POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Optimization of cyanobacteria cultivation under laboratory conditions: Nostoc sp. and Anabaena sp.

Fátima Rafaela Lança Simões

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Dissertação para obtenção do Grau de Mestre em Biotecnologia dos Recursos Marinhos

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Title: Optimization of cyanobacteria cultivation under laboratory conditions: *Nostoc* sp. and *Anabaena* sp.

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‘’Gratitude is the healthiest of all human emotions’’ || ‘’ No Rain No Flowers”

Resumo

As cianobactérias são um grupo de bactérias fotossintéticas que, embora relativamente discretas, têm uma grande importância no ambiente de água doce e marinho, tanto em termos de ecologia fundamental como em relação aos potencias usos biotecnológicos.
Este trabalho teve como objetivos, estabelecer em laboratório as condições de cultivo de duas estirpes de cianobactérias não totalmente identificadas, realizando estudos preliminares através dos quais se procurou determinar os parâmetros de cultivo ótimos, analisando parte da sua composição bioquímica, como sejam proteínas, pigmentos e exopolissacarídeos. Em seguida teve como objetivo a otimização da produção de biomassa e de exopolissacarídeos pela manipulação de nutrientes, e a otimização da produção de pigmentos pela manipulação das condições de luz.
As espécies foram fornecidas pela Alga2O, localizada em Coimbra.
Na determinação da curva de crescimento, as duas espécies mostraram um bom crescimento e produção de biomassa, tendo o *Nostoc 137* atingido a fase estacionária ao fim de 21 dias de crescimento, quando se registou a taxa de crescimento de 71,84±0,0026 $mg.L^{-1}$ e uma produtividade de 14,29± 0,904 $mg.L^{-1}dia^{-1}$. A concentração máxima de biomassa foi obtida ao dia 25, com 0,47±0,22 $g.L^{-1}$ (peso seco). A *Anabaena* 4*,* atingiu a fase estacionária ao fim de 21 dias de crescimento, quando se registou um pico na concentração de biomassa e se obteve uma taxa de crescimento de 52,12 ±36,82 $mg.L^{-1}$ e uma produtividade de 15,31±2,77 $mg.L^{-1}dia^{-1}$. A concentração máxima de biomassa foi obtida ao dia 25, com 0,43±0,06 $g.L^{-1}$ (peso seco).
O ensaio para a maximização da produção de biomassa, no qual se duplicou a concentração $NaNO_3$ ao meio, observou-se um aumento na biomassa de *Anabaena* 4 de 0,332 ± 0,235 $g.L^{-1}$ para um valor de 0,401±0,284 $g.L^{-1}$, enquanto *Nostoc* 137 sofreu decréscimo na produção de biomassa de 0,096 ± 0,0678 $g.L^{-1}$ para 0,025 ± 0,0180 $g.L^{-1}$.
Na determinação do teor de proteína, foi a *Anabaena* 4 que obteve valores mais elevados, com 12,87±0,616%, enquanto no *Nostoc* 137 a média foi de 6,98±1,551 %.
Na quantificação de pigmentos foi também na *Anabaena* 4 que se verificou valores mais elevados de ficocianina, aloficocianina e ficoeritrina, com 105,72±55,960 μg.ml, 29,77±22,300 μg.ml e 19,82±9,706 μg.ml respetivamente.
Sob a influência de luz branca LED, a ficocianina atinge o valor máximo de 84,49±25,073 μg.ml. Já no *Nostoc* 137 houve uma redução na produção de pigmentos sob a luz branca LED de cerca de 3%.
Finalmente, quanto à produção de exopolissacarídeos (EPS), uma vez mais a *Anabaena* 4 mostrou apresentar uma concentração bastante elevada, com

876,5±91,005 mg.L^{-1} enquanto para *Nostoc* 137, não foram obtidos EPS. Foi induzido o stress nas culturas, para tentar aumentar a concentração de EPS, através da adição de bicarbonato de sódio e cloreto de sódio (NaCl).
Para *Anabaena 4* não se registou esse aumento, enquanto para *Nostoc* 137 obtiveram-se 5,67±0,006 mg. L^{-1} no meio com NaCl.
Neste estudo é possível determinar os parâmetros otimizados para o crescimento em laboratório destas estirpes. Demonstrou-se que a luz e a adição de novos compostos ao meio de cultivo podem afetar o crescimento, a produtividade e a composição bioquímica da biomassa.

Palavras-chave: Cianobactéria, cultivo em *batch*, *Nostoc* sp., *Anabaena* sp., curva de crescimento, exopolissacarídeos, ficobiliproteinas, conteúdo proteico.

Abstract

Cyanobacteria are a group of photosynthetic bacteria that, although relatively discrete, are of great importance in the freshwater and marine environment, both in terms of fundamental ecology and potential biotechnological uses.

The aims of this work were to establish in the laboratory the culture conditions for two strains of cyanobacteria that had not been fully identified, carrying out preliminary studies to determine the optimum culture parameters and analyzing part of their biochemical composition, such as proteins, pigments, and exopolysaccharides. Then aimed at optimizing the production of biomass and exopolysaccharides by manipulating the nutrients and optimizing the production of pigments by manipulating the light conditions. The species were supplied by Alga2O, located in Coimbra.

In the determination of the growth curve, both species showed good growth and biomass production, with *Nostoc* 137 reaching the stationary phase after 21 days of growth, when a growth rate of 71.84 ± 0.0026 $mg.L^{-1}$ and a productivity of 14.29 ± 0.904 $mg.L^{-1}day^{-1}$ was recorded. The maximum biomass concentration was obtained at day 25, with 0.47±0.22 $g.L^{-1}$ (dry weight). *Anabaena* 4, reached the stationary phase after 21 days of growth, when an increase in biomass concentration was recorded and a growth rate of 52.12 ± 36.82 $mg.L^{-1}$ and a productivity of 15.31 ± 2.77 $mg.L^{-1}day^{-1}$ was obtained. The maximum biomass concentration was obtained at day 25, with 0.43±0.06 $g.L^{-1}$ (dry weight).

The assay for maximizing biomass production, in which the NaNO3 concentration in the medium was doubled, determined an increase in the biomass of *Anabaena* 4 from 0.332 ± 0.235 $g.L^{-1}$ to a value of 0.401 ± 0.284 $g.L^{-1}$, while *Nostoc* 137 suffered a decrease in biomass production from 0.096 ± 0.0678 $g.L^{-1}$ to 0.025 ± 0.0180 $g.L^{-1}$.

When determining the protein content, it was *Anabaena* 4 that obtained higher values, with 12.87 ± 0.616%, while in *Nostoc* 137 the average was 6.98 ± 1.551%.

In the pigment quantification it was also in *Anabaena* 4 that the highest values of phycocyanin, allophycocyanin and phycoerythrin were found, 105.72±55.960 μg.ml, 29.77±22.300 μg.ml e 19.82±9.706 μg.ml respectively. Under the influence of white LED light, phycocyanin reaches a maximum value of 84.49 ± 25.073 μg.ml. Whereas in *Nostoc* 137 there was a reduction in pigment production under LED white light of about 3%.

Finally, regarding the production of exopolysaccharides (EPS), once again *Anabaena* 4 showed very high concentration with 876.5 ± 91.005 $mg.L^{-1}$ while for Nostoc 137, no EPS were obtained. Cultures were subjected to stress conditions in an attempt to increase

EPS concentration by adding sodium bicarbonate and sodium chloride (NaCl) to the culture medium.

For *Anabaena* 4 no such increase was recorded, while for *Nostoc* 137 5.67 ± 0.006 mg.L^{-1} was obtained in the medium with NaCl.

In this study, it was possible to determine the optimal parameters for the growth of these strains in the laboratory. It was shown that light and the addition of new compounds to the culture medium can affect the growth, productivity, and biochemical composition of the biomass.

Key-words: Cyanobacteria, *Nostoc* sp., *Anabaena* sp., growth curve, exopolysaccharides, phycobiliproteins, protein content

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Abbreviations

N	Nitrogen
EPS	Exopolysaccharides
C-PE	Cyanobacterial Phycoerythrin
C-APC	Cyanobacterial Allophycocyanin
C- PC	Cyanobacterial Phycocyanin
$NaHCO_3$	Sodium Bicarbonate
NaCl	Sodium Chloride
NaNO3	Sodium Nitrate

1. Introduction

Algae are widely present in freshwater environments, such as lakes, rivers, and oceans where they are typically present as micro-organisms – visible only with the aid of a light microscope. Although relatively inconspicuous, they have a major importance in the freshwater and marine environments, both in terms of fundamental ecology and in relation to human use of natural resources. The term 'algae' is not a taxonomic term but is used as an inclusive label for a number of different phyla that fit the broad description noted above. These organisms include both prokaryotes (cells lacking a membrane bound nucleus) and eukaryotes (cells with a nucleus plus typical membrane-bound organelles) (Bellinger & Sigee, 2010).

1.1. Cyanobacteria

Almost 3.5 billion years ago, primitive prokaryotic organisms began to appear on Earth, where carbon dioxide was largely available in the atmosphere and seawater. These organisms began to convert carbon dioxide into organic materials through the process of photosynthesis releasing oxygen, and played a key role in the evolution of organisms on Earth (Eltanahy & Torky, 2021). These first photosynthetic organisms were primitive Cyanobacteria.

Cyanobacteria are a special group that is characterized by a mixture of characteristics of prokaryotic organisms and eukaryotic algae. They are considered non-motile gram-negative eubacteria and are capable of photosynthetic processes (Eltanahy & Torky, 2021). They resemble eukaryotic algae by the presence of chlorophyll *a* and their ability to perform photosynthesis, a very important evolutionary characteristic since they are in the most ancient group of organisms. Because of this feature, they represent the primary producers in many aquatic habitats, both marine and freshwater (Sciuto & Moro, 2015).

Photosynthetic process requires pigments, which are present in the form of chlorophyll *a*, the primary photosynthetic pigment, and accessory pigments, which, in these organisms are phycobiliproteins, called phycocyanin, allophycocyanin and phycoerythrin, responsible for its distinctive blue-green color (Eltanahy & Torky, 2021). These pigments are usually located in phycobilisomes on the external surface of the

thylakoids and instead of being stacked, they are dispersed freely in the cytoplasm (Barsanti & Gualtieri, 2014).

Cyanobacteria are found in habitats from freshwater springs to salt lakes, with tolerance for a broad range of pH, temperature, turbidity, O_2, and CO_2 concentration (Borowitzka, 2018). They can be benthic organisms, if they are attached to the bottom of a substrate or found within sediments, or planktonic organisms, such as most unicellular species, that live suspended in the water column (Barsanti & Gualtieri, 2014).

Due to biotic and abiotic stress factors, these microorganisms have been forced to develop various characteristics in order to survive. At the morphological level, the most evolved filamentous species have developed specialized cells, namely heterocysts and akinetes[1], which are responsible for molecular nitrogen fixation and are resistance cells, respectively. Some species are also capable of producing important bioactive compounds, including toxins, called cyanotoxins. These characteristics can have a positive or negative impact on the environment (Sciuto & Moro, 2015).

1.1.1 Cell structure

As for their structural diversity, cyanobacteria can range from unicellular to branched or unbranched filaments to unspecialized colonial aggregations (Barsanti & Gualtieri, 2014).

At the cellular level, these species have significantly larger cell dimensions than most bacteria (~ 4µm), and they also vary in shape, being spherical, bacciloid, or spindle shaped. On the other hand, the cells of filamentous species can vary from discoid to barrel-shaped (Soule & Garcia-Pichel, 2019).

The structure of their cell envelope is typical of Gram-negative bacteria, including both a peptidoglycan layer and an outer membrane. However, the peptidoglycan layer of the cyanobacterial envelope is thicker (10–700 nm) than that of other Gram-negative bacteria (2–6 nm), and more similar to that of Gram-positive bacteria (**Figure 1**). The outer membrane also has a unique composition, different from that of Gram negative bacteria (Hoiczyk & Hansel, 2000).

[1] These cells are described in detail in section 1.4.

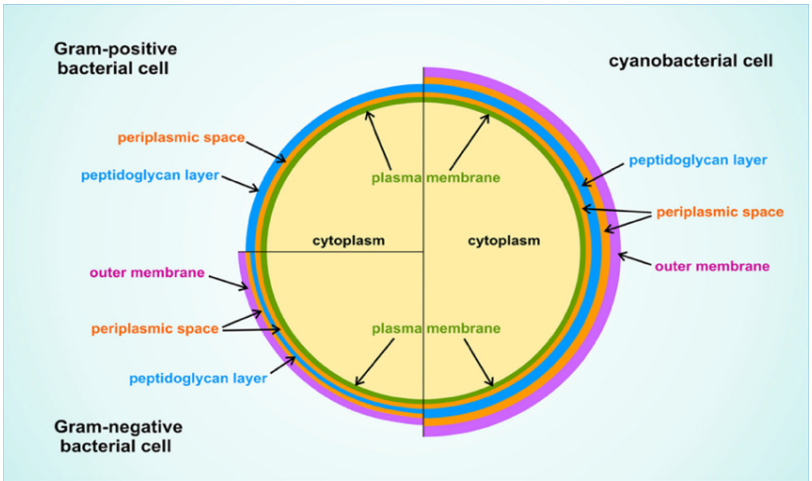

Figure 1- Schematic representation of the cell structure of a cyanobacteria cell compared to the cell structure of a Gram positive and a Gram negative. From (Sciuto & Moro, 2015)

1.1.2. Taxonomy

The taxonomy of cyanobacteria has been the target of complex and persistent study over the years. In the 1970s, a taxonomic system based on the International Code of Nomenclature of Bacteria was developed. This system is based on studies of strains grown under sterile conditions and, furthermore, it incorporates morphological and cytological information, as well as some genetic and physiological information.

The current state of the bacteriological taxonomy of the cyanobacteria is described in the second edition of the Bergey's Manual of Determinative Bacteriology (Soule & Garcia-Pichel, 2019), and is reproduced in **Table I**.

The development of the taxonomy of these species was based on morphological criteria and the observation of samples over time. There are currently two types of parallel taxonomy (Anagnostidis & Komárek, 1988). The first system, an adaptation and constant modification of the Anagnostidis/Komarek' system that is most accepted worldwide, is called the 'Geitlerian' system, encompassing approximately 1300 species grouped in 145 genera and three orders. The second system, the Drouet system, represented an attempt to simplify the previous one, recognizing only 62 species in 24 genera (Sarma, 2013).

Table I-Diagnostic key to the subsections of the Cyanobacteria, according to the Bergey's Manual of Determinative Bacteriology

Subsection	Definition criteria
Subsection I (Chroococcales)	Unicellular. Cells occurring individually or in aggregates.
Subsection II (Pleurocapsales)	Unicellular. Cells occurring individually or in aggregates.
Subsection III (Oscillatoriales)	Filamentous. No heterocysts or akinetes formed.
Subsection IV (*Nostoc*ales)	Filamentous. Uniseriate trichomes without true branching. Heterocysts formed when combined nitrogen is low.
Subsection V (Stigonematales)	Filamentous. Multiseriate trichomes, truly branched. Heterocysts formed when combined nitrogen is low.

Although the classification of cyanobacteria is based mostly on morphological characteristics, certain species, such as the genus *Nostoc*, have characteristics in the natural environment that may be altered in laboratory cultivation, where the optimal conditions may not be achieved. To overcome these limitations due to identification based on morphological characteristics, molecular markers have been applied to make the genetic or biochemical identification of these species easier and more efficient (Teneva et al., 2012).

The most common DNA marker to identify species in the phylum Cyanobacteria is the 16S rRNA gene, although this gene is highly conserved among closely related species and is unable to distinguish species at a lower taxonomic level (Brito, 2015).

1.2. Cyanobacteria *Nostoc*

The genus *Nostoc* belong to the family Nostocaceae. This genus encompasses filamentous, heterocyst, nitrogen-fixing cyanobacteria that thrive in a variety of environmental niches. In their natural environment, they can form macrocolonies that have a wide morphological diversity, such as irregularly spreading, spherical, or hair like colonies and can also form microscopic colonies (Dodds et al., 1995).

As for their structure, the cells of *Nostoc* species can assume a wide variety of shapes. They can be spherical, barrel-shaped or oval, forming unbranched filaments (**figure 2**).

The trichomes (filaments) may be associated with specialized cells, such as heterocysts (thick-walled, non-pigmented, nitrogen-fixing cells) and akinetes (thick, large, resistance cells) (Mollenhauer et al., 1999).

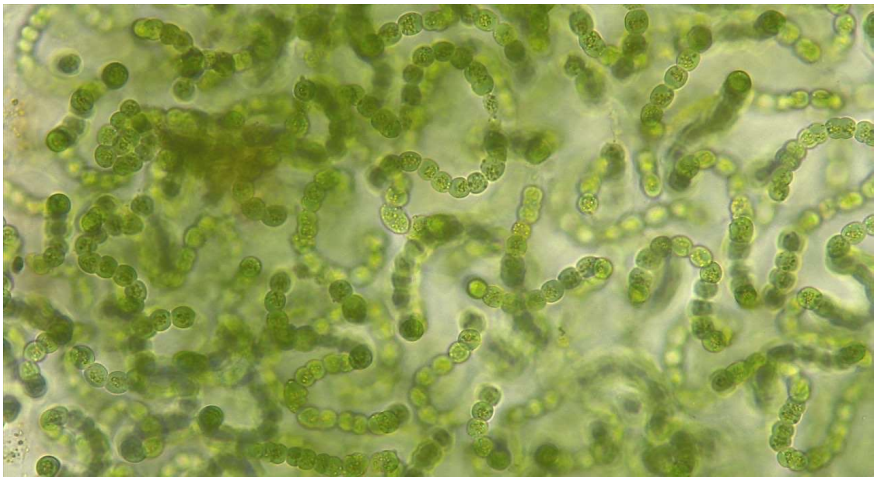

Figure 2- Microscopic observation of strain Nostoc 137 (ZEISS Axiostar plus binocular microscope, microscopic magnification 400x)

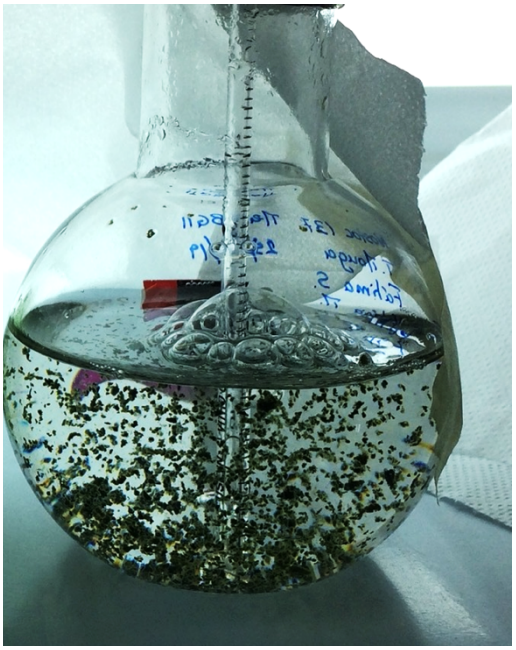

Figure 3-Macroscopic observation of a maintenance balloon of the species Nostoc 137, with visible colonies, formed by filaments.

There are species that can form a specific type of spheres, which resemble pearls (**figure 3**). In these colonies the trichomes are surrounded by a thick layer of exopolysaccharides that also contain proteins and have the function of protecting the cells from external environmental conditions (Camilios Neto & Pinotti, 2004).

Figure 4 shows the exopolysaccharide external layers of the colonies stained with China ink, which measured approximately 30 mm wide.

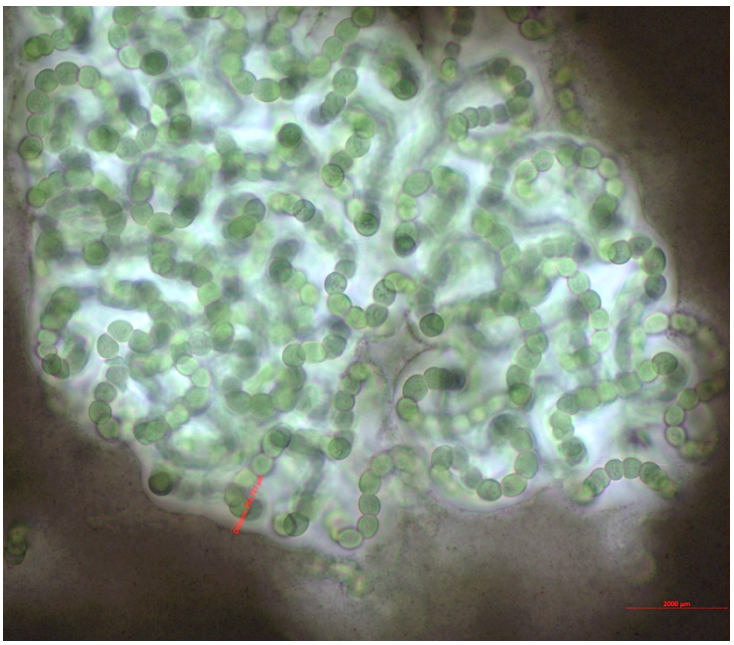

Figure 4-Exopolysaccharide external layers of the Nostoc trichomes.

In terms of reproduction, this it can occur in several ways:

1. Large colonies can break and, from a single *Nostoc* cell, new filaments can develop; this process is a type of vegetative reproduction;

2. Through hormogonia (small filament fragments) that disperse and form new filaments (also a type of vegetative reproduction through fragmentation);

3. Akinetes are formed when environmental conditions are unfavourable for the individual. When these conditions become favourable again, the akinetes begin to germinate releasing their stored contents, nutrients, through pores and from there, new filaments can develop (Borowitzka, 2018).

Dried *Nostoc* microalgae typically contain 46–63% protein, 8–17% carbohydrates, and 4–22% lipids (Rosales Loaiza et al., 2016) , as well as a wide range of vitamins and other bioactive compounds such as peptides and pigments. Due to these properties, these organisms have numerous applications, in both food and pharmaceuticals (Rosales

Loaiza et al., 2016), presenting antitumoral, antioxidant, and anti-inflammatory activities (Rasmussen et al., 2009).

1.3. Cyanobacteria *Anabaena*

Anabaena is a filamentous cyanobacteria, belonging to the family Nostocaceae. It is commonly found in freshwaters, but there are some marine species. The genus *Anabaena* is identified by the presence of uniform trichomes, the absence of a coating or the presence of a soft mucilage coating (Prasanna et al., 2006) Unlike the *Nostoc* genus which has a solid gelatinous coating, this genus has an aqueous gelatinous envelope (McGuire, 1984).

These filamentous cyanobacteria are composed of vegetative cells., Due to environmental conditions, or to cell to cell interaction, they can also produce akinetes (Cruz, 2019).

The ability to fix nitrogen through the heterocysts, combined with photosynthesis makes these organisms able to live in a wide range of environments.

The genus *Anabaena* is documented as an ecological relevant organism since it can thrive under eutrophication conditions.

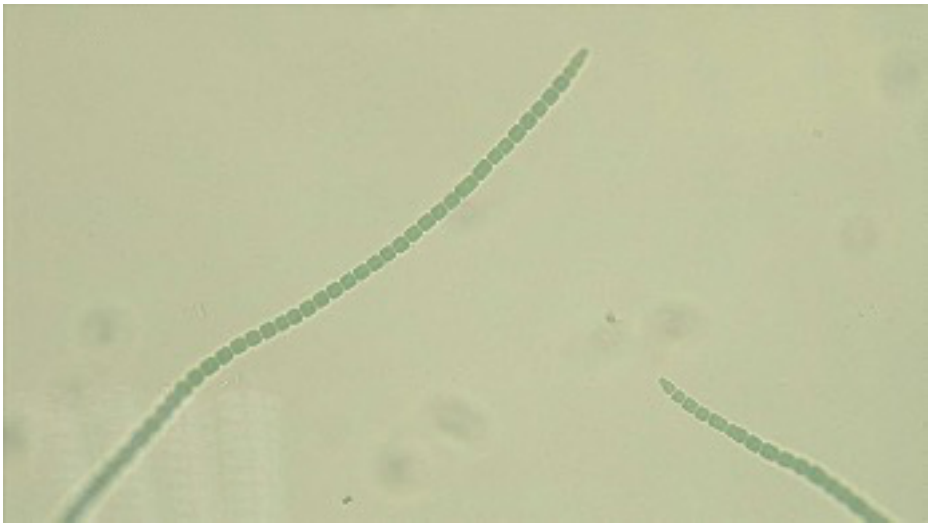

Figure 5-Microscopic observation of strain Anabaena 4 (ZEISS Axiostar plus binocular microscope, microscopic magnification 400x).

Strains of this genus are the most common to form blooms. These blooms pose a health risk, due to the production of various toxins such as microcystins, anatoxins and saxitoxins produced by some species (Hao Wang et al., 2012).

1.4. Specialized cells

As stated, cyanobacteria have evolved to produce specialized structures, such as heterocysts for nitrogen fixation, akinetes for survival in stressed conditions, and hormogonia for dispersal and vegetative multiplication (Gupta et al., 2013).
Specialized cells, such as heterocysts and akinetes can occur in the filaments of both *Nostoc* and *Anabaena* species.

1.4.1. Heterocysts

Heterocysts are cells that are functionally different from vegetative cells by having morphological distinctive features. They are normally round and larger in size than vegetative cells (Adams & Duggan, 1999). At the structural level they have less granular cytoplasm, a thicker cell wall and slight depigmentation (Singh et al., 2020).
These cells have a specific envelope that is made up of different layers: (1) the innermost is the laminated layer consisting of glycolipid [heterocyst glycolipid], (2) is the homogeneous layer consisting of polysaccharide [heterocyst envelope polysaccharide], (3) outermost is the fibrous layer which is probably uncompacted strands of the same polysaccharide (Flores et al., 2019; Nicolaisen et al., 2009). This thick envelope serves to keep the cytoplasm in an anaerobic environment necessary for the functioning of the enzyme nitrogenase, responsible for converting atmospheric nitrogen into ammonia, supplying combined N to the remaining aerobic vegetative cells (Adams & Duggan, 1999).
Many cyanobacterial species are able to fix nitrogen due to these heterocysts, but other unicellular cyanobacteria may also have nitrogenase (functioning during the dark) to convert nitrogen into ammonia (Zehr & Capone, 2020). In the presence of a combined nitrogen source such as nitrate and ammonia, heterocysts are not produced, and nitrogenase is inactive, and thus, long filaments containing only photosynthetic vegetative cells are formed. In the absence of nitrogen there is heterocyst production (Kumar et al., 2010).

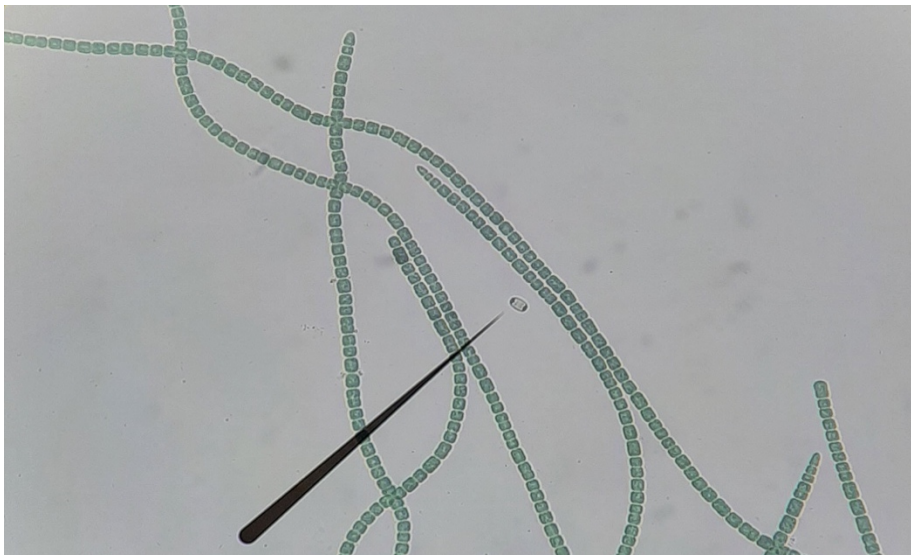

Figure 6-Microscopic observation of Anabaena 4. Showing a heterocyst (ZEISS Axiostar plus binocular microscope, microscopic magnification 400x)

1.4.2. Akinetes

Akinetes are non-motile reserve cells that differentiate from vegetative cells and aid in perennialization, ensuring the long-term survival of the species (Adams & Duggan, 1999). They are larger in size than vegetative cells and have a denser cytoplasmatic content, due to the accumulation of reserve compounds (**Figure 7**).

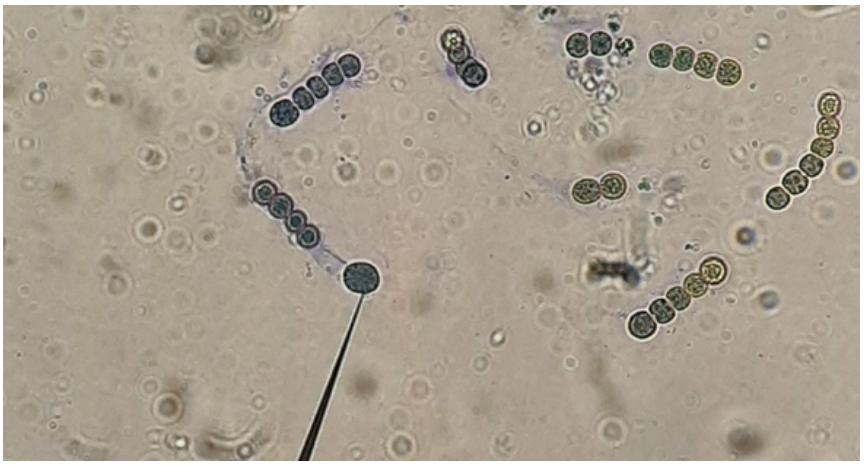

Figure 7-Hormogonia of Nostoc sp. Showing an akinete. 400x. sample stained with methylene blue to highlight the mucilaginous sheath.

The akinete envelope is composed of distinct layers including outermost layer, glycolipid layer, and a mucilaginous layer (Perez et al., 2018). According to Adams & Duggan, (1999b) akinetes are resistant to low temperatures and desiccation, but are sensitive to warmer temperatures, although studies indicate that the accumulation of gluco-glycerol, betaine, and glycine in these resistance cells aid in heat tolerance (Kimura et al., 2017). The presence of hopanoids confers cell rigidity that helps support stress tolerance (Ricci et al., 2016).

The formation of akinetes can be influenced by different factors such as light intensity, light quality, temperature, inorganic nutrients such as phosphate or the ratio of carbon to nitrogen (C:N) or other stress factors (Maldener et al., 2014). In certain species akinete formation occurs when stimulated with different lights, for example green light (Thompson et al., 2009). In the presence of blue light, however, there are studies that show that the production of akinetes is reduced, even when subjected to a small exposure, thus suggesting that this light causes the inhibition of the formation of akinetes in cyanobacteria (Sukenik et al., 2019).

1.5. Pigments

Pigments play an important role in photosynthetic metabolism and pigmentation of microalgae and exhibit antioxidant, anticancer, anti-inflammatory, anti-obesity, and neuroprotective effects (Cuellar-Bermudez et al., 2015; Guedes et al., 2011).

The three classes of pigments found in photosynthetic organisms are chlorophylls, carotenoids, and phycobiliproteins.

The phycobiliproteins are contained in a multi-protein complex (**figure 8**). The chromophores of these pigments are bound to proteins that constitute the photosystem I and photosystem II complexes. There are two major classes of antennae complexes: one, the light-harvesting chlorophyll/carotenoid-binding complexes directly attached to the reaction center in the thylakoids, and two, the phycobilisomes, the supramolecular complexes of phycobiliproteins attached to PSII composed of two or four different phycobiliproteins (phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin) (Morançais et al., 2018).

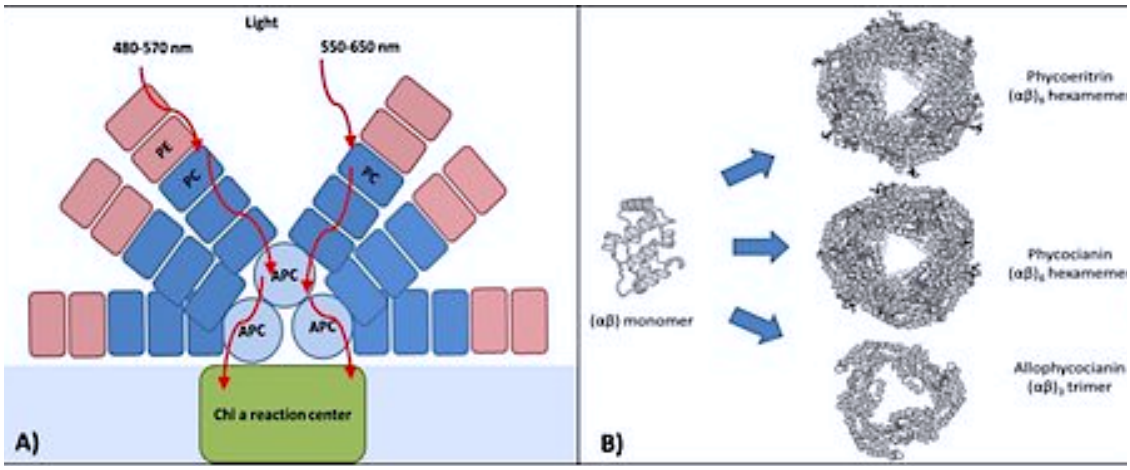

Figure 8-A) The structure of the phycobilisomes includes the accessory pigments phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC); B) The general architecture of phycobiliproteins consist in α and β subunit polypeptides which associate into αβ monomer (Garcia & Olguin, 2020)

1.5.1. Chlorophyll

Chlorophylls are lipid-soluble and are the primary pigment in cyanobacterial species and in all the other photoautotrophic organisms (Morançais et al., 2018).

Different types of chlorophyll may occur in cyanobacteria, chlorophyll *a* being the most abundant, and the pigment responsible for the green colouration of these organisms (Gan & Bryant, 2015). Chlorophylls are used in the pharmaceutical and cosmetic industries for use in deodorants (Ferreira & Sant'Anna, 2017; Morançais et al., 2018) and in the food area as colourants.

1.5.2. Carotenoids

Carotenoids are the most abundant pigments found in the natural environment. They are present in many organisms despite only being synthesized by photosynthetic species (Cerezo et al., 2012). They can be divided into two groups: carotenes and xanthophylls, regarding their structure (Saini et al., 2018).

Carotenoids are fat-soluble pigments and are associated with yellow, red and orange coloration (Morançais et al., 2018).

1.5.3. Phycobiliproteins

Phycobiliproteins are present mainly in Cyanobacteria but also found in Glaucophyta, Rhodophyta and Cryptophyta (Glazer, 1984). According to their absorption properties in the visible spectrum, they are divided into three classes: phycocyanin, phycoerythrin and the allophycocyanin. Some species of Cyanobacteria can also synthesize a fourth class, called phycoerythrocyanin (Dumay et al., 2014).

Phycocyanin has an absorption range between 610-625 nm and is a natural blue pigment (Udayan et al., 2017). Phycocyanin can be sub-divided into three types: C-phycocyanin (615–620 nm), Phycoerythrocyanin (575 nm) and R-phycocyanin (615 nm). It can be used as a food additive, as well as for cosmetics and pharmaceuticals, due to its anti-inflammatory and antioxidant activities (Liu et al., 2013).

Allophycocyanin gives a blue-green coloration, with an absorption zone between 650 and 660 nm. Its use is limited because it is present in smaller quantities in cyanobacteria and due to the lack of an effective method for its purification (Caetano, 2018).

Phycoerythrin has an absorption range between 490-570 nm. Phycoerythrin has a pink coloration and is also classified into different classes according to the first organism from which it was isolated, (1) R-Phycoerythrin, from rhodophytes; (2) B-Phycoerythrin, also from red seaweed, but the most primitive species (Bangiales) and (3) C-Phycoerythrin from cyanobacteria. Their absorption peaks are 565, 545 and 563 nm respectively (Stadnichuk & Tropin, 2017).

Under optimized conditions, the phycobiliprotein content can reach up to 50% of the cellular protein value, and for this reason this work focuses mainly on the three classes of phycobiliproteins presented above (Gupta et al., 2013).

1.6. Exopolysaccharides (EPS)

Exopolysaccharides (EPS) are classified as secondary metabolites and are present mainly in the outer layer of the external membrane. This layer constitution is complex, being composed mainly by polysaccharides, but also proteins, nucleic acids, and lipids with various functional groups (Ozturk & Aslim, 2010). The cell surface associated EPS can be grouped into sheaths, capsules, and slimes, based on their consistency, appearance, and thickness. Sheaths are characterized as a thin, dense layer that covers the cells and needs no staining to be visible under the microscope. Capsules consist of a thick and slimy layer associated with the cell surface. The slime represents mucilaginous material dispersed around the organism but not reflected in the cell shape (Kumar et al., 2018).

One of the functions of these metabolites is to protect the cells from stressful environmental conditions, such as UV radiation, oxidation, temperature, and desiccation. Under these conditions an increase in EPS production occurs as a metabolic strategy response (Camilios Neto & Pinotti, 2004).

EPS have diverse properties such as enhanced water retention capacity in soils, due to their hydrophilic and hydrophobic properties they can absorb and retain water, forming a gelatinous layer around the cells to regulate the inflow and outflow of water, industrial applications as gelling agents and emulsifiers, biomedical applications such as antiviral and antitumor, and bioremediation applications for the removal of toxic metals from wastewater (Camilios Neto & Pinotti, 2004; S. Singh et al., 2016).

The application of EPS originating from cyanobacteria correspond to an interesting alternative to the EPS obtained from plants, because, at the biomass production level, they have higher growth rates and are microorganisms of easier manipulation regarding production optimization (Camilios Neto & Pinotti, 2004). Yet, EPS culture profiles are characterized by being long-lived (up to 30 days) since production is associated with the stationary phase (Delattre et al., 2016). Also, the chemical composition of EPS can be different even in cultures of the same species. Changes in growth condition and age have major influence on the quantity of EPS. Temperature, light intensity, concentration of nutrients such as phosphate and potassium, affect the composition of the EPS. Limitation of some nutrients is the most widely used strategy for increasedincreasing the production and accumulation of exopolysaccharides. However, this strategy has the disadvantage that when the culture is under nutrient limitation, it is under unfavourableunfavourable conditions and its growth will be slower. Therefore, a balance must be found between high cell growth and high EPS production, and generally a batch culture is performed, with an abundant nutrient phase followed by a deprivation phase to increase EPS production (Delattre et al., 2016).

1.1.3. Cultivation methods- Batch Culture

This is one of the most common systems for microalgae production, due to its ease and low cost. This method consists of a closed system, a reactor with a limited volume, in which the respective algal inoculum is added to start cultivation (Richmond, 2004).

In this type of system there is no input or output of biomass or nutrients, this leads to a depletion of nutrients over time.

The cell density will always increase constantly until the exhaustion of some limiting factor for the culture occurs, one of these factors can be the concentration of nutrients, because these decrease over time (Watanabe, 2005).

Once the nutrients have been fully consumed it will be necessary to supply nutrients to the growing medium so that the death of the culture does not occur.

In batch systems, the algal population shows a characteristic growth pattern (**figure 9**), following a sigmoid curve, consisting of different phases characterized by variations in the growth rate (Barsanti & Gualtieri, 2014).

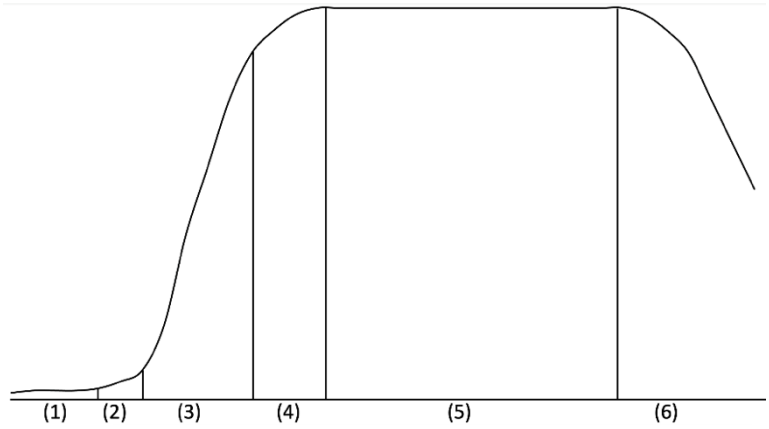

Figure 9-Growth curve of an microalgal population under batch culture conditions. (1)- Lag Phase, (2)- Acceleration Phase, (3)- Exponential Phase, (4). Retardation Phase, (5)- Stationary Phase, (6)- Decline Phase

(1 and 2) Phase Lag and acceleration

After inoculation, cell development does not occur immediately, because most cells are not yet viable and in optimal conditions to divide (Henry, 2004).

The interval required for physiological adaptation to the new culture conditions, such as possible changes in nutrients, light, salinity, among others, until growth begins corresponds to the first phase of the growth curve, called the lag phase (Barsanti & Gualtieri, 2014).

During this phase the growth rate is zero. This phase depends on the concentration and physiological state of the inoculum and can be reduced if the inoculated culture is in its exponential phase.

The acceleration phase is the intermediate phase between the lag phase and the exponential phase, a phase in which the culture is already prepared for algal growth, because adaptation to the new conditions is already complete. During this phase the growth rate increases (Barsanti & Gualtieri, 2014).

(3) Exponential Phase

As the adaptation phase ends and the growth rate increases, the cells begin to multiply. It is also in this phase that the maximum peak cell production and exponential growth rate occur (Watanabe, 2005). In this period, since there is no limitation of nutrients or other factors important for the healthy development of a crop, the growth rate continues to increase at a steady rate, greatly increasing the biomass production (Barsanti & Gualtieri, 2014).

(4) Retardation Phase

When the cell growth rate starts to decrease, it means that the culture is entering the deceleration phase, which can occur as a result of light incidence, nutrient depletion, pH, and other physical or chemical factor that starts to limit growth. At this stage the crop still continues to grow, but the rate slows down until it reaches zero (Barsanti & Gualtieri, 2014).

(5) Stationary Phase

When the growth rate reaches a value of zero, it enters the stationary phase.
At this point the culture remains at its constant maximum concentration, until nutrients are depleted (Barsanti & Gualtieri, 2014).

(6) Decline Phase

The final phase is characterized by a negative growth rate. At this point, the quality of the culture medium deteriorates significantly, mainly due to excessive accumulation of excretion products and nutrient depletion, and thus the growth of the culture cannot be sustained. Cell density decreases dramatically and eventually the culture collapses (Barsanti & Gualtieri, 2014).

2. Cultivation of cyanobacteria

2.1. Environmental parameters

A culture can be defined as an artificial environment in which the organism grows. Theoretically, culture conditions should resemble the organism's natural environment as far as possible (Barsanti & Gualtieri, 2014).

The most important parameters regulating any photosynthetic organism (either a microalga or a cyanobacteria) growth are nutrient quantity and quality, light, pH, turbulence, salinity, and temperature. Optimal parameters as well as the tolerated ranges are species-specific; the different parameters may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another (Barsanti & Gualtieri, 2014).

1.7.1. Temperature

The development and growth of a culture is temperature dependent.

Ideally the temperature at which cultures are kept should be similar to the temperature at which the species was collected (Barsanti & Gualtieri, 2014). An intermediate value of 18°C - 20°C is recommended, as a non-optimal temperature can affect the nutrient medium and species composition (Berg & Sutula, 2015).

This parameter has significant impacts when altered to standard values outside those considered normal for each species under investigation. It has an impact on the growth rate, cell size and chemical and biological composition of the cyanobacteria, and therefore has consequences on the production. For this reason, it is necessary to give due importance to this parameter (Qiang, 2013; Sayegh & Montagnes, 2011).

1.7.2. pH

The pH is an important factor in an algal culture, as it determines the solubility and availability of CO_2 and minerals in the culture. It can also be related directly or indirectly to the metabolism of the culture. In most species, the pH should range between 7.5 and 9.0 (Kong et al., 2007).

As the cell density and age of the culture increases the medium's pH, and tends to increase also due to CO_2 consumption, thus it is sometimes necessary to correct the pH (Silva, 2011).

1.7.3. Aeration

The purpose of aeration is to prevent sedimentation from occurring, thus ensuring that all cell densities are uniformly exposed to light intensity and nutrients, and that the culture is properly homogenized (Öncel & Akpolat, 2006; Tahir, 2014).
Aeration also enhances gas exchange. Aeration, which is too weak causes sedimentation of the culture, preventing correct oxygenation. Too strong aeration may cause damage to cells or to filamentous cyanobacteria (Barsanti & Gualtieri, 2014).

1.7.4. Light

Light is the energy source that drives photosynthetic reactions, which makes this feature one of the limiting factors in the growth of cyanobacteria. Thus, light intensity and photoperiod must be carefully evaluated for they are species specific (Barsanti & Gualtieri, 2014; Öncel & Akpolat, 2006; Oncel & Sukan, 2008). This parameter, like all the other variables, must be adjusted to the needs of each strain. Too strong a light intensity causes photoinhibition, which harms the culture by aging it too quickly. Too low a light intensity causes photo-limitation which inhibits the growth of the culture. Generally, the photoperiod applied is between 12D:12N to 16D:8N cycle and the often employed light intensities range between 100 and 200 $\mu E\ sec^{-1}\ m^{-2}$, which corresponds to about 5–10% of full daylight (2000 $\mu E\ sec^{-1}\ m^{-2}$) (Barsanti & Gualtieri, 2014).

1.7.5. Nutrients

Cyanobacteria need nutrients to grow, that are accessible to the cells through their environment. The composition of the nutrient medium is an important factor in the success of a culture (Markou et al., 2014; Tahir, 2014).
The essential nutrients for biomass growth depend on the species being grown. Nutrient requirements for algal growth are typically divided into macronutrients and micronutrients (Markou et al., 2014). Macronutrients are nutrients required in large quantities and play an important role in the structure and metabolism of the microalgae, whereas micronutrients are required in smaller quantities and assist in enzymatic and structural functions (de la Noue & de Pauw, 1988).

1.7.5.1. Macronutrients

Macronutrients are the most important when it comes to a nutritive culture medium. These include carbon, nitrogen, phosphorus, hydrogen, and oxygen, which are essential compounds for the cyanobacteria.

Carbon is the main nutrient, constituting about 50% of the weight of the algal biomass (Becker, 1994). It is essential for the formation of all primary metabolic compounds such as carbohydrates, proteins, and lipids. Sodium bicarbonate ($NaHCO_3$) can be used as a source of inorganic carbon (Chen et al., 2010; Yeh et al., 2010).

Oxygen constitutes about 20% of biomass and one of the main sources is water.

Nitrogen constitutes about 7-10% of the algal biomass weight and is usually provided in the form of nitrates, ammonium salts or urea.

Nitrogen supply has an effect on growth rate and lipid content, fatty acid composition and nucleic acids content (Chen et al., 2010). The growth stage of the microalgae population also significantly influences its chemical content, as can be seen in **Figure 10**. For example, phycobiliproteins are especially abundant at the end of the exponential phase, while carotenoids are abundant only at the end of the stationary phase. Likewise, polyunsaturated fatty acids (PUFAs) accumulate at the early of stationary phase, and triacylglycerols (TAGs) at the end of the stationary phase.

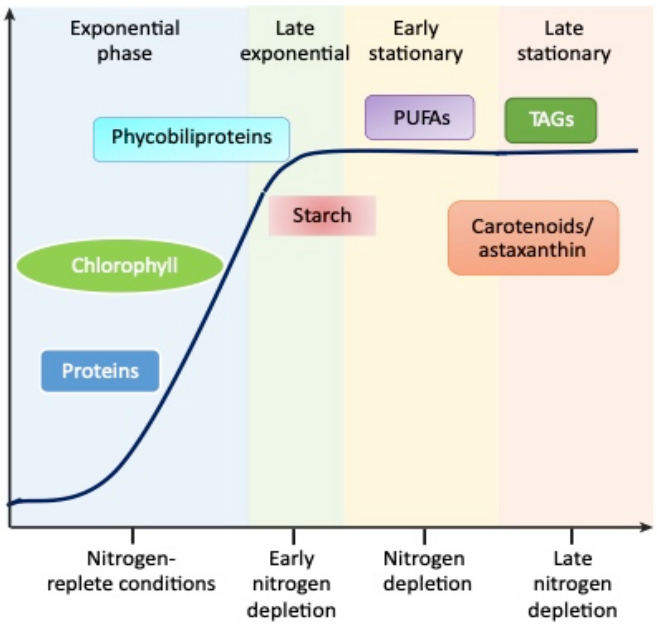

Figure 10- Optimal Conditions for Product Hyperaccumulation Over the Growth Phase as a Function of Nitrogen Concentration. Adapted from (Gifuni et al., 2019)

Phosphorus is an essential compound for metabolism. Even though it constitutes only 1-3% of biomass, this nutrient regulates some aspects of growth, biomass composition and cellular metabolic processes, being fundamental for some lipids (such as phospholipids) and nucleic acids synthesis (Tahir, 2014).

1.7.5.2. Micronutrients

Micronutrients are needed in small amounts compared to macronutrients, although they are essential for the growth and metabolism of the microalga. Some of the most important micronutrients are sulphur, calcium, potassium, magnesium, iron, manganese, zinc, and copper. It is necessary to ensure sufficient supply of these micronutrients to ensure good biomass yield (Grobbelaar, 2004).

1.7.6. Contaminating agents

Biological contaminations are a problem in microalgae and cyanobacteria cultivation because contaminants may enter through aeration or may be present in the water used (Grobbelaar, 2004). Cyanobacterial production can often be affected by unwanted algae, bacteria and fungi (Sheehan et al., 1998). These contaminants can be divided into two groups: the first restricts or destroys cell growth and the second group inhibits or consumes the products produced or excreted by the cyanobacteria (Zhu et al., 2020).
The early detection of these types of contaminants is a very important step in the selection of the most appropriate solution to be adopted in the culture. All the information detected, and characteristics identified in respect to the biological contaminant can provide specific strategies for their control.
The traditional method of microscopic observation can and should be adopted from the beginning and be part of the maintenance routine to allow early contamination detection (Carney & Lane, 2014).

1.7.6.1. Strategies for the control of biological contaminations

To obtain a unialgal culture the species to be cultivated must be isolated from all the rest; three major techniques can be used: Biological control, physical control and environmental control (Barsanti & Gualtieri, 2014).

1.7.6.1.1 Chemical control

Chemical control for a cyanobacteria culture system is usually a viable solution for controlling biochemical contaminants, despite their low specificity. Determining the appropriate agents and concentrations to perform the control is not a simple task, requiring the determination of the minimum inhibitory concentration for contaminants, the tolerance of the desired microalga/cyanobacteria.

Commonly used chemical agents are antibiotics, fungicides, pesticides, salts, aldehydes, and peroxides, among others. The cost of chemical control in mass cultures may be high. Sometimes the combination of chemical agents allows better results, reducing time and costs (Molina et al., 2019).

1.7.6.1.2. Biological control

The introduction of specific pathogens that overcome the effectiveness of chemical methods may be the most appropriate strategy for the control of contaminants in mass cultures. The introduction of this method has to be dependent on the type of contaminant being targeted, and this choice needs detailed information between the type of contaminant and the type of relatedness it has with the strain of cyanobacteria (Carney & Lane, 2014).

1.7.6.1.3. Physical control

One of the most common and effective methods for removing biological contaminants are physical filtration and ultraviolet sterilization, as these methods do not require the introduction of chemicals into the culture and no significant changes need to be made to the infrastructure of the culture system. The removal efficiency by filtration is dependent on cell size, which limits the application of this method (Holm et al., 2008).

1.7.6.1.4. Environmental control

Cyanobacteria have a great ability to adapt in most terrestrial habitats (Kosta et al., 2010). For this reason, several strains of cyanobacteria are tolerant to variations in their normal growing conditions, such as pH, temperature, nutrients, and light intensity.

This method, thus, involves adjusting the environmental conditions to specific values where the cyanobacterial or microalgae cells can survive but the contaminant cannot. Biological contaminations can occur at any stage of cultivation, either chronically or

acutely. Each of these treatments has its weak points and there is no single solution for the total resolution of contaminations. To overcome this problem it is necessary to have very detailed information about the type of contaminants and build a control system with a combination of several strategies based on the physiological characteristics of the contaminants (Hui Wang et al., 2013).

It is often necessary to restart cultivation when none of the above methods have proved successful.

2. Objectives

The main objective of this study was to establish the laboratory routine growth of two strains of cyanobacteria *Nostoc* 137 and *Anabaena* 4, not yet identified to the species level, and to construct a database, including cultivation parameters, and data on the biochemical composition of these species (proteins, pigments, and exopolysaccharides). The aim was to optimize the cultivation parameters for the maintenance of these cultures and for the production of specific compounds, namely exopolysaccharides.
To achieve the aforementioned objective, the following specific objectives were outlined:

(1) Establish the growth curves for *Anabaena* 4 and *Nostoc* 137,
(2) Define growth phases and respective growth rates,
(3) Determine the culture parameters,
(4) Characterize the protein, pigments, and exopolysaccharide content,
(5) Analyze the influence on biomass production, though the manipulation of nutrients in the media,
(6) Analyze the production of pigments, trough the manipulation of light conditions,
(7) Analyze the production of exopolysaccharides, through the manipulation of nutrients in the media.

3. Materials and Methods

3.1. Culture conditions

3.1.1. Pre-treatment and conditions for maintenance of the culture

The cyanobacterial species *Anabaena* 4 and *Nostoc* 137 **(figure 11**), were obtained from the Alga2O Bank, located in Coimbra (N 40º 11.564, W 008º 25.023), on September 10th, 2019, and were transported to the laboratory in test tubes properly covered with foil, to avoid excessive sunlight exposure.

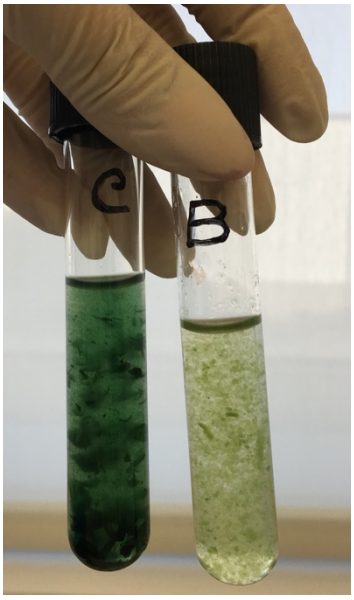

Figure 11-Tubes with cyanobacteria strains upon arrival, C - Anabaena 4; B- Nostoc 137

Before the arrival of the cyanobacteria, we proceeded to the cleaning and preparation of flat bottom flasks to be possible to start the culture. To wash the flasks, a 10% HCl solution was prepared, to eliminate any contaminants. Half of the total volume of the flasks were filled with this solution and left to stand for 30 minutes, after which the flasks were rinsed twice with distilled water type 3 and, finally, distilled water type 2 to eliminate the cleaning solution. After the complete preparation of the flasks, they were autoclaved at 120ºC for 20 minutes.

The culture medium used in the cultures was BG-11 Medium[2] based on Rippka, Deruelles, & Waterbury (1979) with buffer and pH modifications by Yepremian & Pauline Rambur (2019), HEPES was added as a buffering agent to maintain the pH of the cultures (McFadden & Melkonian, 1986). Upon arrival of the cyanobacteria in the laboratory, they were transferred to 250 mL flat bottom flasks with BG11 nutrient medium. The cyanobacteria were kept in constant aeration, during the entire sampling phase, in an acclimatized room (20±2°C) and properly covered with black paper in order to reduce light intensity.

The established photoperiod was 16:8h (Light:Dark) and cultures were irradiated with Daylight cool white fluorescent lamps at 10.8 µmol $m^{-2}s^{-1}$.

Anabaena was covered with paper due to its low concentration in the first two weeks.

After the first few weeks all the flask of the species under study were covered with a layer of black plastic bag to reduce the light intensity incident on the flask from 56 µmol $m^{-2}s^{-1}$ to 10.8 µmol $m^{-2}s^{-1}$, because cyanobacteria are known to prefer low-intensity light (**figure 12**).

3.2. Growth curves

Modified BG11 nutrient medium, 250 (500) mL flat bottom flasks, 1L (2L) flask, and beakers were prepared and autoclaved beforehand.

The day before the experiment started, aerating tubes were placed in a mixture of distilled water and bleach 5%.

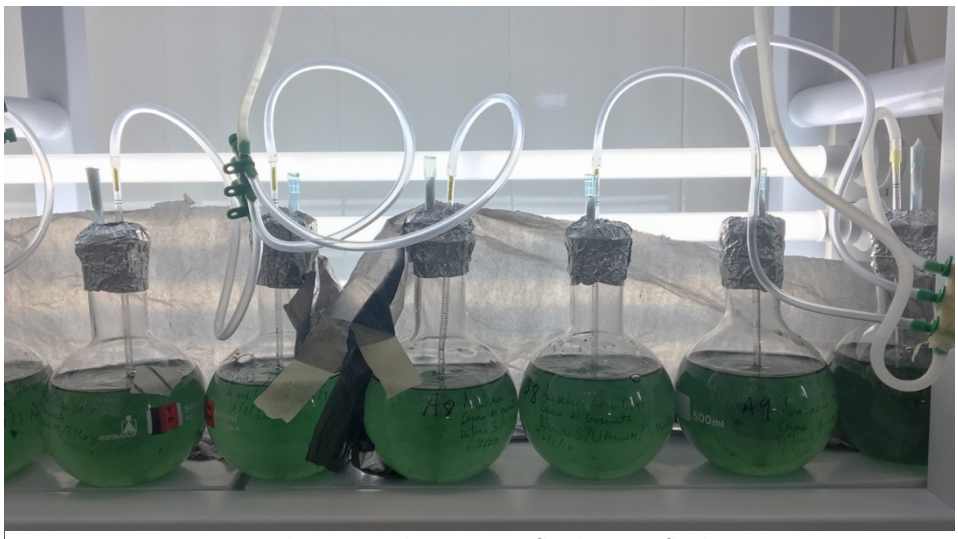

Figure 12 - Setting up Anabaena 4 cultivation on flat bottom flask.

[2] Details on the chemical composition of BG11 are in Attachment A - Table AI and Table AII

On the first day, the tubes were first removed from the mixture of distilled water and bleach, then rinsed with distilled water several times to remove residual bleach , lastly the tubes were set up in the acclimatized room in a location designated for this assay.

The inoculum was distributed into 500mL flasks, as homogeneous as possible. The starter culture used a 20% inoculum, by adding 75mL of cyanobacterial culture to 375mL of nutrient medium. Two replicates were performed, each with 9 flasks, counting time zero.

The flasks were installed in the acclimatized room, the aeration was adjusted to each flask individually, and they were covered with a layer of black plastic bag to reduce light exposure from 56 µmol $m^{-2}s^{-1}$ to 10.8 µmol $m^{-2}s^{-1}$.

Dry weight was determined on day zero, and every two or three days as presented in **figure 13**. The total volume of the flasks was filtered (Prat Dumas 125mm, France) and then the filters were dried in an oven (Binder FD 115) at 105°C for 24 hours.

The next day, dry weights were recorded, after weight stabilization.

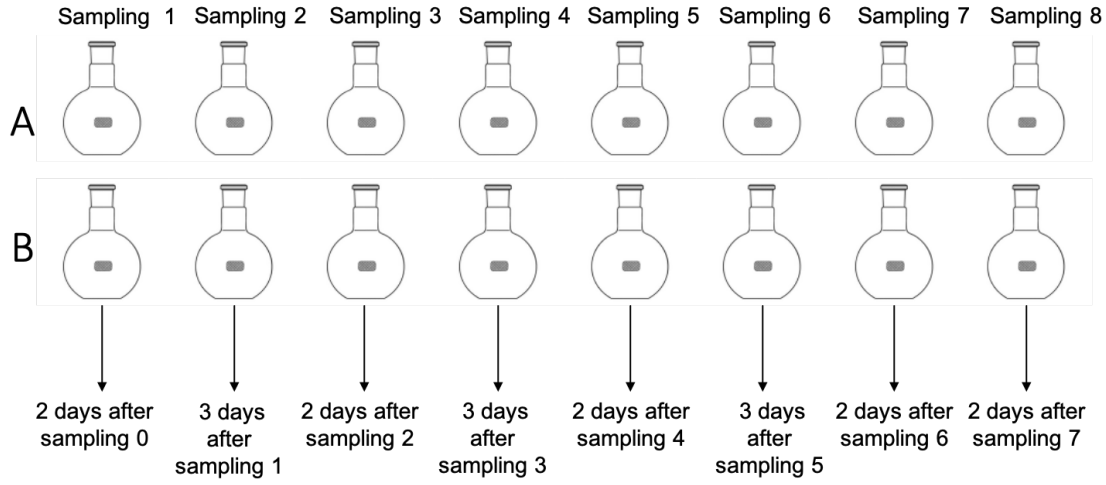

Figure 13 - Schematic representation of the sampling days during the culture growth period (n=2).

The Specific Growth Rate (SGR) (g.day^{-1}) of each culture was calculated according to (Vonsha, 1997) by the formula:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (1)$$

Productivity ($g.L^{-1}$ day^{-1}) was calculated according to the following formula:

$$P = \frac{x_2 - x_1}{t_2 - t_1} \quad (2)$$

Where, m is the specific growth rate, x_1 the initial biomass concentration, x_2 the final biomass concentration, t_1 the initial time, and t_2 the final time. The biomass produced was also analysed for protein, phycocyanin, and exopolysaccharides contents, as described in the chemical analysis section, **section 3.4**.

3.3 Growth Tests Assays

In the following assays, we have changed only one parameter, compared to the control, in an attempt to increase the growth rate or the production of selected compounds. All the assays were performed in triplicate.

3.3.1. Effect of nitrate concentration on biomass production

The purpose of the nitrate assay was to evaluate the growth rate of the culture and, thus, the biomass production. The assay was performed in triplicate and lasted for 15 days.
For this assay, twelve flasks with a volume of 500 mL and an initial inoculum percentage of 20 % were used.
The control was performed with modified BG11 medium, while the parameter to be analysed was performed with modified BG11 medium with the addition of twice the amount of nitrates from 1.5 to 3 $g.L^{-1}$ (Çelekli & Yavuzatmaca, 2009).
The growth rate was analysed at time zero and at time 15. During time 0, the total volume of the flask was filtered (Prat Dumas 125mm, France) and then the filters were dried in an oven (Binder FD 115) at 105°C for 24 hours (**Figure 14**). The next day, the dry weights were recorded until they stabilized. The same process was applied for the end of the assay. SGR and productivities were calculated according to formulas (1 and (2).

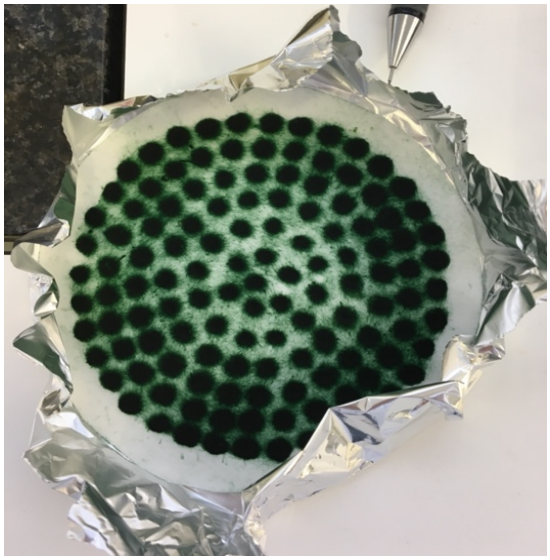

Figure 14- Anabaena 4 sample filtrate.

3.3.2. Effect of LED light on the production of phycobiliproteins

This trial was performed in order to assess the impact of light intensity increase on the production of pigments, namely of phycocyanin.

The trial had a duration of 15 days, the control was performed under the same conditions used in the growth curve assay, that is, under Daylight cool white fluorescent lamps at 10.8 µmol $m^{-2}s$-^{1}, in flasks with a volume of 500 ml with an initial inoculum percentage of 20%. The independent variable used was a white LED light at 14 µmol m^{-2} s^{-1}. The choice for the LLED light was based on the fact that it is a way to obtain more energy efficiency in a more economical way. In relation to the white colour had only to do with the availability in the laboratory. The advantages of using LED lights are explained further in section 5.2.2 of the discussion.

SGR and productivities were calculated according to formulas (1 and (2).

3.3.3. Effect of sodium bicarbonate on exopolysaccharide production

This assay was performed to assess the impact of sodium carbonate on the production of exopolysaccharides.

The assay had a duration of one month, the control was performed in flasks with a volume of 250 ml with an initial inoculum percentage of 20%. The assay was performed under the same conditions with the addition of 0.150 g sodium bicarbonate for 500mL modified BG11 medium (Vergnes et al., 2019).

SGR and productivities were calculated according to formulas (1 and (2).

3.3.4. Effect of sodium chloride on exopolysaccharide production

The salinity assay determined the number of EPS produced under the influence of sodium chloride. The assay was performed in triplicate and the trial lasted for 15 days. For the assay, 250 mL flasks and an initial inoculum percentage of 20 % were used. The control flasks contained modified BG11 medium, and the variable flasks contained modified BG11 medium with 400mM NaCl. (Jo et al., 2020; Nowruzi et al., 2013).
For one liter of medium, 23.38 g of NaCl were added.

SGR and productivities were calculated according to formulas (1 and (2).

3.4 Chemical Tests - Analysis

3.4.2. Protein analysis

Extraction of protein was performed adapting the protocols of (Parimi et al., 2015). About 100 mL of each sample of maintenance flasks were collected (n=3). The sample was centrifuged (Eppenford 5810R) at 8,000 g for 15 minutes at room temperature. The supernatant was then discarded, and the pellet of each sample was placed in an oven at 60°C overnight.

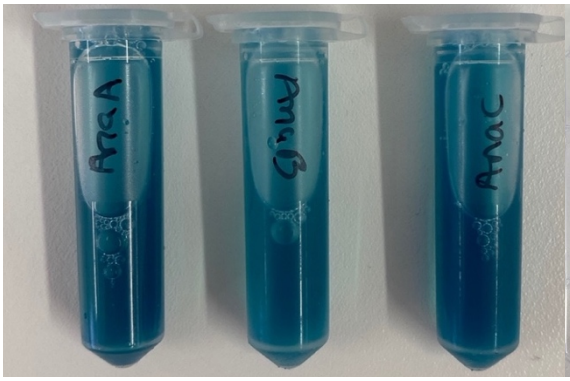

Figure 15-Eppendorfs with the combined supernatants

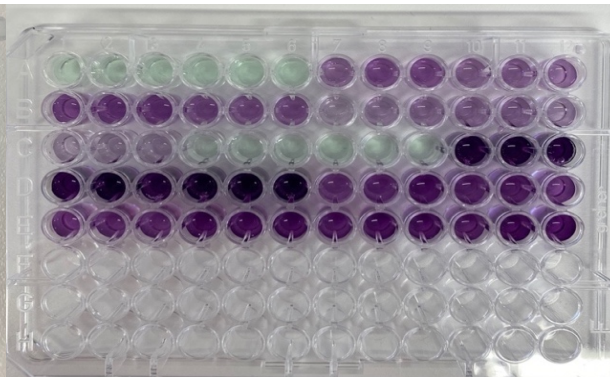

Figure 16-Protein plate assay in Greiner 96 flat bottom

The next day, 40 mg of dried pellet of each sample was weighed into an Eppendorf, where the biomass was macerated to a fine powder texture. Subsequently, it was carried out an extraction of proteins by solubilization at alkaline pH using 1M NaOH, centrifugation was performed at 8,670 g for 35 min, followed by precipitation from the supernatant (obtained from the previous step) at acidic pH using either 1M HCl and finally

another centrifugation under the same conditions as before. after this extraction the two supernatants were combined (**Figure 15)** into a microtube for further analysis by BCA Protein Assay Kit (Thermo Scientific™ 23225) (**Figure 16**). The standards and calibration curve had to be performed first for further protein analysis, for which the Pierce™ BCA Protein Assay Kit instruction guide was used.[3]

Each of the final samples was measured in the spectrophotometer (Thermo scientific, Evolution 201) at an absorbance of 652 nm.

3.4.3. Phycobiliproteins Quantification

Cyanobacterial biomass (1g) was collected from the 3 control culture flasks and from the three white LED light flasks. To this biomass, 20 mL of Milli-Q water was added, and the previously identified sample was frozen, -18ºC. Three freeze-thawing cycles of 12 hours each were performed (Ranjitha & Kaushik, 2005). During this process, the samples were kept on ice and protected from sunlight.

After this period, a centrifugation (Eppendorf, 5804) at 5,000 g for 10 minutes at room temperature was performed. The supernatant was collected in a 15 mL Falcon®, completely covered with foil to protect the samples from solar incidence (Beattie et al., 2018).

Next, measurements were performed in the spectrophotometer (Thermo scientific, Evolution 201), where a scan was performed on each of the samples, according to the target pigments of the study, the phycobiliproteins, thus the wavelength range was 400-900nm.

The pigment content was calculated using formulas deduced by Lobban et al., (1988).

To quantify the extracted phycocyanin concentration based on the spectrophotometric data, equation (3) was used:

$$[PC] = \frac{A(620nm) - 0.72 \times A(650nm)}{6{,}29} \quad (3)$$

Where,

[PC] - Concentration of phycocyanin

A(650nm) - Absorbance of the sample at wavelength of 650 nm

A(620nm) - Absorbance of the sample at wavelength of 620 nm

[3] - Details of the construction of the standards and the calibration curve are in Attachment C.

To quantify allophycocyanin concentration, based on the spectrophotometric data, equation 4 was used:

$$[APC] = \frac{A(650nm) - 0.191 \times A(620nm)}{5.79} \quad (4)$$

In which,

[APC] – Concentration of allophycocyanin

A(620nm) - Absorbance of the sample at wavelength of 620 nm

A(650nm) - Absorbance of the sample at wavelength of 620 nm

To quantify the extracted phycoerythrin concentration, based on the spectrophotometric data, equation 5 was used:

$$[PE] = \frac{A(565nm) - 2.41 \times (CPC) - 1.41 \times (APC)}{13.02} \quad (5)$$

Where,

[PE]- Concentration of phycoerythrin

A 565nm - Absorbance of the sample at wavelength of 565 nm

[APC] - concentration of extracted allophycocyanin

[CPC] - concentration of extracted phycocyanin

3.4.2. Exopolysaccharide's analysis

The extraction followed the method described by Flores & Tamagnini (2019).

On the first day of analysis, 100 ml of sample was collected first. It was centrifuged (Eppendorf, 5804) at 14,610 g for 10 minutes, the supernatant was collected, and the pellet was washed three times with Milli-Q water, with subsequent centrifugations always collecting the supernatant.

Before starting the dialysis process with the 10 KD membranes (Fisher Scientific, Biodesign™ Cellulose Dialysis Tubing Roll), they were activated in a container with 70% ethanol and stored at about 4°C, for 24 hours, before use. After this period, the membranes were washed with type 2 distilled water, filled with the supernatant, and finally tied at the end with a thread. Each membrane was checked for leaks and placed in a goblet with 2 litres of Milli-Q water on a magnetic plate stirrer (Ika, C-Mag HS7) for 24 hours. The water in the goblets was changed after 12 hours (**Figure 17**).

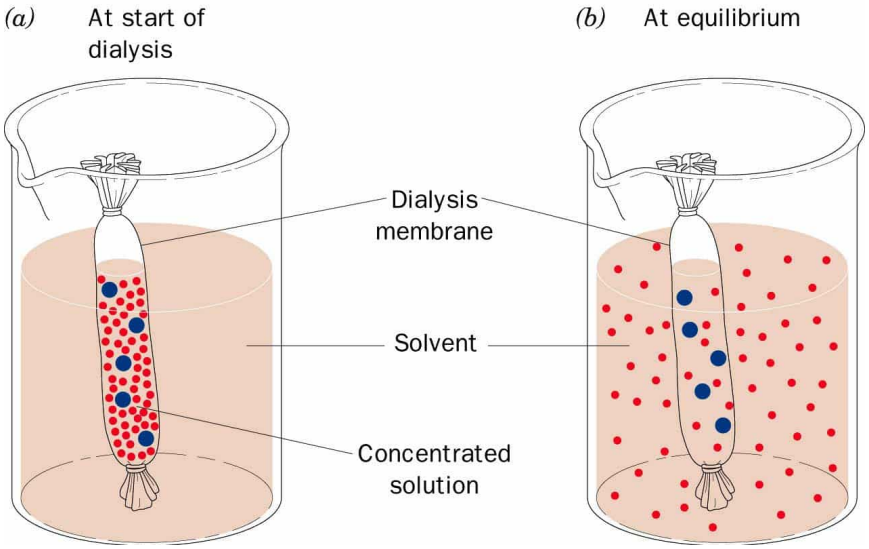

Figure 17-Schematic representation of the dialysis process. From (C. R. Anderson, 2010)

The next day, the contents of the membranes were collected. Samples were then centrifuged (Eppendorf, 5810R) at 18,514 g at 4°C for 15 minutes to remove most contaminants (cell wall material and lipopolysaccharides). Then the supernatant was collected and double the volume of cold 96% ethanol was added (**figure 18**). It was placed in the cold room properly covered and left overnight.

On the third day, the content of the membranes was centrifuged (Eppendorf, 5810R) at 13,000 g, for 25 minutes at 4°C. After this cycle, about 100 ml of cold 96% ethanol were added for further precipitation to occur and left for 48 hours stored in the fridge. After this period the entire contents of the goblet were centrifuged (Eppendorf, 5810R) under the same conditions as previously. Finally, the samples were freeze-dried, and the content was weighed as the final EPS content of the samples (**figure 19**).

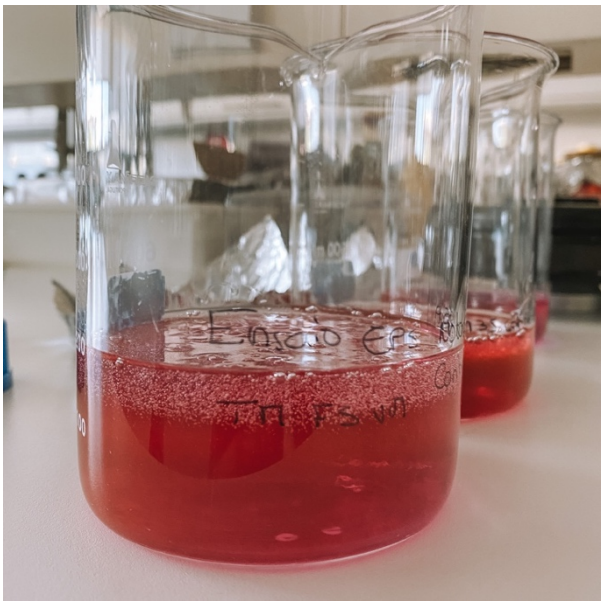

Figure 18-Formation of the exopolysaccharide layer after adding of 96% ethanol.

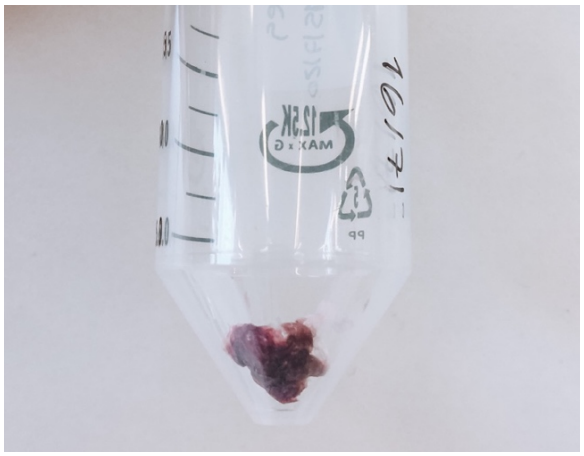

Figure 19-Freeze-dried exopolysaccharide sample.

3.5. Statistical analysis

The data are expressed as mean±standard deviation. All the statistical analyses considered significant were at a level of 5% (p-value < 0.05).

To test normality and variance homogeneity, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used, respectively. If the data fulfil these assumptions, parametric t-student were performed. If the data did not fulfil these assumptions, the non-parametric Mann-Whitney tests were used. Statistical analyses were performed using IBM SPSS statistical software, version 27.0 (IBM Corporation, Armonk, NY, USA).

4. Results

4.1. Growth Tests Assays

4.1.1. Growth curve and growth rate

The growth curve of *Anabaena* 4 was defined over the 28-day period (**Figure 20**).

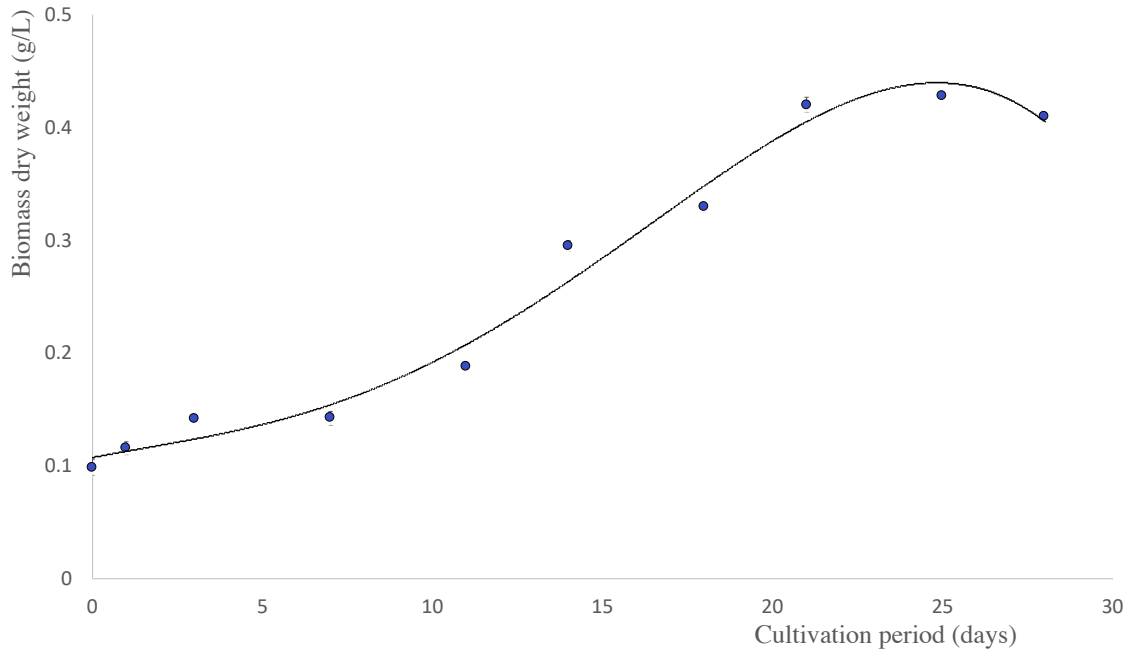

Figure 20-Anabaena 4 (n=2) growth curve for 28 days, in a 500mL flat bottom flask, with a photoperiod of 16h D / 8 h, T=20±2°C with a light intensity of 10.8 µmol $m^{-2}s^{-1}$.

The growth of *Anabaena* 4 follows the pattern of microalgae typical growth curve, although the strain is a slow growing cyanobacteria, with the stationary phase being reached only after 21 days.

Anabaena 4 initiates with an adaptation phase to the environment, lag phase, which lasts until the sixth day of cultivation.

The exponential phase occurs from day 7 to day 21, where the maximum productivity is reached on day 14 with 14.014±8.26 $mg.L^{-1}day^{-1}$.

From day 21 to day 25 a stationary phase occurs, reaching the biomass concentration of 0.4273±0.06 $g.L^{-1}$ (DW). In this phase the growth rate stabilizes and then it begins to decrease until the nutrients are depleted.

From that day on, we can see that there is a decline, or death, phase. This phase is characterized by a negative growth rate and the total exhaustion of nutrients and quality of the culture.

As to *Nostoc* 137, the structure of the filaments aggregating in irregular macroscopic colonies, made the weight measurements very difficult to perform and thus, the results obtained are much more variable and the curve is much more heterogenous. Despite these difficulties, the growth curve of *Nostoc* 137 was defined over the 28-day period (**Figure 21**).

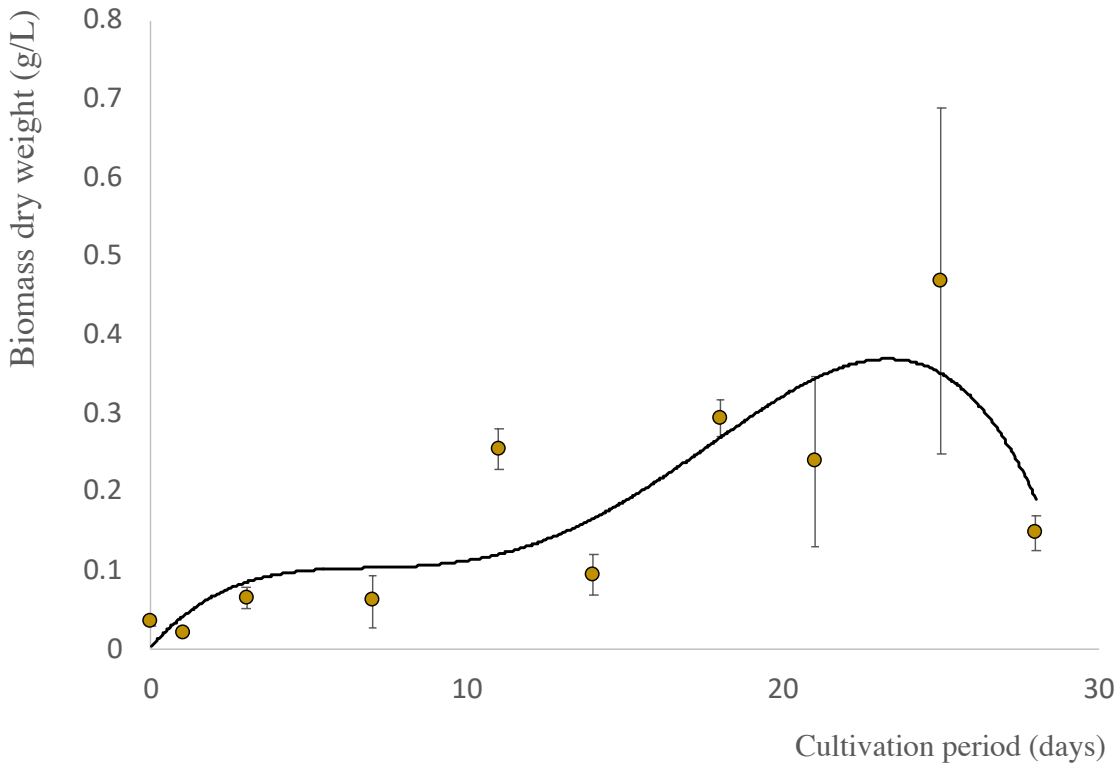

Figure 21- Nostoc 137 (n=2) growth curve for 28 days, in a 500mL flat bottom flask, with a photoperiod of 16h D / 8h N, T=22±4°C with a light intensity of 10.8 1 µmol $m^{-2}s^{-1}$.

There is no evident lag phase, since the inoculum originated from a dense initial culture (20%), thus there was a minimum period of adaptation to a new medium. Thus, an acceleration phase started immediately but the growth stopped for a few days.

Thus, acceleration phase, only started from day 7 to 12. Then, until day 20 the exponential phase occurred, when the highest productivity of the culture is achieved on day 18 with 14.29±0.90 $mg.L^{-1}day^{-1}$.

The stationary phase began until day 25, with maximum biomass concentration of 0.47±0.22 gL^{-1} (DW). And from that day on we can observe that there was a clear declining phase. The growth rate for each strain is shown in **Table II**.

Table II-Growth rate (μ) of the two strains of Cyanobacteria analyzed, in g day -1. Data are expressed as mean ± standard deviation (n=2).

Days	*Anabaena* 4	*Nostoc* 137
0-3	0.30±0.01	0.41±0.02
4-7	0.19±0.00	0.26±0.02
8-10	0.19±0.00	0.28±0.04
11-14	0.23±0.01	0.13±0.02
15-18	0.11±0.06	0.25±0.02
19-21	0.14±0.03	0.13±0.04
22-24	0.07±0.01	0.17±0.03
25-28	0.08±0.02	-0.04±0.19

Nostoc 137 shows a rather heterogeneous growth throughout the duration of the test, which makes it difficult to understand and distinguish the various growth phases. Still, it seems visible that it reaches the highest value of growth rate between days 15 and 18, as shown in **figure 21**. This period also corresponds to the maximum yield reached, with a value of 14.29±0.90 mg. $L^{-1}day^{-1}$ at day 18. Between days 25-28, this strain shows negative values which represents the decline phase observed in **figure 21.**

Anabaena 4 represents a more homogeneous culture, and the different growth phases are perceptible. It reaches the highest value of growth rate on day 14, with 0.23±0.01 $g.day^{-1}$, which corresponds to the exponential phase. Although not visible in Table II, this strain reaches the decline phase on day 25.

4.1.2. Effect of nitrate concentration on biomass production

When trying to increase the productivity through the increase in the nitrate content in the medium, we can observe that when the amount of nitrates (3g) doubled (**Figure 22**), for *Nostoc* 137, there was a decrease in the biomass production while in *Anabaena* 4, there was an increase in the biomass production.

The growth rate, calculated by the difference between day zero of the assay and day 15, obtained values for the strain *Nostoc* 137, of 0.096 ± 0.0678 $g.L^{-1}$ of dry biomass for the control (1.5g of nitrates) and 0.025 ± 0.0180 gL^{-1} of dry biomass for the variable (3g of nitrates). For *Anabaena* 4, the control obtained a dry biomass production of 0.332 ± 0.235 $g.L^{-1}$ while with the 3g nitrate supplement a value of 0.401 ± 0.284 $g.L^{-1}$ was obtained.

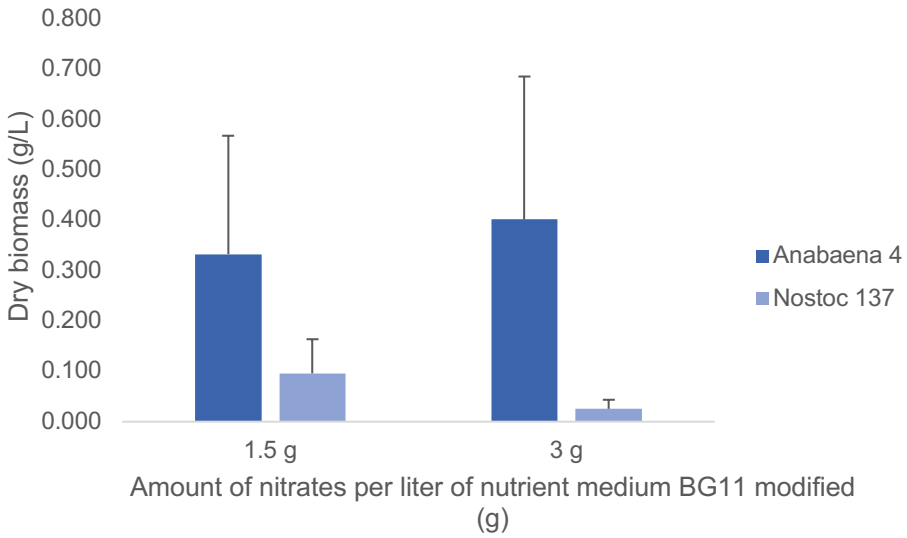

Figure 22- Biomass quantification with two variables: modified BG11 medium and BG11 2x Nitrates medium in Anabaena and Nostoc culture. Results presented as mean ± standard deviation (n=3).

These values can be compared with those obtained with the assay of the growth curve (**figures 20 and 21, section 4.1**). Comparing the same period of *Anabaena* 4 growth curve and the value obtained with double nitrate (3 g of nitrate) is much higher than that obtained in the growth curve. The highest values obtained in this assay, 0.401 ± 0.284 $g.L^{-1}$ in a control, were only obtained around day 20/21 as can be seen in figure 20. Comparing the same period of *Nostoc* 137 growth curve, the value obtained for the control (1.5g of nitrate) is very similar, which is not observed when the nitrate is doubled. In fact, the value obtained in this assay is much lower than the one obtained for the same period (15 days), in the growth curve (**figure 21**).

Although the sample was small, non-parametric tests were performed in order to verify if there are statistically significant differences between the two culture media.

The Mann-Whitney tests showed that there were no statistically significant differences among the two locations (U = 61.50, ρ = 0.7). Regarding the test with *Anabaena* 4, the condition 3g Nitrates was the most favourable, showing a more homogeneous distribution, i.e., the values between replicates are higher, do not disperse much and are close to the median. The control condition (1.5 g), on the other hand, showed much more dispersed and far from the median, values (**figure 23**). Also, there is still some boxplot overlapping, indicating that the true medians do not differ significantly.

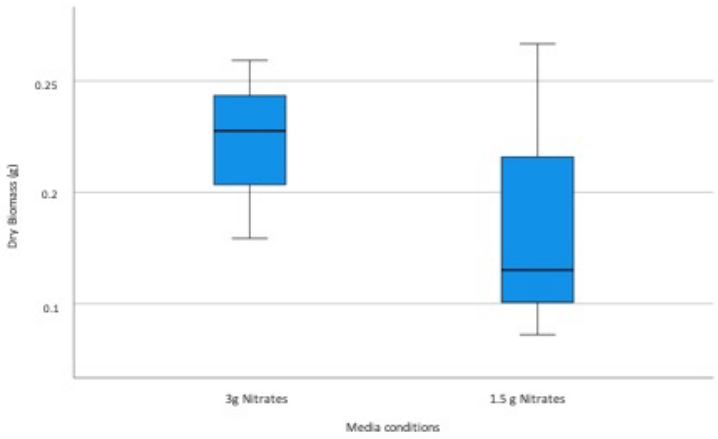

Figure 23-Boxplots of the nitrate assay for Anabaena 4.

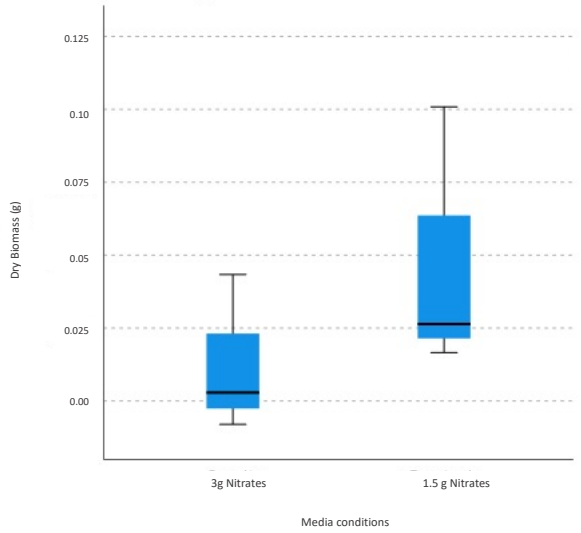

Figure 24-Boxplots of the nitrate assay for Nostoc 137.

By observing the **figure 24**, it is possible to understand the distribution of the data from this test for *Nostoc* 137. The figure clearly shows that the data are very heterogenous, with the distribution of the data scattered, showing thus great variability, mostly for the

control. Besides, although the medians are distinct, the boxplots are overlapping, within the 95% confidence interval, clearly indicating that the true medians do not differ.

Yet, although there are no statistically significant differences, there is a trend of increased biomass production in the control, regarding the 3g Nitrate, indicating that this is not a mean to increase the biomass of *Nostoc* 137.

4.2. Results -Chemical Analysis

4.2.1. Protein Content

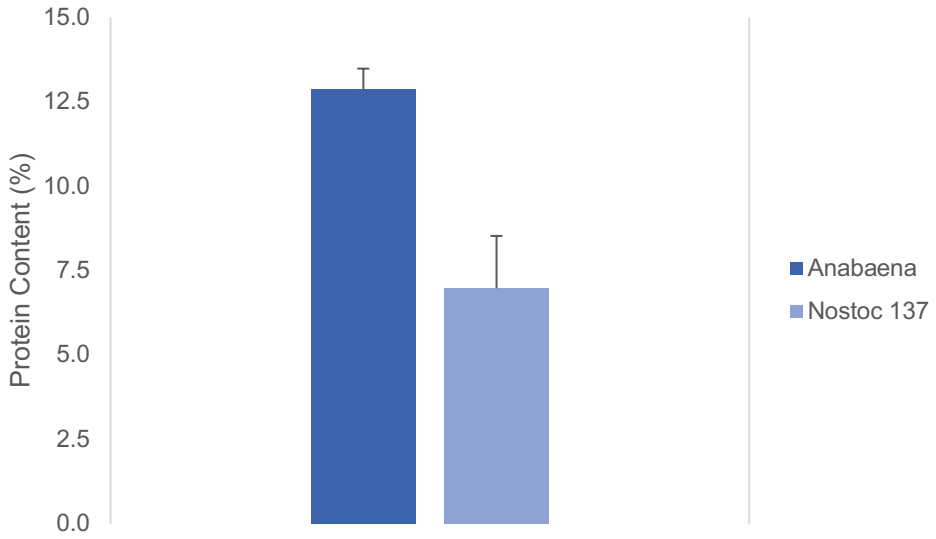

Figure 25-Protein quantification of Anabaena 4 and Nostoc 137 (n=3), grown in modified nutrient medium BG11, photoperiod 16h D/8h N, T=20±2ºC with a light intensity of 10.8 µmol $m^{-2}s^{-1}$. Results presented as mean ± standard deviation (n=3).

The biomass harvest (n=3) for the protein assay was performed using flat bottom flasks under modified nutrient medium BG11, photoperiod 16h D/8h N, T=22 ±4ºC with a light intensity of 10.8 µmol $m^{-2}s^{-1}$.

By observing **figure 25**, we can state that the strain *Nostoc* 137 shows low extraction values compared to the values that are associated with cyanobacteria (between 30% and 50%) (López et al., 2010), as does *Anabaena* 4. Yet *Anabaena* 4 has more uniform

protein content values and a higher extraction rate. *Anabaena* 4 has an average of 12.87% ± 0.6162 % protein content while *Nostoc* 137 has an average protein content of 6.98% ± 1.5505 %.

4.2.2. Effect of LED light on the production of phycobiliproteins

Table IV shows the values obtained for the quantification of pigments *Anabaena* 4 strain under standard conditions and when submitted to LED light, in the initial of the trial and after 15 days.

In the control, under the influence of daylight cool white fluorescent lamps, there is a decrease over the test period in phycocyanin (C-PC) production and phycoerythrin (C-PE) production. Allophycocyanin (C-APC) showed a slight increase in production. Inversely, observing the values in Table IV, it can be seen that a large increase in the production of all the pigments, C-PC, C-APC and C-PE occurred when grown under LED light.

Table III-Quantification of pigments C-PC, phycocyanin; C-APC, allophycocyanin and C-PE, phycoerythrin for the species Anabaena 4 under different light conditions. Results presented as mean ± standard deviation (n=3).

	Strain	C-PC (μg.ml)	C-APC (μg.ml)	C-PE (μg.ml)
Time 0	*Anabaena* Control	162.47 ± 35.518	19.44 ± 7.595	28.89 ± 4.664
	Anabaena LED	74.74 ± 24.965	22.55 ± 7.770	14.10 ± 4.891
Time 15	*Anabaena* Control	105.72 ± 55.960	29.77 ± 22.300	19.82 ± 9.706
	Anabaena LED	223.06 ± 66.351	56.10 ± 19.266	43.69 ± 12.348

To better understand the pattern of photosynthetic pigments, the spectrum was scanned between the 400 and 900 nm zones, to cover all photosynthetic pigments under analysis, namely the phycobiliproteins. Figure 26 shows that there is a peak in the 620 nm zone that corresponds to the phycocyanin, which is responsible for the bluish coloration.

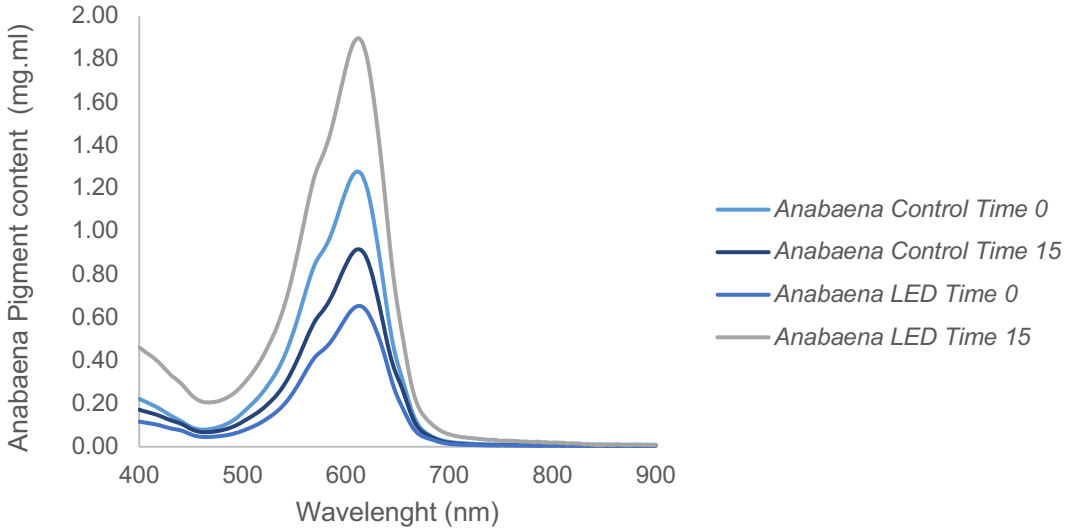

Figure 26-Spectrum scan between the 400-900 nm zone for Anabaena 4 under the influence of two different light conditions, at the beginning and the end of the experiment.

Table V shows the values obtained for the quantification of pigments for strain *Nostoc* 137, in much smaller concentrations than those obtained for *Anabaena* 4.

In the control, under the influence of daylight cool white fluorescent lamps, we see that there is an increase over the test period in the production of all the pigments under study, C-PC, C-APC and C-PE.

Inversely, under the influence of white LED, it can be seen that there was a decrease in the production of all the pigments analysed, C-PC, C-APC and C-PE.

Table IV-Quantification of pigments C-PC, phycocyanin; C-APC, allophycocyanin and C-PE, phycoerythrin for the strain Nostoc 137 under conditions of two light variables. Results presented as mean ± standard deviation (n=3)

	Strain	C-PC (μg.ml)	C-APC (μg.ml)	C-PE (μg.ml)
Time 0	*Nostoc 137* Control	11.75 ± 11.470	1.85 ± 2.214	6.10 ± 4.801
	Nostoc 137 LED	19.40 ± 6.456	4.90 ± 1.320	13.67 ± 6.470
Time 15	*Nostoc 137* Control	26.12 ± 26.257	10.99 ± 12.269	22.77 ± 21.249
	Nostoc 137 LED	5.59 ± 0.710	1.66 ± 0.285	3.80 ± 0.697

The following figure (**figure 27**) shows the absorption spectrum for the pigments under analysis. The scan was made between 400 and 900 nm to cover all the desired wavelengths.

For *Nostoc* 137 we can see that all the spectra have a peak (black arrow) around 610/620 nm which corresponds to the C-PC.

We also observe a peak (red arrow) also in the 560/570 nm zone, which is associated with the pigment phycoerythrin that gives the red coloration. This result is consistent with the macroscopic colour of the strains which present a brownish colour.

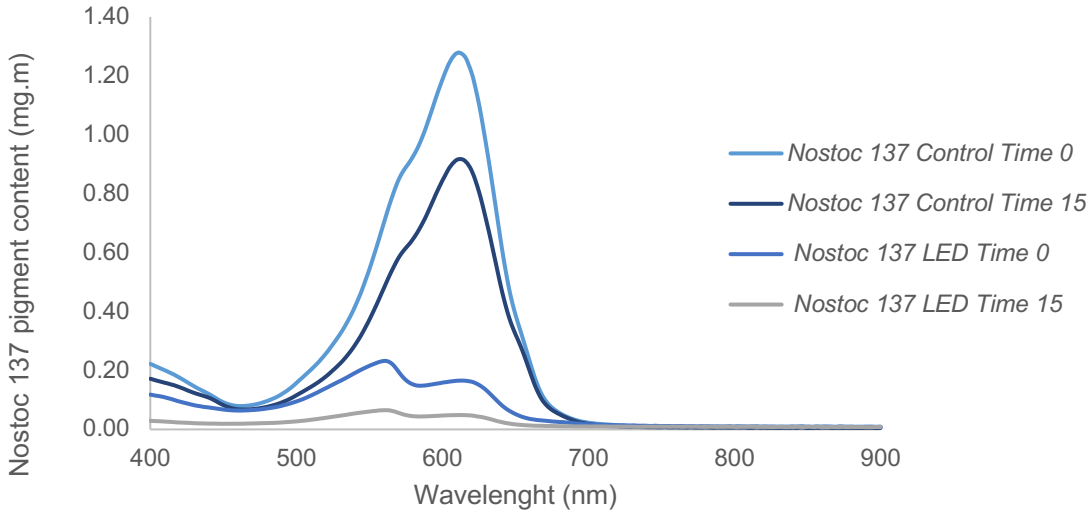

Figure 27-Spectrum scan between the 400-900 nm zone for the strain Nostoc 137 under the influence of two different light conditions.

To verify whether the different culture conditions (light) have statistically significant differences between them. For *Anabaena* 4 parametric t-student tests were performed to verify the equality of means. There were statistically significant differences for both phycoerythrin and phycocyanin pigments (*p=0.012 for C-PE and p=0.009 for C-PC, p<0.05*).

For the *Nostoc* 137, t-student tests were performed for the phycoerythrin pigment and there were no statistically significant differences found between the two culture conditions (p=0.073, p>0.05). For the phycocyanin pigment as data normality was not verified, non-parametric tests were applied, where it was verified that there are no statistically significant differences (U=0,00; p=0.1; Mann-Whitney test)

Nevertheless, and because the sample size is small, there is a tendency, in the test with *Anabaena* 4, for pigment production to increase under the influence of LED light and for pigment production to decrease under the influence of fluorescent light. For the strain *Nostoc* 137, there is an increasing trend of pigment production under the influence of fluorescent light and a decreasing trend under the influence of LED light.

4.2.3. Effect of sodium bicarbonate on exopolysaccharide production

Regarding the exopolysaccharide extractions, very different values were obtained for the two strains. By observing Table VI, we understand that the quantification values are null or close to zero for *Nostoc* 137. Yet, a much higher concentration was obtained for *Anabaena* 4, mainly under BG11 nutrient medium.

Table V-EPS quantification with the addition of sodium bicarbonate. Results presented as mean ± standard deviation (n=3)

Nutrient medium BG11		BG11 nutrient medium with added $NaHCO_3$	
Strain	Freeze dried mass ($mg.L^{-1}$)	Strain	Freeze dried mass ($mg.L^{-1}$)
Anabaena 4	876.50 ± 91.005	*Anabaena* 4	135.70 ± 21.25
Nostoc 137	0 ±0	*Nostoc* 137	1.33 ± 0.153

For *Anabaena*, in the case of the control there is only one duplicate (n=2) due to the fact that of one of the replicates suffered a contamination and didn't reach the end of the cultivation period.

To determine whether there are statistically significant differences between the two conditions, the non-parametric Mann-Whitney test was performed, for the strains *Anabaena* 4 and *Nostoc* 137. The Mann-Whitney tests showed that there were no statistically significant differences among the two locations (U = 6.5, ρ = 0.2), for *Anabaena* 4 and (U = 1.5, ρ = 0.2) for *Nostoc* 137.

4.2.4 Effect of sodium chloride on exopolysaccharide production

When inducing stress to the strains, trying to increase exopolysaccharides production, again, very different results were obtained. In this case there are only data for the *Nostoc* strain, because all the *Anabaena* strain trials performed were not successful, the culture always ended up dying after 10/15 days, never completing the 30-day trial. We can, therefore, conclude that this strain is intolerant to salt, unlike the *Nostoc* strain analysed. By examining Table VII, we observed zero values in the control test, as in the previous trial, the extraction process for this species was insufficient. In the variable with the addition of NaCl, the value was 5.67±0.603 $mg.L^{-1}$.

Table VI- EPS Quantification with the addition of Sodium Chloride. Results presented as mean ± standard deviation (n=3)

Nutrient medium BG11		BG11 nutrient medium with added NaCl	
Strain	Freeze dried mass ($mg.L^{-1}$)	Strain	Freeze dried mass ($mg.L^{-1}$)
Nostoc 137	0 ± 0	*Nostoc* 137	5.67 ± 0.603

At first it was thought to be due to the addition of sodium chloride, for it could be a species that did not tolerate the introduction of salinity, but it turned out to be a contamination by an unidentified microalga in all maintenance flasks of this species, which were used for this trial. Thus, since the culture was already under stress, the additional stressor prevented the growth and survival of the culture.

In order to determine whether there are statistically significant differences between the two conditions, a non-parametric Mann-Whitney test was performed, for the strain *Nostoc* 137. The Mann-Whitney tests showed that there were statistically significant differences among the two locations ($U = 7.5$, $\rho = 0.2$).

5. Discussion

5.1. Growth Tests Assays

5.1.1 Growth curve and growth rate

The purpose of this assay was to obtain the growth curve of two strains of cyanobacteria and their growth rate, and also to try to understand what the duration of each of the subsequent assays would have to be.

Compared to the species *Nostoc flagelliforme* (Yu et al., 2009), under photoautotrophic conditions, for the *Nostoc* and *Anabaena* strains we analysed, we obtained almost the same concentration of biomass (Yu et al., 2009). For a trial duration of 20 days the authors obtained about 0.4 $g.L^1$, while in the *Nostoc* strain at the end of the same period the amount of dry biomass was about 0.32 $g.L^1$ and in the *Anabaena* strain about 0.4 $g.L^1$ of dry biomass.

In another study of Rosales Loaiza et al., (2016) also with *Nostoc* and *Anabaena* higher concentrations of 1.32 gL^{-1} for Nostoc and 1.86 g/L for *Anabaena* (values obtained in the stationary phase) were obtained. The maximum biomass production for *Anabaena* 4 was 0.4273 $g.L^{-1}$ and for *Nostoc* 137 was 0.4674 gL^{-1}, respectively. Therefore, it can be seen that with this test with biomass extraction in the same phase, much less biomass was obtained than in the study mentioned above.

El Shafay et al., (2021), shows that in a 24-day culture with *Anabaena variabilis* and *Nostoc muscorum*, the stationary phase ends on day 14 for the *Anabaena* species and on day 16 for the *Nostoc* species with a maximum biomass of 0.69 gL^{-1} and 0.64 gL^{-1}, respectively. In comparison to this trial, we can see that both species showed a slower growth rate, because the stationary phase does not occur until day 21 for both *Anabaena* 4 and *Nostoc* 137.

Despite having achieved the full growth of both strains, we had some difficulties in the filtration, because mainly the *Nostoc* strain is a very heterogeneous culture, forming agglomerates of different sizes as can be seen in figure 3, and, although we have tried to homogenize the culture before starting the assay, it was impossible to obtain the same number of initial cells in all test flasks, which certainly had an impact on the growth curve of this strain.

For *Anabaena*, the culture presents a more homogeneous aspect, but this strain, after a short period without aeration, tends to deposit at the bottom of the flask. Another problem could have been that the filters used for filtration were not the most appropriate. Thus, we could have used filters with a lower retention of particles or used two types of filters to avoid losing biomass.

5.1.2. Effect of nitrate concentration on biomass production

Among the nutrients required for the healthy growth of a cyanobacterial culture, nitrogen is a key element for the growth and to produce cellular compounds. Changes in the level of this nutrient can alter the biochemical composition of the cyanobacteria (Çelekli & Yavuzatmaca, 2009) besides changes in the growth rate and, thus, the yield of the cultivation.

In this case, we wanted to verify if the addition of a higher amount of this nutrient would influence the concentration of the two filamentous cyanobacteria under study.

The data obtained showed that there is a tendency of increase in biomass production compared to the control for *Anabaena* 4, while with *Nostoc* 137 there is a trending decrease in biomass production.

The previous study of Crnkovic et al., (2018), show that low nitrate levels significantly reduce biomass production in the filamentous species, including cyanobacteria that produce heterocysts and are able to fix N_2, as is the case with the target species in this work.

Eliminating the nitrogen source from the culture medium is a well-established technique and some strains are able to grow in these environments with low nitrogen concentration conditions (Ramus, 1981), while developing higher number of heterocysts that will convert N_2 into ammonia. Our results show that the *Nostoc* strain is able to grow in environments with low concentrations or even in deprivation of this nutrient, because adding nitrogen had a negative effect on the growth of the strain. On the contrary, although *Anabaena* is also a heterocystous species, its biomass production is limited at low sodium nitrate concentrations.

Our results are in line with the study by Rosales Loaiza et al. (2016), where *Nostoc* strains produce higher biomass at low sodium nitrate concentrations, while *Anabaena* 4 strains showed better biomass production when increasing $NaNO_3$ concentration. The higher biomass production at low nitrate levels observed in *Nostoc* 137, may be due not to increased cell production or metabolite accumulation, but rather to the increased exopolysaccharide (EPS) production, which is normal to occur in cyanobacteria under stress conditions. When filtration occurs in dry weight determination EPS are also accounted for (Otero & Vincenzini, 2004).

Despite several studies showing that cyanobacteria grow better in environments with higher levels of nitrogen (Loreto et al., 2003; Rosales et al., 2006), it should be noted that this characteristic may not be present in all cultures, as it depends on the culture medium provided.

The research of El Shafay et al., (2021) showed that the highest amount of biomass and productivity were observed at the highest nitrogen concentrations, 0.79 ± 0.026 g/L for *Anabaena variabilis* and 0.74 ± 0.002 $g.L^{-1}$ *for Nostoc muscorum* followed by the control, which in this study was also found for *the Anabaena* 4 that increased its biomass production to 0.401 ± 0.284 $g.L^{-1}$ (DW). This increased production at higher nitrogen concentrations can be attributed to the role of nitrogen in photosynthesis, since the availability of this nutrient is one of the most important factors in determining the growth of cyanobacteria.

The ability to develop and grow in different concentrations of nitrogen shows the cyanobacteria's physiological versatility in adapting to various environments, even when nitrogen concentration is limited or absent. As stated, this is mainly due the N_2-fixing heterocysts present in these strains.

5.2. Chemical Analysis

5.2.1. Protein Content

The protein content obtained in our study for the two strains was smaller than that obtained by El Shafay et al., (2021), which reached protein content values of about 80 $mg.L^{-1}$ for *Anabaena variabilis* and about 100 mgL^{-1} *for Nostoc muscorum*. In the study by López et al., (2010) for *Arthrospira* (formerly *Spirulina*) a protein content of about 52% was obtained, while in this study an average protein content of 12.87% ± 0.6162 was obtained for *Anabaena* 4 and an average of 6.98% ± 1.5505 for *Nostoc* 137.

The total extraction of a specific component from microalgae or cyanobacteria is often incomplete due to the intrinsic rigidity of the cell wall that covers the cell membrane. Overcoming this barrier requires a first cell disruption operation to facilitate the extraction process and access to all the target components.

Pre-treatment, such as mechanical mechanisms or chemical treatment, have been shown to result in improved extraction due to partial or complete degradation of the cell wall, allowing better accessibility to intracellular components. Since solubility is pH-dependent, high acid and alkaline conditions improve the solubility of algal protein (Parimi et al., 2015; Safi et al., 2014).

In this assay, the initial aim was to use freeze-dried biomass, but due to technical problems in the laboratory it was not possible to use this process. Alternatively, the sample pellet was dried in an oven overnight at a 60ºC, which may have impaired the result of this test. In the study of Desmorieux & Hernandez (2004) the freeze-dried biomass had a lower percentage of protein loss (less than 10%), while in the 60ºC oven this loss corresponded to 10-20%. In this study, the authors also observed that at 60ºC there was a damage to the *Arthrospira* filaments.

The growth phase of the culture also has an impact on the ease of cell disruption, cells in the exponential phase are less robust cells and therefore easier to rupture and release their contents than cells in the stationary, slow-growth phase, here the cells are more robust and therefore need more effective pre-treatments (López et al., 2010).

In the paper by El Shafay et al., (2021), perform an assay in which they verify the influence of nitrogen deprivation on protein content, and it is verified that the protein content reduces about 50% in relation to the control, values of about 50 $mg.L^{-1}$ were observed for *Anabaena variabilis* and 45 $mg\text{-}.L^{-1}$ for *Nostoc muscorum*, which may suggest that the samples analysed in this study could be at this stage of culture, thus not allowing the quantification of higher concentrations of protein.

As a pre-treatment we used manual maceration, may not have been very efficient because the *Nostoc* forms agglomerated colonies of filaments which become very stiff when drying, Enabling the final goal that was to obtain a fine powder. One pre-treatment that could be used in the future is the maceration with ceramic particles, which is effective for breaking more robust cells and could have shown better results in this strain under study (López et al., 2010).

5.2.2. Effect of LED light on the production of phycobiliproteins

Different lights were tested in order to specify the most appropriate cultivation condition aiming to optimize the production of phycobiliproteins.

Light plays a very important role in the production and accumulation of pigments in cyanobacteria. Most of these species have the ability to control the amount of photosynthetic antenna pigments, and their phycobiliproteins, based on the light spectrum they are in, which is known as chromatic adaptation. When exposed to changes in the coloration of light or its intensity, cyanobacteria change their pigment composition in response to the specific exposure they are in (Khatoon et al., 2018).

In an indoor cultivation, artificial light is a critical factor during cyanobacterial cultivation. Traditionally, fluorescent lamps have been the main source of light, however they are energy inefficient and show wide range of emission spectra, including wavelengths with low photosynthetic activity.

For this reason, traditional light sources are being replaced by light emitting diodes (LEDs), which demonstrate several advantages, such as **(1)** lower heat emission compared to conventional light; **(2)** longer lifetime; **(3)** higher conversion efficiency than fluorescent lamps (Chen, et al., 2011); **(4)** zero toxic elements

such as mercury (Atta, et al., 2013); and **(5)** minimal variability of biomass rate and biochemical composition due to the simultaneous control between light quality and quantity (Park & Dinh, 2019).

The study of Prates, et al. (2018), compared the use of white fluorescent lamps and white LED on the growth and chemical properties of Cyanobacteria and showed that there was an increase in the production of phycobiliproteins under the influence of white LED compared to the white fluorescent, showing a considerable increased from 46.36 $mg.g^{-1}$ biomass to 83.76 $mg.g^{-1}$ biomass.

In the assay with the *Nostoc*, an increase in phycobiliprotein production could not be confirmed as described by Prates et al., (2018) study. Although there are no statistically significant differences between the two conditions, the assay shows that the concentration obtained under the influence of the white fluorescent lamp is higher than under LED light, being always low despite the conditions. To optimize the phycobiliprotein production in the *Nostoc* species, new experimental design should be performed, namely using coloured LED, such as with green coloration, since it is the condition that in the study that (Prates et al., 2018) conducted had the greatest influence on phycobiliprotein production. The emission spectrum of green LED light is between 500-600 nm, which coincides with the absorption spectrum of some phycobiliprotein, such as phycoerythrin.

Yet, an increase in phycobiliprotein production was observed with *Anabaena* where a trend of more than twice the amount obtained in the control was confirmed, achieving statistically significant differences between the two cultivation conditions ($p<0.05$). This assay shows that *Anabaena* can be easily manipulated to increase the production of phycobiliproteins under LED light, which is an important result, given the fact that phycocyanin is a well-known and sought-after pigment.

5.2.3. Effect of sodium bicarbonate on exopolysaccharide production

In this trial, the influence of adding sodium bicarbonate concentration to the medium was evaluated regarding the exopolysaccharide production.

The exopolysaccharides assays are quite long since require cultivation periods of 30 days. This is due to the fact that the cyanobacteria growth curve is long and

the production of exopolysaccharides is associated with the stationary phase (Flores & Tamagnini, 2019).

Bicarbonate is a crucial factor influencing the growth and the production of metabolites. In cyanobacterial species that live in more alkaline environments, the amount of dissolved CO_2 is reduced, so bicarbonate is added as a carbon source and assimilated due to the energy the cells capture from light (Vergnes, et al., 2019).

Previous studies showed that several factors influenced the production of EPS, such as light, temperature, nitrogen deprivation, phosphate, pH, and aeration (Flores & Tamagnini, 2019), i.e., the highest amounts of exopolysaccharides were obtained when the culture was exposed to some stress condition. The study of Vergnes et al. (2019), was the first known to have positive results for the influence of bicarbonate on EPS production in *Arthrospira platensis* and more generally in cyanobacteria.

There is a positive influence of bicarbonate on EPS production since cells need carbon to grow. When the cell is under favourable conditions, it uses the remaining carbon for EPS production.

Studies show that the higher the concentration of bicarbonate used in the medium, the higher is also the production of EPS (Vergnes et al., 2019). As verified with this assay with *Nostoc*, although there were no statistically significant differences (ρ >0.05) between the two variables, there is a tendency for an increase in EPS production in the presence of sodium bicarbonate.

The low amount of EPS obtained for *Nostoc* 137 may be due to the fact that both cultures had already passed the stationary phase by day 30, and this was more evident for *Nostoc* 137 than for *Anabaena* 4 (**Figure 20 and 21**).

Since the culture in this assay was performed in batch mode, it would be expected that over time it would lead to a shortage of nutrients such as nitrogen and complementing with the addition of bicarbonate would be sufficient to maximize the production of EPS, for this reason only the addition of bicarbonate was assumed.

Research of El Shafay et al. (2021), suggests that when comparing the control with nitrogen deprivation there is an increase in carbohydrate production from about 150 $mg.L^{-1}$ to 225 mgL^{-1} for *Anabaena variabilis* and from 220 $mg.L^{-1}$ to about 300 $mg.L^{-1}$ *for Nostoc muscorum*. Considering the results obtained in this

study, nitrogen deprivation should have been combined with the addition of sodium bicarbonate.

As to *Anabaena* 4 there was a tendency for a *decrease* in EPS production in the presence of sodium bicarbonate (table V), although, again, this did not show statistically significant differences (ρ >0.05). Tiwari et al. (2015) found a range of EPS production for different bacteria between 60 and 1,580 $mg.L^{-1}$, meaning that *Anabaena* produced a large concentration of EPS. Although, we were unable to induce stressful conditions that would increase this production through sodium carbonate, as in *Nostoc* 137.

5.2.4. Effect of sodium chloride on exopolysaccharide production

The aim of this assay was to evaluate the influence of the stress caused by increased salinity, on the production of exopolysaccharides.

Several factors affect the production and biochemical composition of algal biomass. As stated before, these include various physical parameters such as pH, temperature, light intensity, photoperiod, and availability of nutrients such as carbon, nitrogen, phosphate, and vitamins (Markou et al., 2014).

Salinity is a complex stressor that influences various physiological and biochemical mechanisms associated with algal growth and development (Jo et al., 2020). Since sodium chloride is a cheap and readily available compound, adjusting the concentration of NaCl can be an easy enhancement of the production of various compounds such as EPS.

Being under the influence of salinity stress, cyanobacteria alter their metabolism to adapt to extreme environments. They can be grouped as halophiles (salt requiring for optimum growth) and halotolerant (having response mechanisms that allow them to survive in saline environments), in both cases there is the production of some metabolites for protection against damage that is caused by this stress and also to make the osmotic balance (Rao et al., 2007).

Salinity stress can lead to three types of stress: **ionic stress**, which results from an ionic homeostasis imbalance; **osmotic stress**, where the presence of salt causes a lowering of the osmotic potential occurring a reduction in water uptake and finally **oxidative stress**, where salinity stress causes an imbalance in the

generation of reactive oxygen species (ROS) causing oxidative stress (Chokshi et al., 2017).

The effect of NaCl on EPS production in cyanobacteria is not yet fully understood. Yet, studies showing positive results regarding the influence of salinity on EPS production, show an increase in EPS production under the influence of high salinity (400 mM) in three cyanobacterial species when compared to control. The EPS production seems to act as a barrier between the cell wall and extreme environments to ensure the cell survival (Ozturk & Aslim, 2010).

In this assay with the strain *Nostoc*, it also showed a tendency to increase the production of EPS under the influence of high salinity (400 mM) compared to the control (0 mM), since the values increased from 0 mg to 5.67±0.60 $mg.L^{-1}$ under the influence of NaCl, although there were no statistically significant differences due to the presence of salt in the media ($\rho > 0.05$).

With *Anabaena* 4 we couldn't obtain any data regarding the tolerance to the presence of NaCl since this species was contaminated by an unidentified microalga which hindered the success of this assay. The *Anabaena* 4 cultivation was then submitted to a decontamination process by altering its nutrient medium, totally depriving the culture from nitrogen, allowing *Anabaena*'s survival through the heterocysts, but removing the contaminant microalga. New trials with the healthy *Anabaena* 4 would allow to understand the cyanobacterial tolerance to high salinity and this factor influence on the production of EPS. As mentioned in the previous trial, these results could also have been increased if we had shortened the trial period and collected the cultivation earlier. Another option was also to perform the trial with different salinities to see which would be the best option for the optimization of EPS production, which was not possible due to lack of time. These trials are of long duration and Covid19 didn't allow us to extend the trials.

6. Conclusion

In summary, this work allowed to understand the performance of *Anabaena* 4 and *Nostoc* 137 strains under laboratory conditions, to study part of the chemical composition, as well as to understand, although in a preliminary way, how to manipulate some conditions to alter the growth rate and the chemical composition.

The initial and proposed objectives for the implementation of growth curves, for the definition of growth phases and respective growth rates, for the determination and optimization of culture parameters, for the quantification of pigments and their optimization, and for the optimization of biomass through nutrient manipulation were achieved.

Despite some difficulties faced throughout these assays, the results obtained showed us that the growth of these species is slower than in other studies mentioned here.

The quantification of protein had higher and more uniform results for *Anabaena* than for *Nostoc*. The method for protein extraction was not the most suitable for this type of biomass, and low protein values were obtained.

Studies have shown that LED light may bring numerous advantages both economically and in the biochemical composition of these two strains. Nevertheless, it only had a positive impact on *Anabaena* 4 and, although there were no statistically significant differences, the tendency to increase the production of these pigments is evident in this organism.

The proposed objectives for the analysis of EPS production through nutrient manipulation were partially achieved, but better results can be obtained in the future with some modifications described throughout this investigation.

Studies show that there is a higher production of EPS under the influence of sodium bicarbonate, but when it comes to sodium chloride it is still not well understood, although showing a positive influence when subjected to high salinities. *Nostoc* 137 is in line with previous studies, since there was a tendency of higher EPS production under the influence of both sodium bicarbonate and sodium chloride.

Although this study provides a better understanding of these strains and their behaviour under laboratory cultivation, further studies, mostly regarding *Anabaena,* the strain that showed greatest potential for commercial exploitation need to be developed to understand which environmental stressors trigger growth and specific metabolites production.
And for this reason, with the conclusion of this study, it is evident that there is still much work to be done in order to better understand these two new strains.

It is suggested that in future work the following changes be performed:

(1) Performance of a growth curve, using filters with different porosities for biomass extraction to achieve smaller standard deviations.

(2) Studying different pre-treatments for protein extraction, until the most suitable one is obtained, using freeze-dried biomass;

(3) Evaluate the influence of biomass production with more different nitrate concentrations, among other nutrients;

(4) Evaluate the influence of coloured LED lights on the production of the target pigments;

(5) Conduct trials with different and various concentrations of sodium bicarbonate and sodium chloride, and other stressful conditions, combined with nitrogen deprivation to maximize EPS production.

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Attachments

Attachment A. *Composition and preparation of the modified BG11 medium (modified at BUFFER concentration and pH) by C.Yepremian & Pauline Rambur) used for cyanobacterial culture.*

Stock number	Compounds	For 0.5L Mass (g)
1 (Not Autoclave)	MgSO4.7H20	3,25
	CaCl2.2H2O	1,8
2	K2HPO4.3H2O	2
	EDTA	0,05
3	$C_6H_8O_7$	0,3
	C6H11FeNO7	0,3
4	H3BO3	1,430
	MnCl2.4H20	0.905
	ZnSO4.7H20	0,110
	Na2.MOO4.2H20	0,195
	CuSO4.5H20	0,010
	Co(No3)2.6H20	0,025

Table AI -Composition of the modified BG11 medium for cyanobacterial culture

Table AII- Preparation of the modified BG11 medium for cyanobacterial

Stock number	Volume of stock to be added per litter of medium (ml)
-	Add 1.5g of NaNO3
1	10
2	10
3	10
4	2
-	Add 1.19g of HEPES

***Attachment B**. Pigment assay results for Anabaena 4 and Nostoc 137 (n=3). The control was performed under cool daylight white fluorescent lamps at 10.8 µmol $m^{-2}s^{-1}$ and a variable was performed under a white LED light at 14 µmol $m^{-2}s^{-1}$, modified nutrient medium BG11, photoperiod 16h D/8h N, room temperature 22 ±4°C.*

Table BI - Pigment results for Anabaena species

	Strain	C-PC (mg.ml)	C- APC (mg.ml)	C- PE (mg.ml)
Day 0	*Anabaena* Control (1)	0.05987	0.02085	0.04885
	Anabaena Control (2)	0.04320	0.01124	0.03980
	Anabaena Control (3)	0.06771	0.02623	0.05668
	Anabaena LED (1)	0.03923	0.02895	0.03069
	Anabaena LED (2)	0.02812	0.02479	0.02166
	Anabaena LED (3)	0.01999	0.01390	0.01534
Day 15	*Anabaena* Control (1)	0.01875	0.00850	0.01636
	Anabaena Control (2)	0.03885	0.02784	0.03127
	Anabaena Control (3)	0.06457	0.05298	0.04800
	Anabaena LED	0.11034	0.06863	0.08423
	Anabaena LED	0.06027	0.03391	0.04853
	Anabaena LED	0.08284	0.06575	0.07539

Where, C-PC- Phycocyanin; C-APC- Allophycocyanin; C-PE- phycoerythrin

Table BII- Pigment results for Nostoc species

	Strain	C-PC (mg.ml)	C- APC (mg.ml)	C- PE (mg.ml)
Day 0	*Nostoc* 137 Control (1)	0.00891	0.00439	0.01361
	Nostoc 137 Control (2)	0.00085	0.00034	0.00142
	Nostoc 137 Control (3)	0.00288	0.00082	0.00746
	Nostoc 137 LED (1)	0.00601	0.00508	0.02261
	Nostoc 137 LED (2)	0.01006	0.00612	0.01475
	Nostoc 137 LED (3)	0.00598	0.00350	0.01035
Day 15	*Nostoc* 137 Control (1)	0.02370	0.02514	0.05333
	Nostoc 137 Control (2)	0.00516	0.00448	0.01318
	Nostoc 137 Control (3)	0.00366	0.00334	0.01030
	Nostoc 137 LED (1)	0.00186	0.00140	0.00391

	Nostoc 137 LED (2)	0.00235	0.00162	0.00413
	Nostoc 137 LED (3)	0.00230	0.00197	0.00525

Where, C-PC- Phycocyanin; C-APC- Allophycocyanin; C-PE- phycoerythrin

***Attachment C**. Preparation of standards and calibration curves for Anabaena 4 and Nostoc 137 for protein analysis using the Pierce™ BCA Protein Assay Kit.*

Table CI - Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µl)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	*0*	300 of Stock	*2000*
B	*125*	375 of Stock	*1500*
C	*325*	325 of Stock	*1000*
D	*175*	175 of vial B dilution	*750*
E	*325*	325 of vial C dilution	*500*
F	*325*	325 of vial E dilution	*250*
G	*325*	325 of vial F dilution	*125*
H	*400*	100 of vial G dilution	*25*
I	*400*	0	*0 = Blank*

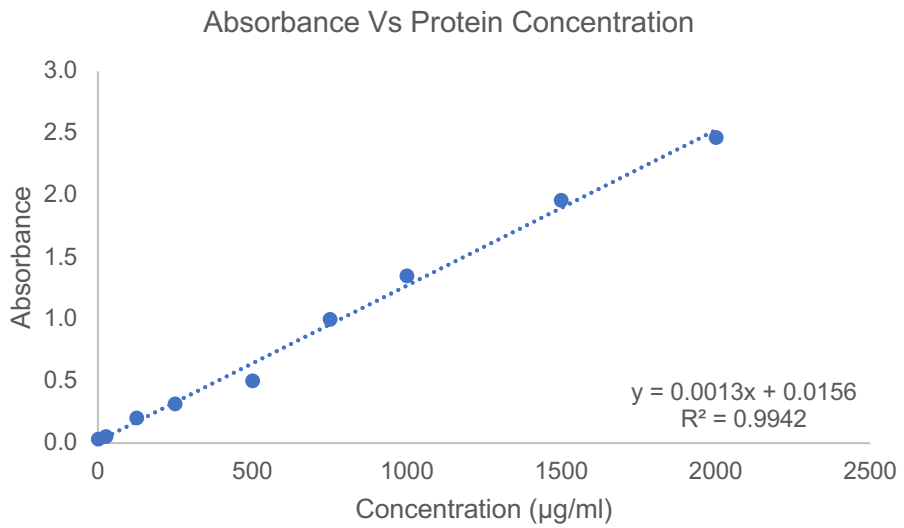

Figure C1 - Calibration curve for the specie Anabaena 4

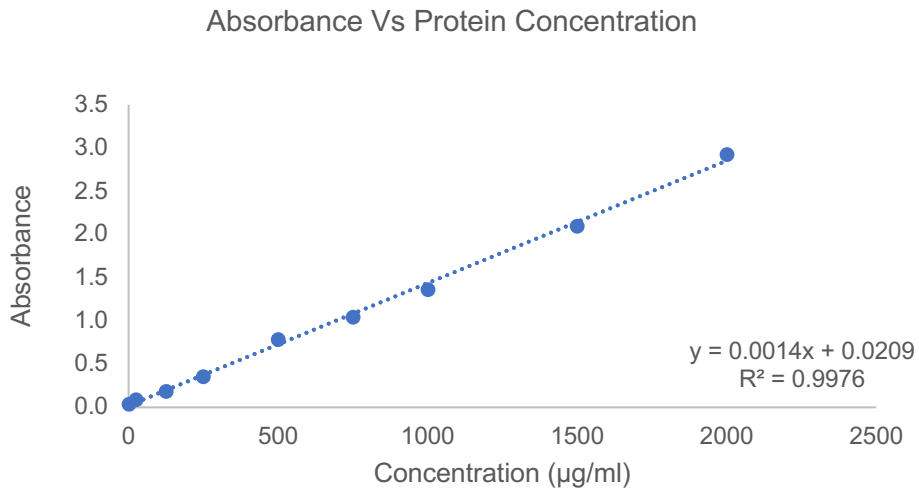

Figure C2 - Calibration curve for the specie Nostoc 137