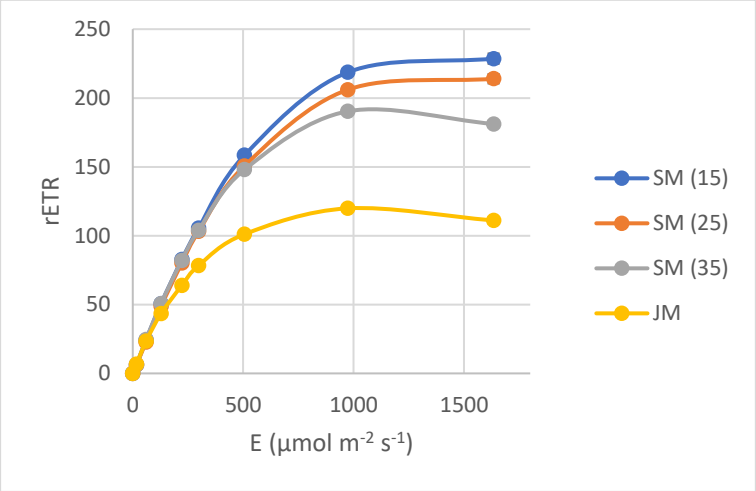

Figure 14 Light-response curves of rETR for the tested growth media (average data).

Figure 14 summarizes the variation of rETR vs E curves with medium salinity. The initial slope of the curve, α, appears not to vary significantly between treatments. In accordance with Fig. 15, rETRm reached its highest value with medium SM(15), followed by SM(25), SM(35), and with the lowest, JM. The photoacclimation parameter, E_k, and the optical intensity, E_{opt}, generally followed the pattern of variation of $rETR_m$ (Fig. 15).

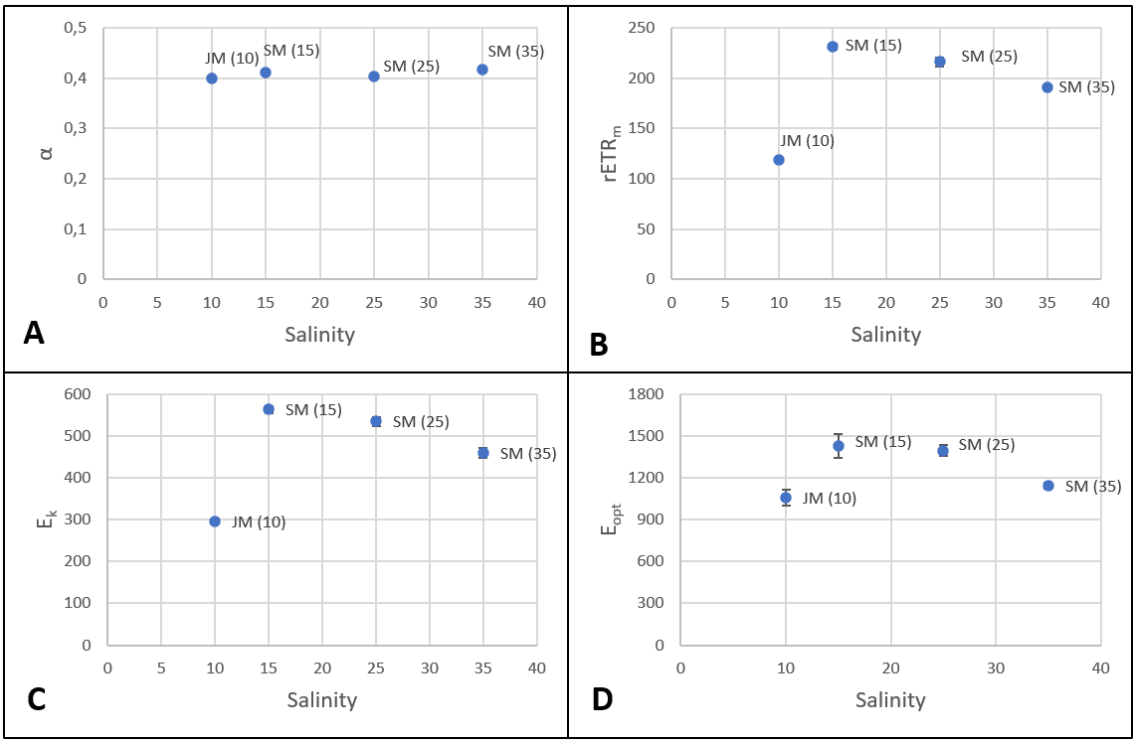

Figure 15 RLC parameters with salinity test, at growth temperature (35°C): α (A), $rETR_m$ (B), E_k (C), and E_{opt} (D).

Phase 2

Growth curves

1) Optical density and dry weight

In this phase the presented results regard both strain 1 and 2 cultures, growing in "*Spirulina medium,* Modified". Figure 16 displays the variation of OD over 12 days of culture growth. Both start with a similar OD, however, from day 4 on, it is visible that strain 2 starts to grow faster and that OD reaches higher OD values than strain1.

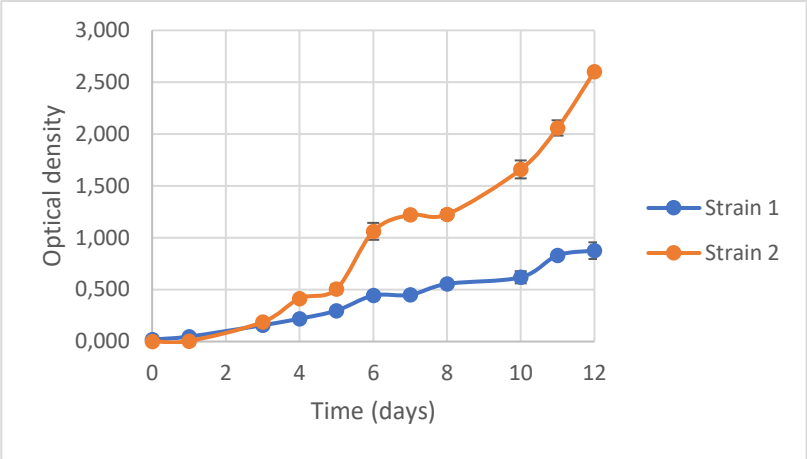

Figure 16 Variation of Optical density of the cultures of strain 1 and 2 over 12 days (average data for technical replicates).

In Figure 17 the DW vs OD results can be seen, and in table 7, the corresponding trendline equations. Strain 2 showed a higher R^2 than strain 1. Strain 1 seems to be able to reach a higher DW, as OD increases.

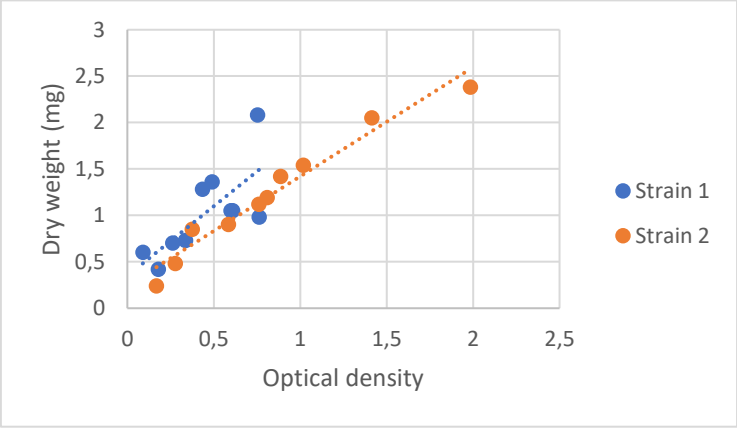

Figure 17 Dry weight vs optical density of strain 1 and 2.

Table 7 Trendline DW equations and R^2 for strain 1 and 2.

	Equation	
Strain 1	y = 1,4999x + 0,3487	R^2 = 0,5418
Strain 2	y = 1,1759x + 0,2443	R^2 = 0,958

2) Chlorophyll *a* extraction

At day 6 the OD was higher for strain 1 than for strain 2 (Fig. 18), meaning that the culture was less concentrated in this stage of growth, in accordance with Figure 17. Even though this is true, strain 1 showed a higher amount of chlorophyll *α* per mL (Fig. 19). Chlorophyll *a* per DW was not predicted.

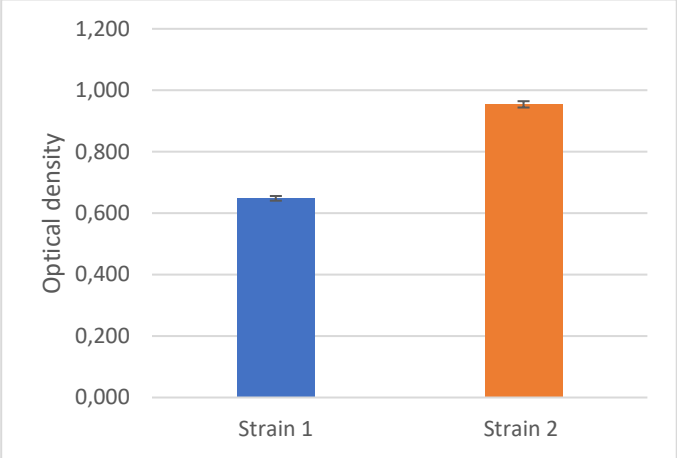

Figure 18 Optical densities of strain 1 and 2 cultures on day 6, before extraction (average data of technical and biological replicates).

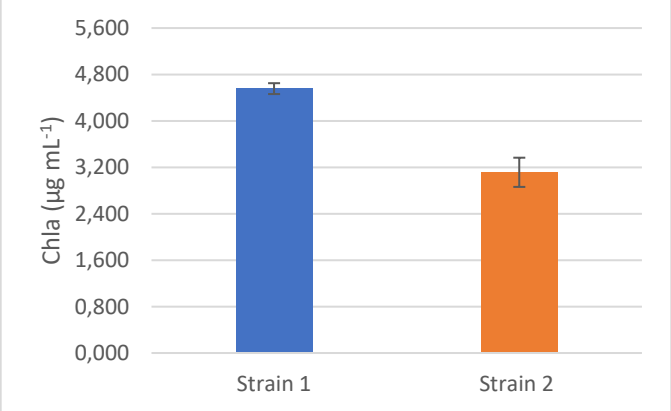

Figure 19 Chlorophyll *a* per mL of strain 1 and 2 cultures (average of technical and biological replicates).

Rapid Light Curves

In Figure 20, regarding strain 1, some of the tested temperatures are illustrated, so the trend of the responses would be perceptible, with higher and lower degrees of this acute test. The highest temperatures result in rETR values even higher than the growth temperature

ones (at 35°C), while lowest than that, show a lower rETR. The same happens with strain 2, in Figure 21, but with overall lower values of rETR.

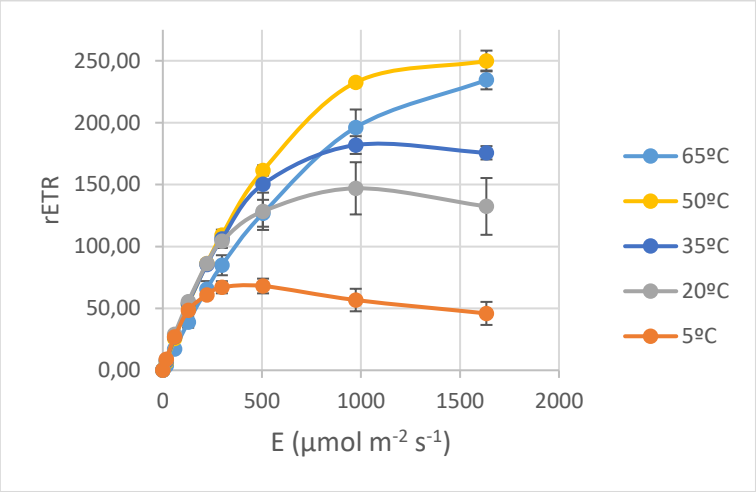

Figure 20 Light-response curves of rETR for the tested temperatures of [°C]: 5, 20, 35, 50, 65 – strain 1.

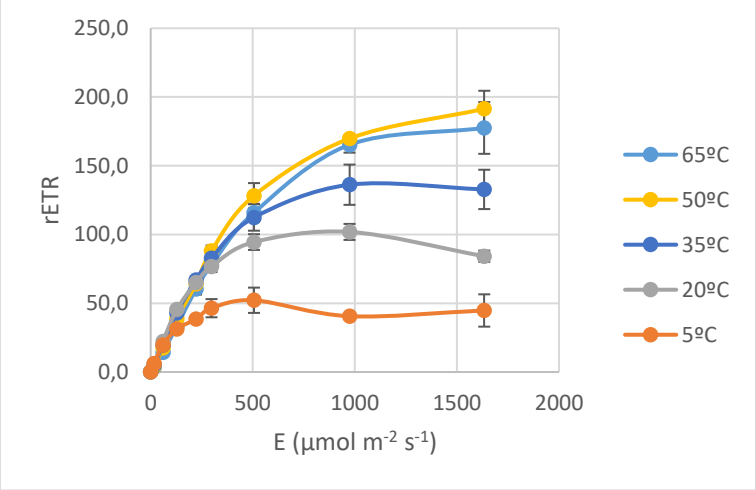

Figure 21 Light-response curves of rETR for the tested temperatures of [°C] 5, 20, 35, 50, 65 – strain 2.

In Fig. 22, the parameters summary of all the tested temperatures in strain 1 can be seen. The initial slope of the curve (α) appears to have a descending tendence, as the temperature rises. On the other hand, all the other parameters, have an ascending tendence, the peak of ETR_m being at 55°C. E_k and E_{opt} seem to have its highest value at 65°C, however, as the standard deviation is higher at those temperatures, the conclusion is not very precise. Strain 2, in Fig. 23 have similar tendence, however, the measurements have a higher standard deviation than in strain 1 results, making them harder to analyze.

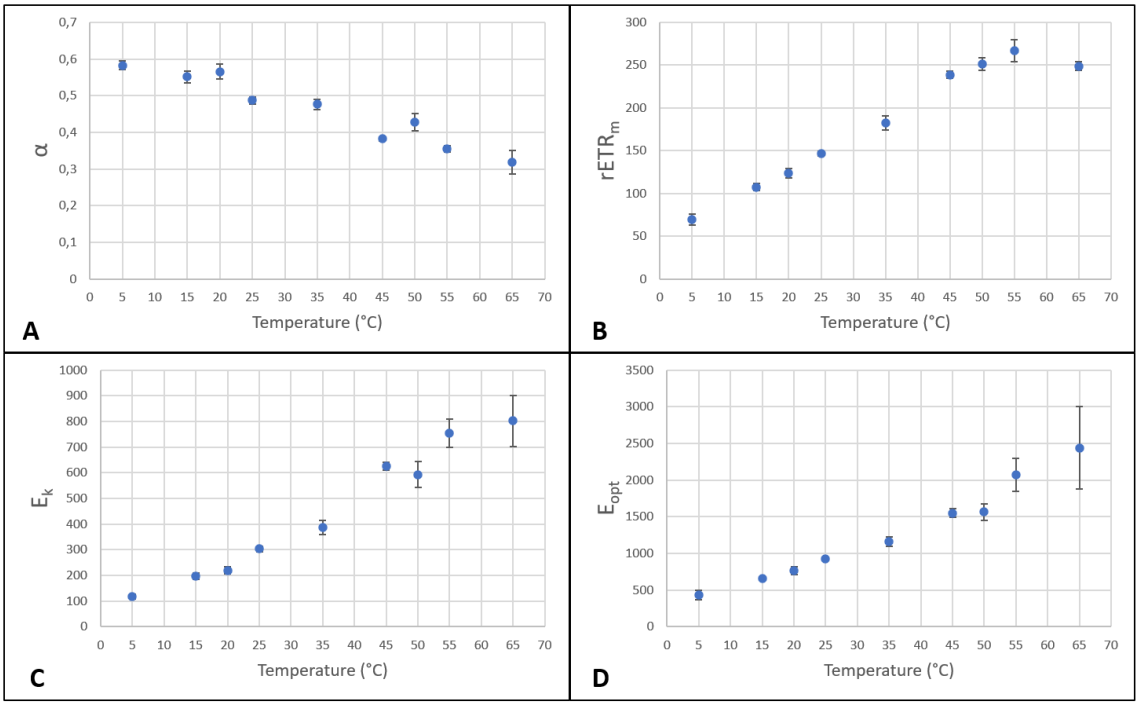

Figure 22 RLC parameters with temperature test in strain 1 [°C] 5, 15, 20, 25, 35, 45, 50, 55, 65. A (A), $rETR_m$ (B), E_k (C), and E_{opt} (D).

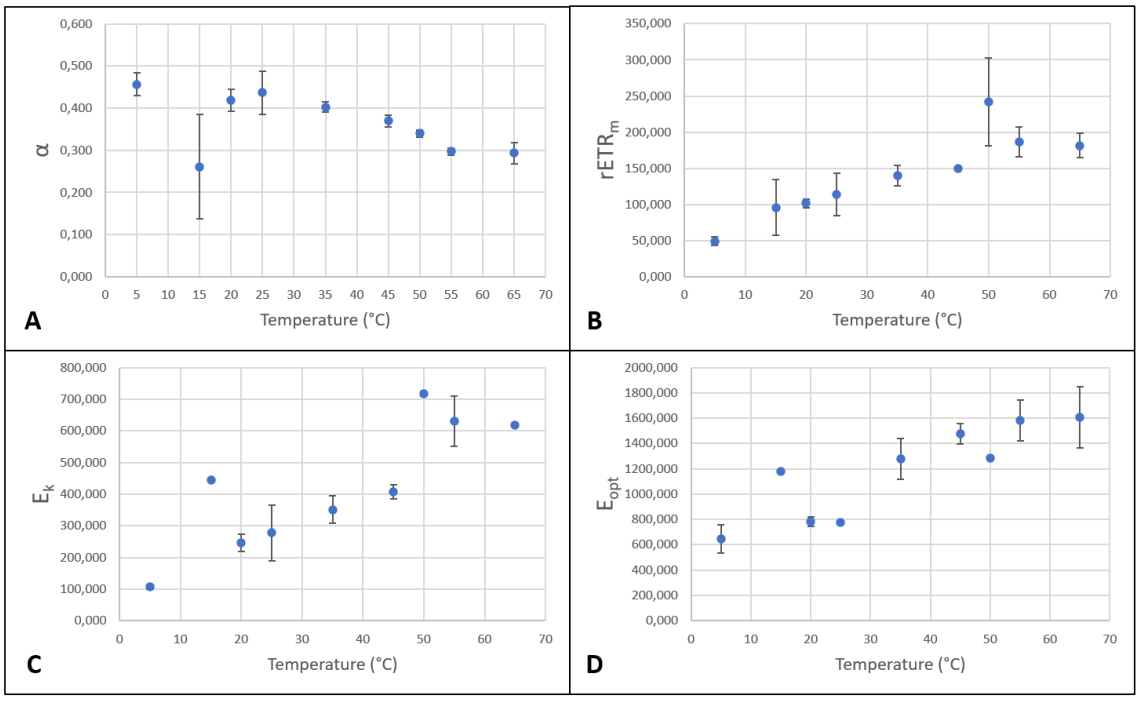

Figure 23 RLC parameters with temperature test in strain 2 [°C] 5, 15, 20, 25, 35, 45, 50, 55, 65. A (A), $rETR_m$ (B), E_k (C), and E_{opt} (D).

Strain identification

On Fig. 24 and 25 the resulting bands for both strains and primer pairs don't give very precise information: CYA106F / CYA781R primer pair bands results look quite faint, while 16S27F / 23S30R appear more intense, but both strains look like they are not a single band.

A second try was carried out, (with the thermocycler and cycles described in materials and methods), and the resulting profile electrophoresis gel analysis is in Fig. 26. Here, the results for both used primer pairs and strains, clearly have more than one band and CYA106F / CYA781R shows a positive negative control, indicating contamination with DNA, therefore the DNA products were not reliable for sequencing.

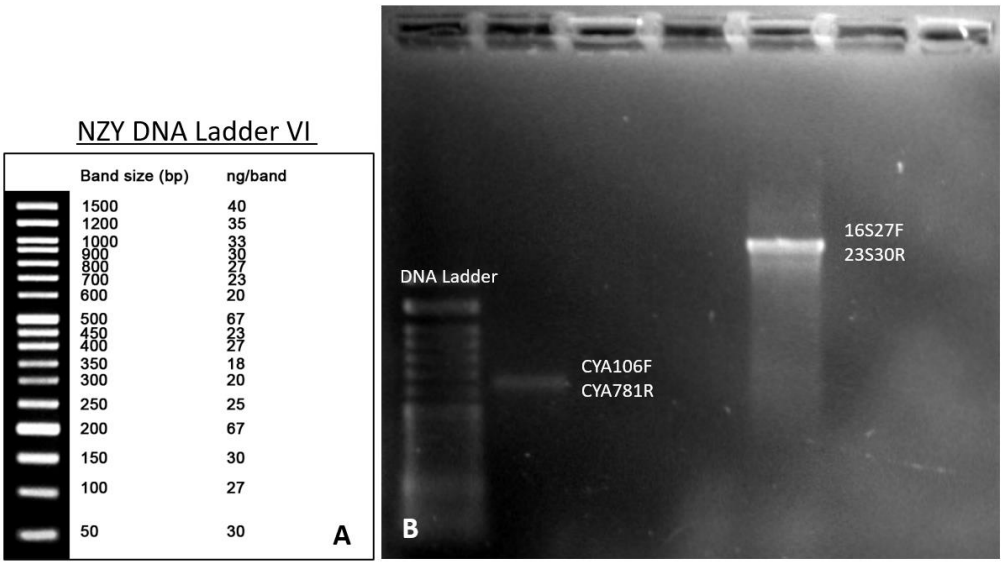

Figure 24 PCR product detection profile in BioRad with Image Lab™ software results with first described cycles, for strain 1, A- NZY DNA Ladder VI; B- Strain 1 with primer pairs CYA106F / CYA781R and 16S27F / 23S30R.

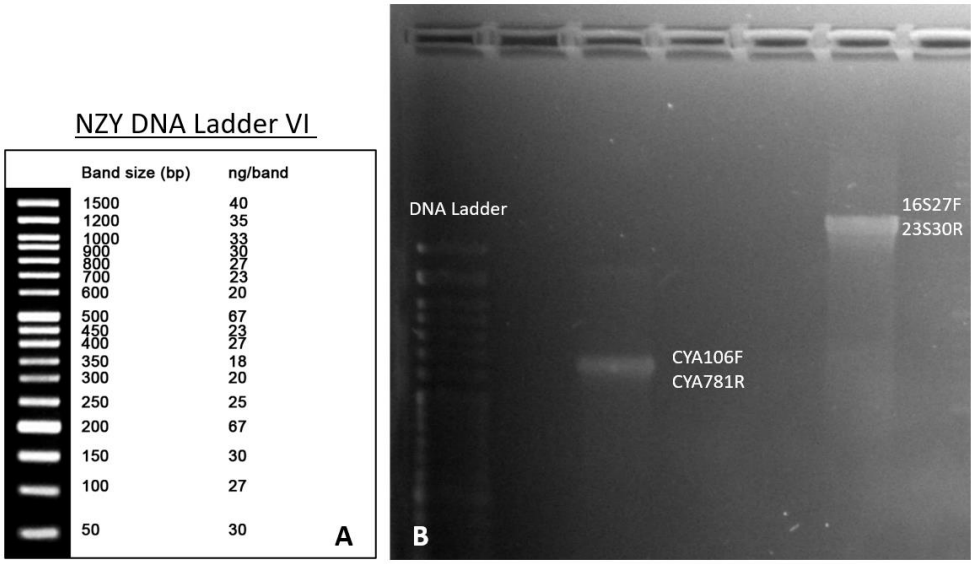

Figure 25 PCR product detection profile in BioRad with Image Lab™ software results with first described cycles, for strain 2, A- NZY VI DNA Ladder VI; B- strain 2 with primer pairs CYA106F / CYA781R and 16S27F / 23S30R.

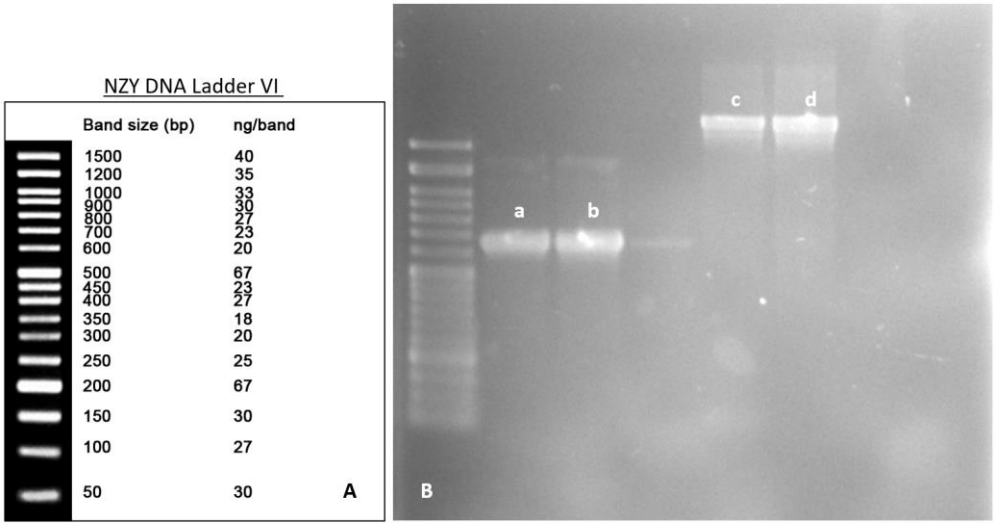

Figure 26 PCR product detection profile in BioRad whith Image Lab™ software results with second, modified, described cycles, for strain 1 and 2: A- NZY VI DNA Ladder; B- a) strain 1 with primer pair CYA106F / CYA781R; b) strain 2 with primer pair CYA106F / CYA781R; c) strain 1 with primer pair16S27F / 23S30R; strain 2 with primer pair16S27F / 23S30R.

DISCUSSION

Phase 1- Growth curves and photophysiology

It is remarkable that the cultures grew and adapted quite well to the all the media and salinities tested in this study, considering the standard laboratory media being 15 and industrially used being 10. The results showed that SM (15) yielded the lowest maximum OD, which can be due to the low amount of sodium bicarbonate in the medium, when compared to SM (25) and SM (35). As for JM, even though its salinity is the lowest (10), the presence of urea in this medium makes it with a more concentration of salts, hence its higher OD. This also happens in the DW correlation results shown in Figure 13, in which the mediums with higher salt reaching the highest DW vs OD. We can assume that the denser the medium, the higher weight will result, but not necessarily means a greater number of cells. It also doesn't mean that the cultures are healthier or have more pigments than the others, as it's visible on the result for this phase of the study.

This is confirmed by the chlorophyll *a* content, crucial for photosynthesis to occur. JM resulted in the lowest chlorophyll *a* content, even though apparently having one of the highest growths, per mL and per mg of DW. On the other hand, OD and DW results for SM (15) cultures could lead us to think that the cells do not produce as much pigment as the others, however, happens to be the medium that provides the best chlorophyll *a* content per mg powder, closely followed by SM (25). It is impressive to realize that the medium used in industry happens to be the one that yields the lowest pigment content per cell of all the tested treatments. Even seawater salinity like medium SM (35) yielded a higher chlorophyll *a* content. Comparing chlorophyll *a* content per mL and per mg of DW, differences can be seen, for example, SM (25) shows higher pigment per mL then per DW, and SM (35) higher chlorophyll *a* per mL than SM (15), but a lot lower for DW results, then again it had a higher OD in this stage of growth, and DW results were an extrapolation, independent of growth stage. Looking at the results, SM (15) and SM (25) look very similar in terms of chlorophyll *a* content. A criticism to this, the DW extrapolation might not be true for all phases of growth, because the growth is not linear, as well as chlorophyll *a* content, it might vary. In the future, to improve these results, more chlorophyll *a* extraction experiments tests should be done, in different stages of growth. Also, a very important thing is to maximize the replicates to biological ones, apart from technical.

If doubts about healthiness of cultures remained, RLC gave the extra information needed to confirm that SM (15) was the best medium in terms of chlorophyll *a* content, hence the best one in supporting photosynthetic activity. The results presented in Figures 14 and 15

show that the efficiency of PSII has its highest values with SM (15) but is very close to SM (25). SM (35) is not behind by far and appears to be more efficient than the industrial one (JM), that happens to be, again the one with lowest values. All the parameters showed this same trend. This data would benefit from statistical data analysis, to understand if the mediums with best results are significantly different, and if we can confidently say that the best growth medium is SM (15). However, we can state that it is indeed possible to grow *A. platensis* (at least strain 1) with seawater, adjusting nutrient parameters, which is a very important conclusion, to reduce freshwater intake from industries. It would be important to carry out a salinity test in strain 2, to evaluate if it behaves like strain 1. Salinity stress studies have been carried out in the past. Al Mahrouqi, et al. (2015) did an experiment very similar to the one carried out in this study, and concluded that this cyanobacteria shows an interesting potential to grow in seawater (Al mahrouqi et al., 2015), same conclusion in the present study. Vonshakl, el al. (1996) also tested different salinities, and concluded that growth rates decreased, however this also happened in the present study, and it does not mean that seawater cannot still be used, even mixed with freshwater, still reducing its intake. Faucher, et. al (1979), also claimed that saltwater functions as good as laboratorial medium for another species of *Arthospira, Arthrospira maxima.* But there is still room for more testing, as there are few studies on this.

Phase 2 - Growth curves and photophysiology

The growth rate in phase 2 is also interesting to look at (Fig. 16). Here there are two strains, both growing in SM medium. Strain 1 grew identically to SM (15) in phase 1, as expected, but on the other hand, strain 2 was a lot faster in reaching higher OD values and appears that would be growing as fast after the 12 days of measurements, while strain 1 seemed to be reaching a steady state. But it is important to have in consideration that strain 2 comes from laboratory, while strain 2 comes from industrial facilities, hence having to adapt to laboratory conditions, strain 2 was already adapted. To improve these results, and come to more accurate conclusions, the measurements could have been carried out during a longer period. There is an interesting information however, coming from the laboratory which provided strain 2, that it grew exponentially for at least 100 days (Inês Macário, et. al, unpublished data). Nevertheless, we can say that strain 2 is more interesting to produce than strain 1, in terms of growth rate.

Regarding the biomass experiment, from DW, (Fig. 17), there is not much that can be compared, because the experiment with strain 1 did not work as expected and the results are not very reliable, due to low R^2 (although it cannot be confirmed confidently due to lack of statistical analysis), however it does look that strain 1 would reach higher DW amounts than

strain 2 for the same OD, but there is also to consider that the growth is not linear, hence DW results can lead to some misinterpretations. More tries would have to be carried out to get better results and generate more reliable curves.

Even without considering DW results, the chlorophyll *a* content results show that, clearly, strain 1 reaches higher a biomass than strain 2, because even though it had lower OD in that growth stage, it had higher pigment per milliliter. So, this leads to the conclusion that, as mentioned above, strain 2 could be better for reaching higher biomass quicker, but not necessarily the best quality in pigment, which is an important asset for industry to enhance the value of the product.

As chlorophyll *a* content of strain 1 culture is higher, this also means that the photosynthetic apparatus will have a higher efficiency, which is confirmed by the comparison of RLCs on Figures 20 and 21: strain 1 shows overall higher rETR values than strain 2.

The variation of RLCs with the temperature are the big surprise of this study. The results show a clear preference for higher temperatures than the established optimum of 35°C. It is impressive to realize that, from 5 to 65°C the cultures survive and maintain some photosynthetic efficiency. The peak being 55°C for strain 1 and 50°C for strain 2, even considering the standard errors (Figs. 22 and 23). The results of the parameters $rETR_m$, E_k and E_{opt} show for both strains an increasing tendency towards higher temperatures, and α decreasing tendency, although a higher standard error in strain 2 indicates that the measurements were harder to perform. This is true, there is difficulty to generate RLCs with both these strains of *A. platensis*: strain 1 has a very pronounced floating behavior, and even with a stir magnet the culture was not always homogenic. Strain 2 has a behavior that, if there is no agitation it forms thick aggregations of filaments, which also makes it difficult to analyze, because stir magnet agitation inside the MC-PAM was not strong enough to prevent from this. For industrial production, this characteristic of strain 2 might not be so interesting, because the agitation of cultures has to be constant, increasing energy costs, opposite of strain 1, that even though it floats, it does not aggregate so it is very easy to mix. The floating behavior is described for *A. platensis* (Kim et al., 2005, Kim, et al. 2007), but not per strain, and this study proved that the behavior of both strains is completely different.

From the author's research results, there are almost no studies subjecting *Arthrospira* cultures to such high temperatures as in this study. Soni, et al. (2019) studied the growth between summer and winter, and concludes the best growth occurs in summer, but does not state its degrees. Colla, et al. (2015), however carried out a long-term temperature tolerance test, with 25, 40 and 50^{o}C and came to the conclusion that at 40 and 50 the growth decreases after 50 days of exposure (Colla et al., 2015). Also, Chaiklahan, et. al. (2007) compared

photophysiology of cultures of *A. platensis* grown at 35 and 43°C, and concluded that the higher temperatures decrease photosynthetic activity These studies are a good complement of information to the author, because the conclusions of the present study show a preference for higher temperatures, nevertheless there is no information about the strain in the mentioned study, so we do not know if the same would happen to the author's experiment.

The light intensity effect is a parameter that was not explored in this study, but published data show that this factor has a major role in growth (Nosratimovafagh et al., 2022). This is a topic not yet very explored, because of this, would be interesting to study.

Genetic identification and analysis

Amplification of the target genes (X and Y) did not not result in PCR products ready to sequence. However, with more time to investigate and explore of cycle profiles, this primer pairs should work, as they have published data of *Arthrospira* strain identification (Taton et al., 2003; Srivastava et. al. , 2015; Nübel et al., 1997). Unfortunately, the second try showed contamination in the negative control, that could have happened due to DNA contamination of the compounds used, or even the materials. Ideally, the results should have shown an unquestionably single band, to then use the PCR product for nucleotide sequencing, to identify the strain. Even though the author has information about strain 2 (UTEX LB2340), there is no published strain identification for strain 1, the *Chad strain*. To go deeper on genetical identification study on thus would be of maximum importance, as is one of the most used in industry. In fact, there is very little information about genetic identification of *Arthrospira / Spirulina* and primer pairs choice was harder than expected.

CONCLUSION

Considering the market role this supplement has, it is impressive that there is not much information about genetical identification. In fact, people never know which strain of the organism they are consuming, and for the little results in this study, it is visible that different strains have, for example different chlorophyll content, and presumably they could vary in other aspects, like phycocyanin, carotenoid and even overall protein, so it is obvious that this topic requires more attention.

This study would benefit of statistical analysis to better interpret the results. This being stated, the medium that showed to be the best in photosynthetic efficiency as well as amount of chlorophyll *a* pigment is the original laboratory one, "*Spirulina* medium, Modified" for both strain 1 and 2, nevertheless, the same medium composition with salinities of 25 and 35, show that strain 1 of *A. platensis* easily adapts to higher salinity changes (at least until 35), and still showing to be photosynthetically efficient. Both strains have shown to resist to a wide range of temperatures in an acuity test, from 5 to 65^{o}C, even preferring higher temperatures than its optimum described in literature, 35^{o}C, this would benefit of further investigation with long term exposure. Strain 2 have shown to be the best choice if the goal is to produce high volumes of biomass quicker, with the downside of having to be agitated all the time. However, strain 1 shows to be the one capable of maintaining best photosynthetic efficiency and pigment composition, also with the advantage of being easiest to mix, so better for production energy costs on that matter.

The author considers that, although there is a lot of information published, further research is needed in terms of medium improvement and strain identification. *A. platensis* is highly valuable source of protein, with endless health benefits. Its study and industrial application to make the best quality product is essential, so it can be consumed at its peak of benefits and hopefully help in a greener economy.

BIBLIOGRAPHY

Publications

Almahrouqi, H. A., Naqqiuddin, M. A., Achankunju, J., Omar, H., & Ismail, A. (2012). Different salinity effects on the mass cultivation of *Spirulina (Arthrospira platensis)* under sheltered outdoor conditions in Oman and Malaysia. *Journal of Algal Biomass Utilization*, *28*(1), 9227.

Andersen, R. A., Berges, J. A., Harrison, P. J., & Watanabe, M. M. (2005). Recipes for Freshwater and Seawater Media. *Algal Culturing Techniques*, *January*, 429–538. https://doi.org/10.1016/b978-012088426-1/50027-5

Araújo, R., Vázquez Calderón, F., Sánchez López, J., Azevedo, I. C., Bruhn, A., Fluch, S., Garcia Tasende, M., Ghaderiardakani, F., Ilmjärv, T., Laurans, M., Mac Monagail, M., Mangini, S., Peteiro, C., Rebours, C., Stefansson, T., & Ullmann, J. (2021). Current Status of the Algae Production Industry in Europe: An Emerging Sector of the Blue Bioeconomy. *Frontiers in Marine Science*, *7*(January), 1–24. https://doi.org/10.3389/fmars.2020.626389

Averina, S., Senatskaya, E., & Pinevich, A. (2020). *Far-Red Light Absorbing Photosynthetic Pigments in Cyanobacteria: Steady-State Fluorescence Detection, Time-Resolved Fluorescence Spectroscopy, and Confocal Laser Scanning Microscopy* (N. Grigoryeva (ed.); p. Ch. 2). IntechOpen. https://doi.org/10.5772/intechopen.92776

Blankenship, R. E. (2010). Early evolution of photosynthesis. *Plant Physiology*, *154*(2), 434–438. https://doi.org/10.1104/pp.110.161687

Chaiklahan, R., Khonsarn, N., Chirasuwan, N. Ruengjitchatchawalya, M., Bunnag, B. & Tanticharoen, M. (2007). Response of Spirulina platensis C1 to high temperature and high light intensity. *Kasetsart Journal- Natural Science, 21,* 123-129.

Colla, L. M., Bertol, C. D., Ferreira, D. J., Bavaresco, J., Costa, J. A. V., & Bertolin, T. E. (2015). Estabilidade térmica e fotoestabilidade do potencial antioxidante de *Spirulina platensis* em pó. *Brazilian Journal of Biology*, *77*(2), 332–339. https://doi.org/10.1590/1519-6984.14315

Demoulin, C. F., Lara, Y. J., Cornet, L., François, C., Baurain, D., Wilmotte, A., & Javaux, E. J. (2019). Cyanobacteria evolution: Insight from the fossil record. *Free Radical Biology and Medicine*, *140*(November 2018), 206–223. https://doi.org/10.1016/j.freeradbiomed.2019.05.007

Dillon, J. C., Phuc, A. P., & Dubacq, J. P. (1995). Nutritional Value of the Alga Spirulina. In *World*

Review of Nutrition and Dietetics (pp. 32–46). https://doi.org/10.1159/000424464

Faucher, O., Coupal, B., & Leduy. (1979). Utilization of seawater-urea as a culture medium for *Spirulina maxima. Canadian journal of Microbiology, 25*(6), 752-759. https://doi.org/10.1139/m79-109

Genty, B., Briantais, J.-M., & Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *990*(1), 87–92. https://doi.org/https://doi.org/10.1016/S0304-4165(89)80016-9

Gershwin, M. E., & Belay, A. (2007). *Spirulina in Human Nutrition and Health*. CRC Press. https://books.google.pt/books?id=Vz7SpjEsE3IC

Habib, M. A. B., Parvin, M., Huntington, T. C., & Hasan, M. R. (2008). A review of culture, production and use of Spirulina as food for humans and feeds for domestic animals and fish. In *FAO Fisheries and Aquaculture Circular. No. 1034. Rome, FAO. 33p.* (Issue 1034).

Heierli, U., & Weid, D. Von Der. (2007). Sustainable approaches to combat malnutrition. *Osborn, P. Médiateurs*, 1–84. www.antenna.ch/medias/spirulina-casestudy.pdf

Herdman, M., Janvier, M., Waterbury, J., Rippka, R., Stainer, R., & Mandel, M. (1979). Deoxyribonucleic Acid Base Composition of Cyanobacteria. *Microbiology-Sgm*, *111*, 63–71. https://doi.org/10.1099/00221287-111-1-63

INE. (2021). Estatísticas da Pesca 2020. In *Estatísticas da Pesca 2020*.

Kadri, K. (2019). Polymerase Chain Reaction (PCR): Principle and Applications. In M. L. Nagpal, O. M. Boldura, C. Baltă, & S. Enany (Eds.); *Synthetic Biology* (pp.147-165). IntechOpen. https://doi.org/10.5772/intechopen.86491

Karkos, P. D., Leong, S. C., Karkos, C. D., Sivaji, N., & Assimakopoulos, D. A. (2011). *Spirulina* in Clinical Practice: Evidence-Based Human Applications. *Evidence-Based Complementary and Alternative Medicine*, *2011*, 531053. https://doi.org/10.1093/ecam/nen058

Kim, S.-G., Choi, A., Ahn, C.-Y., Park, C.-S., Park, Y.-H., & Oh, H.-M. (2005). Harvesting of *Spirulina platensis* by cellular flotation and growth stage determination. *Letters in Applied Microbiology*, *40*(3), 190–194. https://doi.org/https://doi.org/10.1111/j.1472-765X.2005.01654.x

Kim, T., Choi, W.-S., Ye, B.-R., Heo, S.-J., Oh, D., Kim, S., Choi, K.-S., & Kang, D.-H. (2018). Cultivating *Spirulina maxima*: Innovative Approaches. *Cyanobacteria*. https://doi.org/10.5772/intechopen.74265

Masuda, K., Inoue, Y., Inoue, R., Nakamura, A., Chitundu, M., Murakami, J., Ota, Y., & Matsugami, J. (2014). Spirulina Effectiveness Study on Child Malnutrition in Zambia. *Institute of Development Studies*, *September*, 49–56.

Mayers, J., Ekman Nilsson, A., Svensson, E., & Albers, E. (2016). Integrating Microalgal Production with Industrial Outputs—Reducing Process Inputs and Quantifying the Benefits. *Industrial Biotechnology*, *12*. https://doi.org/10.1089/ind.2016.0006

MERCK. (2000). Microbiology Manual. *Unitech Communications, Faisalabad*, 237.

Mobin, S., & Alam, F. (2017). Some Promising Microalgal Species for Commercial Applications: A review. *Energy Procedia*, *110*, 510–517. https://doi.org/https://doi.org/10.1016/j.egypro.2017.03.177

Murchie, E. H., & Lawson, T. (2013). Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983–3998. https://doi.org/10.1093/jxb/ert208

Narala, R. R., Garg, S., Sharma, K. K., Thomas-Hall, S. R., Deme, M., Li, Y., & Schenk, P. M. (2016). Comparison of microalgae cultivation in photobioreactor, open raceway pond, and a two-stage hybrid system. *Frontiers in Energy Research*, *4*(AUG), 1–10. https://doi.org/10.3389/fenrg.2016.00029

Nosratimovafagh, A., Fereidouni, A. E., & Krujatz, F. (2022). Modeling and Optimizing the Effect of Light Color, Sodium Chloride and Glucose Concentration on Biomass Production and the Quality of Arthrospira platensis Using Response Surface Methodology (RSM). *Life*, *12*(3), 1–19. https://doi.org/10.3390/life12030371

Office, A. S., & Elvet, O. (2000). *CHARACTERIZATION OF ARTHROSPIRA (SPIRULINA) STRAINS BY A thesis submitted to the Department of Biological Sciences University of Durham In accordance with the requirements for the degree of Doctor of Philosophy*.

Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research*, *89*(1), 27–41. https://doi.org/10.1007/s11120-006-9065-9

Sánchez-Bayo, A., Morales, V., Rodríguez, R., Vicente, G., & Bautista, L. F. (2020). Cultivation of Microalgae and Cyanobacteria: Effect of Operating Conditions on Growth and Biomass Composition. *Molecules (Basel, Switzerland)*, *25*(12). https://doi.org/10.3390/molecules25122834

Schirrmeister, B. E., Gugger, M., & Donoghue, P. C. J. (2015). Cyanobacteria and the Great Oxidation Event: evidence from genes and fossils. *Palaeontology*, *58*(5), 769–785. https://doi.org/https://doi.org/10.1111/pala.12178

Schreiber, U. (2004). Chlorophyll a Fluorescence Advances in Photosynthesis and Respiration. In *Advances in Photosynthesis and Respiration*.

Schreiber, U., Schliwa, U., & Bilger, W. (1986). Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation

fluorometer. *Photosynthesis Research*, *10*(1), 51–62. https://doi.org/10.1007/BF00024185

Shih, P. M., Wu, D., Latifi, A., Axen, S. D., Fewer, D. P., Talla, E., Calteau, A., Cai, F., Tandeau De Marsac, N., Rippka, R., Herdman, M., Sivonen, K., Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K. W., Han, C. S., Rubin, E. M., … Kerfeld, C. A. (2013). Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(3), 1053–1058. https://doi.org/10.1073/pnas.1217107110

Srivastava, A., Tiwari, R., Srivastava, V., Singh, T. B., & Asthana, R. K. (2015). Fresh water cyanobacteria *Geitlerinema sp.* CCC728 and *Arthrospira sp.* CCC729 as an anticancer drug resource. *PLoS ONE*, *10*(9), 1–18. https://doi.org/10.1371/journal.pone.0136838

Taton, A., Grubisic, S., Brambilla, E., De Wit, R., & Wilmotte, A. (2003). Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): A morphological and molecular approach. *Applied and Environmental Microbiology*, *69*(9), 5157–5169. https://doi.org/10.1128/AEM.69.9.5157-5169.2003

Vincent, W. F. (2009). *Cyanobacteria* (G. E. B. T.-E. of I. W. Likens (ed.); pp. 226–232). Academic Press. https://doi.org/https://doi.org/10.1016/B978-012370626-3.00127-7

Zhang, F., Yu, J., Zhang, L., Sheng, J., Yuan, M., Lu, Y., & Wang, J. (2017). UV-Vis Spectrum Characteristics of Phycocyanin Purification in Water from Chao Lake. *Guang pu xue yu guang pu fen xi = Guang pu*, *37*(3), 806–810.

Legislation

Commission Decision (EU) 2022/1244 (2022). https://eur-lex.europa.eu/eli/dec/2022/1244/oj

Directive 2002/46/EC of the European Parliament and of the Council (2002) https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32002L0046&from=PT

Instituto Nacional de Estatística - Estatísticas da Pesca: 2021. Lisboa: INE, 2022 https://www.ine.pt/xurl/pub/36828280

Regulation (EU) No 1169/2011 of the European Parliament and of the Council (2011). https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32015R2283&from=PT

Websites

Algaebase. (2019, December 18). *Arthrospira platensis Gomont 1892.* https://www.algaebase.org/search/species/detail/?species_id=24749

European Comission. *Novel Food. What is a Novel Food?* https://food.ec.europa.eu/safety/novel-food_en , accessed on September 2022.

ESA. (2016, October 3). *A comida dos astronautas ajuda pessoas na Terra.* The European Space Agency.https://www.esa.int/Space_in_Member_States/Portugal/A_comida_dos_astronautas_ajuda_pessoas_na_Terra

Fédération des Spiruliniers de France (2022, September). *La Spiruline.* https://www.spiruliniersdefrance.fr/ , accessed on September 2022.

Gerretsen, I. (2022, January 13). *Could our understanding of the deep ocean help unlock the mysteries of our space? Nasa's space mission is leading us to unexplored depths of our own planet*. BBC. https://www.bbc.com/future/article/20220111-why-nasa-is-exploring-the-deepest-oceans-on-earth

Lambers, H., Bassham, Alan, J. (2022, August 13). *Photosynthesis.* Encyclopedia Britannica. https://www.britannica.com/science/photosynthesis

NOAA. *How much oxygen comes from the ocean.* National Ocean Service. https://oceanservice.noaa.gov/facts/ocean-oxygen.html, accessed on September 2022.

Windley, B. (2021, August 2). *Proterozoic Eon*. Encyclopedia Britannica. https://www.britannica.com/science/Proterozoic-Eon