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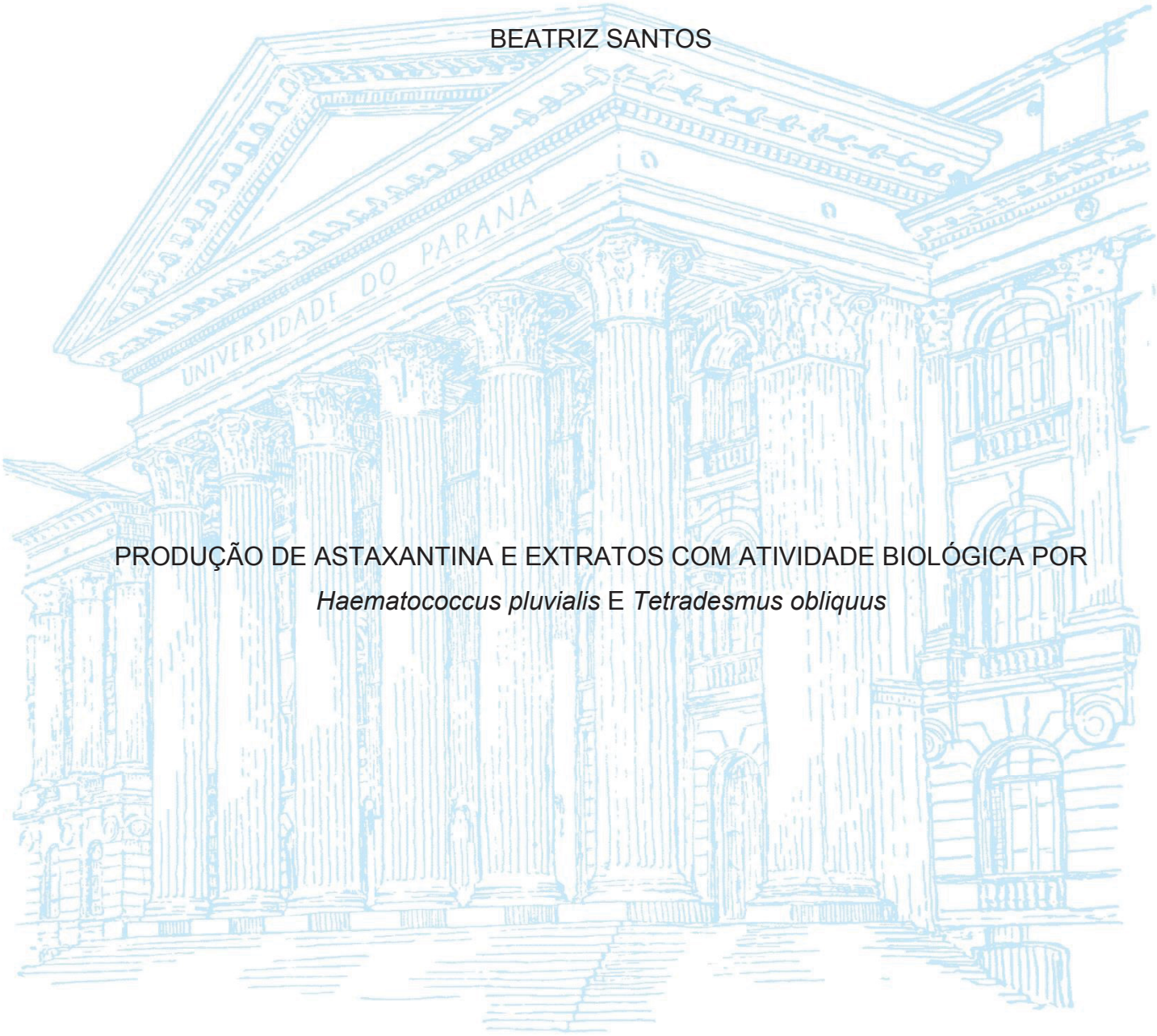
BEATRIZ SANTOS



PRODUÇÃO DE ASTAXANTINA E EXTRATOS COM ATIVIDADE BIOLÓGICA POR *Haematococcus pluvialis* E *Tetradesmus obliquus*

CURITIBA

2020

BEATRIZ SANTOS

Produção de astaxantina e extratos com atividade biológica por *Haematococcus pluvialis* e *Tetradesmus obliquus*

Tese apresentada ao Programa de Pós-Graduação em Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Genética

Orientadora: Profª. Drª Vanessa M. Kava
Coorientador: Prof. Dr. José V. C. Vargas
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A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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I was taught that the way of progress was neither swift nor easy.

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RESUMO

O cultivo de microalgas tem atraído a atenção de pesquisadores e indústrias de biotecnologia muitas vezes devido ao potencial valor agregado da sua biomassa. Ácidos graxos poli-insaturados, carboidratos, proteínas e carotenoides estão presentes nesta biomassa e muitos desses compostos possuem ação antioxidante, atividade antimicrobiana e bioestimulante de crescimento vegetal. No intuito de melhorar a obtenção destes bioprodutos, diferentes estratégias de cultivos podem ser avaliadas. A utilização de luz azul em conjunto com a privação de nitrogênio é uma estratégia conhecida para o aumento da produção de astaxantina, por exemplo. A indução de crescimento mixotrófico, com a adição de compostos orgânicos ao meio de cultura, também pode resultar no aumento de produção destas substâncias pelas microalgas. O estudo de genes expressos diferencialmente em diferentes condições de cultivo pode apontar para estratégias de melhoramento genético, incluindo modificações genéticas. Nesse sentido, o objetivo desse estudo foi avaliar a produção de carboidratos, lipídeos, proteínas e astaxantina em diferentes condições de cultivos das microalgas *Haematococcus pluvialis* e *Tetradesmus obliquus.* A expressão de genes carotenogênicos (fitoeno sintase, licopeno β-ciclase, β-caroteno cetolase e β-caroteno hidroxilase) foi avaliada em cultivos de *H. pluvialis,* expostos à luz azul, vermelha ou branca, sendo esta última o controle. Também foram obtidos extratos da biomassa de *H. pluvialis* e *T. obliquus* para avaliar a atividade antimicrobiana e a indução de crescimento vegetal. Sob a luz azul, *H. pluvialis* produziu 8 vezes mais astaxantina em comparação aos cultivos com luz branca ou vermelha. Transcritos de genes carotenogênicos confirmaram a resposta à luz azul, após terem sua expressão aumentada. Neste experimento também foi observado que as diferentes condições luminosas não resultaram em diferenças no acúmulo de lipídeos. Por outro lado, os cultivos em luz branca produziram o maior teor de proteínas, alcançando 32%, enquanto os cultivos em luz vermelha acumularam o maior teor de carboidratos, chegando a 29%. Em cultivos mixotróficos de *H. pluvialis*, a adição de 1 g L^{-1} de glicerol aumentou significativamente o acúmulo de lipídeos, carboidratos e astaxantina. Já nos cultivos de *T. obliquus*, a concentração de glicerol de 5 g L^{-1} proporcionou uma produção de 1 g L^{-1} de biomassa com 23% de lipídeos, após 10 dias. Extratos brutos de *H. pluvialis* e *T. obliquus,* obtidos com água ou acetona, com concentrações variando de 6,7 mg L^{-1} a 0,1 µg L^{-1} não inibiram o crescimento de *Acinetobacter baumannii* e *Enterococcus* Resistente à vancomicina. Por outro lado, o extrato aquoso (TW) obtido da biomassa de *T. obliquus* cultivada sem glicerol apresentou atividade bioestimulante de crescimento vegetal. A partir destes resultados foi possível concluir que a luz azul e a adição de glicerol são alternativas viáveis à produção de biomassa e compostos com valor agregado. Além disso, a luz azul com intensidade de 20 $\mu mol_{fótons}$ m^{-2} s^{-1} em conjunto com a privação de nitrogênio foi suficiente para aumentar o número de transcritos dos genes relacionados com a síntese de astaxantina. Embora o extrato TW tenha respondido com sucesso como bioestimulante de crescimento vegetal, devem ser avaliadas novas estratégias de extração e a utilização de concentrações maiores de extratos.

Palavras-chave: Luz azul, Expressão gênica. Glicerol. Atividade antimicrobiana. Bioestimulante vegetal.

ABSTRACT

The cultivation of micro-algae has attracted the attention of researchers and biotechnology industries, often due to the potential added value of their biomass. Polyunsaturated fatty acids, carbohydrates, proteins, and carotenoids are present in this biomass and many of these compounds have antioxidant action, antimicrobial activity, and bio-stimulant for plant growth. To improve the obtaining of these bio-products, different cultivation strategies can be evaluated. The use of blue light in conjunction with nitrogen deprivation is a known strategy for increasing the production of astaxanthin, for example. The induction of mixotrophic growth, with the addition of organic compounds to the culture medium, can also result in increased production of these substances by micro-algae. The study of genes differentially expressed in different culture conditions may point to strategies for genetic improvement, including genetic modifications. In this sense, the objective of this study was to evaluate the production of carbohydrates, lipids, proteins, and astaxanthin under different cultivation conditions of the micro-algae *Haematococcus pluvialis* and *Tetradesmus obliquus*. The expression of carotenogenic genes (phytoene synthase, lycopene β-cyclase, β-carotene ketolase, and β-carotene hydroxylase) was evaluated in cultures of *H. pluvialis*, exposed to blue, red or white light, the latter being the control. Extracts from the biomass of *H. pluvialis* and *T. obliquus* were also obtained to evaluate antimicrobial activity and induction of plant growth. Under blue light, *H. pluvialis* produced 8 times more astaxanthin compared to crops with white or red light. Transcripts of carotenogenic genes confirmed the response to blue light after their expression was increased. In this experiment, it was also observed that the different light conditions did not result in differences in the accumulation of lipids. On the other hand, white light crops produced the highest protein content, reaching 32%, while red light crops accumulated the highest carbohydrate content, reaching 29%. In mixotrophic cultures of *H. pluvialis*, the addition of 1 g L^{-1} of glycerol significantly increased the accumulation of lipids, carbohydrates and astaxanthin. In the cultures of *T. obliquus*, the glycerol concentration of 5 g L^{-1} provided a production of 1 g L^{-1} of biomass with 23% lipids, after 10 days. Crude extracts of *H. pluvialis* and *T. obliquus*, obtained with water or acetone, with concentrations ranging from 6.7 mg L^{-1} to 0.1 µg L^{-1} did not inhibit the growth of *Acinetobacter baumannii* and Vancomycin-resistant *Enterococcus*. On the other hand, the aqueous extract (TW) obtained from the biomass of *T. obliquus* grown without glycerol showed bio-stimulating activity of plant growth. From these results it was possible to conclude that blue light and the addition of glycerol are viable alternatives to the production of biomass and compounds with added value. In addition, the blue light with an intensity of 20 µmol m^{-2} s^{-1} together with nitrogen deprivation was sufficient to increase the number of gene transcripts related to astaxanthin synthesis. Although the TW extract has responded successfully as a plant growth stimulant, new extraction strategies and the use of higher concentrations of extracts should be evaluated.

Keywords: Blue light, Gene expression. Glycerol. Antimicrobial activity. Plant grow biostimulant.

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1 INTRODUÇÃO

Microalgas representam um grupo de microrganismos fotossintetizantes, em sua maioria, presentes em ambientes aquáticos ou úmidos. A estrutura principal das microalgas é denominada talo e sua morfologia é bastante diversa, podendo ser encontradas espécies unicelulares, coloniais, formadoras de cenóbios ou, ainda, espécies filamentosas. O termo microalga foi proposto por Lineu e inclui microrganismos eucariontes e procariontes, sendo, portanto, desprovido de valor taxonômico. No entanto, em vista dessa diversidade, é possível encontrar diferentes compostos de interesse nos diferentes grupos de microalgas.

O consumo de microalgas foi amplamente realizado por tribos indígenas durante séculos. Chineses já consumiam espécies dos gêneros *Nostoc*, *Spirulina* e *Aphanizomenon*, há centenas de anos (JENSEN; GINSBERG; DRAPEAU, 2001). Os primeiros trabalhos com microalgas tinham como objetivo compreender a metodologia para cultivo e estiveram ligados a estudos bacteriológicos. No entanto, após 1940, os experimentos com cultivos de microalgas saíram dos estudos básicos e passaram para os relacionados às aplicações biotecnológicas com cultivos em grandes escalas (BOROWITZKA, 1999). Atualmente, dada a importância econômica das diferentes aplicações para a biomassa de microalgas, os estudos estão voltados para o aumento da produtividade de biomassa, lipídeos para produção de biocombustíveis e produtos de alto valor agregado.

Os compostos que agregam valor à biomassa de microalgas incluem os ácidos graxos poli-insaturados, os carboidratos, as proteínas e os carotenoides. A literatura relata diversas aplicações desses compostos, especialmente voltadas para a indústria alimentícia, agrária, farmacêutica e cosméticos. Esses setores têm interesse principalmente, mas não apenas, nas atividades biológicas presentes nesses compostos, como, por exemplo, atividade antitumoral, antioxidante, antimicrobiana e bioestimulante de crescimento vegetal. Diante desse potencial biotecnológico, diversos estudos que abordam estratégias de cultivos para aumentar o acúmulo de compostos bioativos, bem como métodos de extração com diferentes solventes e fracionamento das moléculas bioativas, têm sido relatados. Dentre as

espécies de microalgas estudadas, pode-se destacar duas: *Haematococcus pluvialis* e *Tetradesmus obliquus*.

H. pluvialis é uma Chlorophyceae de água doce, unicelular e biflagelada. De acordo com as condições ambientais, ou de cultivo, pode-se observar quatro morfologias celulares distinguíveis: microzooide, macrozooide (zoósporos), palmelar e hematocisto (aplanósporo). Macrozooides e microzooides possuem dois flagelos e uma parede celular delgada. Essas formas celulares são encontradas durante condições ótimas de cultivo, no entanto, microzooides são observados quando ocorre gametogênese nos hematocistos. Porém, sob condições desfavoráveis, como baixa concentração de nutrientes e alta intensidade luminosa (GIANNELLI et al., 2015; IMAMOGLU et al., 2009; ZHAO et al., 2018), os macrozooides perdem seus flagelos e a célula aumenta seu tamanho, adotando a morfologia palmelar. Com o agravamento da condição de estresse, as células palmelares passam por algumas mudanças, transformando-se em hematocistos. Essas células possuem parede celular trilaminar mais espessa e mais resistente. Nesse estágio, observa-se um acúmulo de carotenoides, especialmente astaxantina, que desperta o interesse das indústrias farmacêuticas e cosméticas, devido ao alto poder antioxidante dessa molécula. Estudos mostram que astaxantina auxilia na prevenção de doenças cardiovasculares, previne o desenvolvimento de alguns tipos de tumores, e possui ação protetora contra doenças neurológicas causadas por estresse oxidativo (FANG et al., 2019; SATHASIVAM; KI, 2018). Além desses benefícios, astaxantina é utilizada na alimentação de peixes, principalmente salmonídeos, para pigmentação da musculatura (JIANG et al., 2019; YOUNG et al., 2017). Além da astaxantina, a biomassa de *H. pluvialis* possui outros potenciais biotecnológicos. Dados da literatura mostram o potencial dos extratos de *H. pluvialis*, obtidos com diferentes solventes, em inibir o crescimento de bactérias, fungos e vírus (SANTOYO et al., 2009, 2012). Outro exemplo de atividade encontrada é a de bioestimulante de crescimento vegetal. Dados da literatura mostram os efeitos dos sobrenadantes de culturas de *H. pluvialis*, mantida em vinhaça, na expansão celular e na formação de raiz de *Nidularium procerum* (bromélia) (GOLLO et al., 2016). Entretanto, pouca informação sobre o potencial das moléculas presentes em extrato aquoso de *H. pluvialis* tem sido publicada, não sendo observado nenhum trabalho avaliando atividade bioestimulante dos extratos aquosos dessa espécie.

T. obliquus (sinônimos homotípicos: *Scenedesmus obliquus* e *Acutodesmus obliquus*) é uma Chlorophyceae de água doce, formadora de cenóbio que compreende duas, quatro ou mais células. Essa espécie é capaz de acumular até 35% do seu peso seco em lipídeos e mais de 55% de carboidratos, dependendo da condição de cultivo (ISLAMI; ASSAREH, 2019; SILVA et al., 2018). Além disso, é possível aliar a produção de macromoléculas em cultivos de *T. obliquus* com o tratamento de emissões (CORREA et al., 2017) e efluentes (SELESU et al., 2016). *T. obliquus* é uma das mais promissoras espécies de microalgas para produção comercial, pois, além da produção de macromoléculas de interesse, essa espécie é resistente a diferentes condições ambientais e a alguns protozoários, devido à formação de cenóbio (DI CAPRIO et al., 2018; WU et al., 2014).

Diante disso, novos modelos de cultivos e novas aplicações para a biomassa de *H. pluvialis* e *T. obliquus*, têm sido relatados na literatura científica, como a utilização de luz com diferentes comprimentos de onda ou intensidade (ABOMOHRA et al., 2019; GONÇALVES et al., 2019; ZHU et al., 2018) e a utilização de carbono orgânico em cultivos mixotróficos (ZHANG et al., 2020; ZHU et al., 2018). Além disso, há um esforço coletivo em elucidar quais e como genes de interesse estão relacionados com a produção de compostos biotecnológicos para, assim, direcionar as estratégias de cultivos. Um exemplo é a escolha da melhor fase de crescimento celular para induzir a produção de determinado composto, ou, em qual gene é possível realizar uma engenharia genética (KATHIRESAN et al., 2015; VO et al., 2016). Essas escolhas são facilitadas quando genes e o seu padrão de expressão são conhecidos.

Essa habilidade de crescimento em condições mixotróficas, em águas residuais e alimentadas com emissões, possibilita a integração entre diferentes processos de produção, aproveitando ao máximo a potencialidade desses microrganismos. Portanto, novas estratégias que promovam o aumento da produção de biomassa e dos compostos de valor agregado devem ser avaliadas. Por essa razão, pesquisas que possuem como objetivo o desenvolvimento de estratégias para produção de biomassa de microalgas é essencial para o avanço dos conhecimentos nessa área.

2 JUSTIFICATIVA

Devido à alta demanda por produtos naturais e ao alto valor agregado dos compostos bioativos, com atividade bioestimulante e antimicrobiana, pesquisadores têm criado estratégias para aumentar a produtividade destes compostos. Otimização dos meios de cultivos, luz e carbono orgânico têm sido utilizados na busca por maiores produtividades. Além disso, há pouca informação em relação à atividade bioestimulante de crescimento vegetal de extrato aquoso de *H. pluvialis* e como genes relacionados à síntese de astaxantina são afetados na presença de luz azul com intensidade de 20 μmol m^{-2} s^{-1}.

Como base no potencial biotecnológico da biomassa de microalgas, como fonte de astaxantina, e dos extratos com atividade antimicrobiana e bioestimulante vegetal reportados na literatura é de fundamental importância expandir o conhecimento já estabelecido por meio do emprego de novas metodologias de cultivos e obtenção de extratos.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o potencial biotecnológico de *Haematococcus pluvialis* e *Tetradesmus obliquus* e os efeitos da luz azul, luz vermelha e glicerol, na expressão gênica e no acúmulo de astaxantina e macromoléculas.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar a produção de astaxantina, carboidratos, lipídeos e proteínas, bem como quantificar a expressão dos genes fitoeno sintase, licopeno β -ciclase, β-caroteno hidroxilase, β-caroteno cetolase, fototropina e um gene da família de proteínas PII em cultivos autotróficos de *H. pluvialis* sob iluminação azul e vermelha na privação de nitrogênio;
- Avaliar o efeito de diferentes concentrações de glicerol em cultivos mixotróficos de *H. pluvialis* e *T. obliquus* em relação à produção de astaxantina, carboidratos, lipídeos e proteínas.
- Avaliar o potencial antimicrobiano e bioestimulante vegetal de extratos obtidos com água e acetona das biomassas de *H. pluvialis* e *T. obliquus* produzidas com e sem glicerol.

4 ESTRUTURA DA TESE

A estratégia experimental consistiu na avaliação de diferentes métodos no acúmulo de astaxantina, carboidratos, proteínas e lipídeos e obtenção dos extratos para avaliação quanto à atividade antimicrobiana, antileishmania e bioestimulante vegetal. Os resultados serão apresentados na forma de capítulos, cada um referente a um artigo científico em língua inglesa, sendo intitulados:

- CAPÍTULO 1. Light color modulation of differential gene expression in *Haematococcus pluvialis* and its consequences in astaxanthin production

- CAPÍTULO 2. Lipids, proteins and pigments production in mixotrophic cultures of *Haematococcus pluvialis* and *Tetradesmus obliquus*.

- CAPÍTULO 3. Microalgae extract bioactivity: from plant biostimulant to antileishmania properties

CAPÍTULO 1 – Light color modulation of differential gene expression in *Haematococcus pluvialis* and its consequences in astaxanthin production

Light color modulation of differential gene expression in *Haematococcus pluvialis* and its consequences in astaxanthin production

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Abstract

Haematococcus pluvialis can accumulate high levels of astaxanthin under unfavorable conditions such as nitrogen starvation, high light incidence, or different light colors. The biomass composition of *H. pluvialis* may change during astaxanthin synthesis so that different products can be obtained. The present study evaluated the effect of blue, red, and white light on the expression of carotenogenic genes (*psy*, *lyc*, *bkt*, and *crtr-r*), and two other genes, one related to nitrogen metabolism (*glb-1*) and the second one, related to the capture of blue light (*phot*). In addition, analysis of both promoter and light-oxygen-voltage (LOV) domain from phot gene was performed. Those surveys support the light responsiveness of phot gene by both gene expression and functional response. Besides, changes in astaxanthin synthesis and macromolecule accumulation were also evaluated. Blue light and red light were able to over-regulate all genes within 96 hours, including the *phot* gene. Astaxanthin concentration increased under all conditions. At the end of the stress period, blue light cultures accumulated 2-fold more astaxanthin than red light cultures and 8-fold more than cultures kept in white light. On the last day of cultivation, the biochemical composition of the biomass was evaluated, and the highest protein content was found in the cultures kept in white light. In the other hand, the high carbohydrate content was observed in red light cultures. Lipid content was statistically equal under all conditions. Our results show different compounds accumulate at different times of cellular growth of *H. pluvialis*, as well as are affected by the incident wavelength.

Keywords: Pigments; Microalgae; Carotenogenesis; Lipids; Carbohydrates; Proteins; LOV domain

1 Introduction

Astaxanthin (3,3'-dihydroxycarotene-4,4'dione) is a ketocarotenoid with reddish-colored antioxidant properties produced by some species of yeast, bacteria,

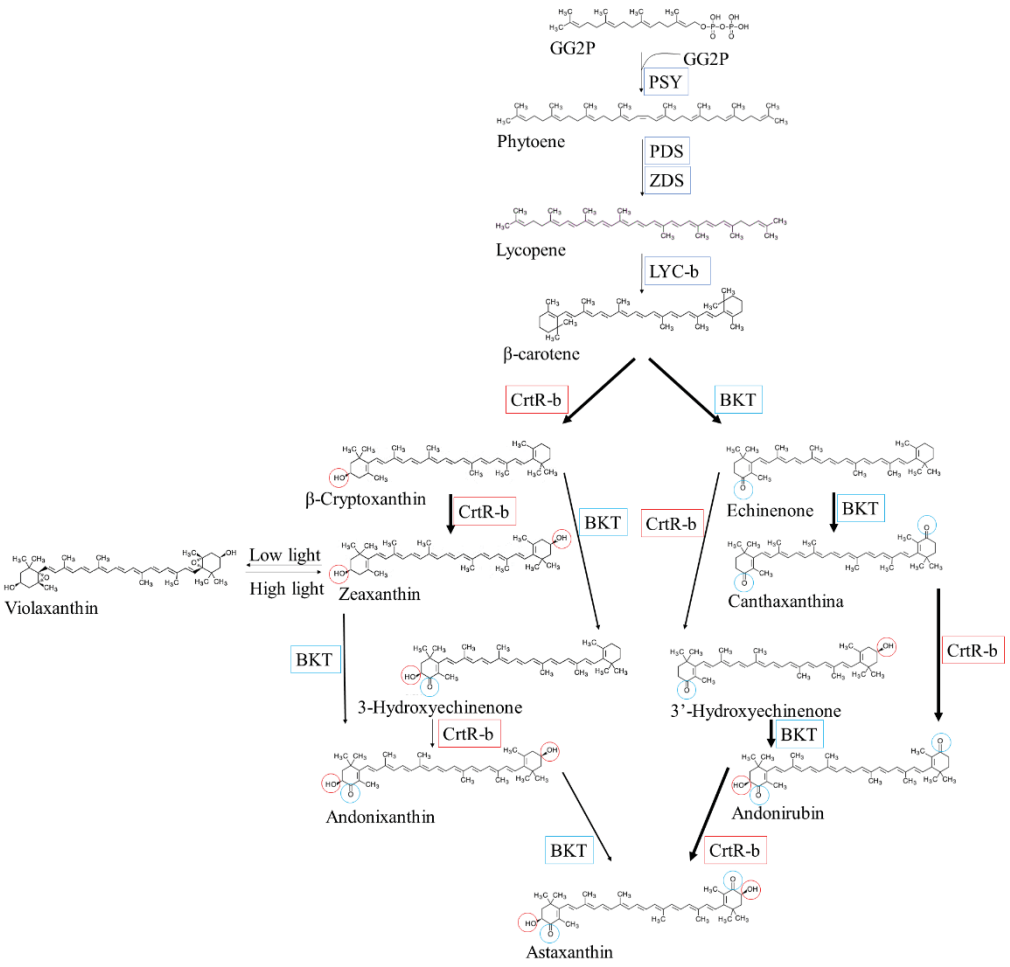
plants, and microalgae. This pigment attracts the interest of researchers because astaxanthin has several benefits to human health (GUERIN et al., 2003). Although synthetic astaxanthin production still represents the largest share of the commercial market, the demand for natural products has been driving the search for natural sources of astaxanthin (HIGUERA-CIAPARA et al., 2006).

One of the main sources of natural astaxanthin is the microalgae *Haematococcus pluvialis* (Flotow, 1844), a unicellular freshwater chlorophyte (BOUSSIBA; VONSHAK, 1991). During the life cycle of this species, it is possible to observe phenotypic variations in response to environmental stress (BRINDA et al., 2004). The production of astaxanthin in *H. pluvialis* can reach about 5% of its dry weight under high light and nitrogen deprivation (SHAH et al., 2016). In addition to the quantity, the luminous quality plays a fundamental role in the accumulation of pigments in microalgae. Red wavelengths (600 nm – 700 nm) provide sufficient energy to elevate the electron of chlorophyll *a* and *b* to their first excited state, while blue light (440 nm – 500 nm) can excite electrons to their second excited state. Such that excess energy may not be interesting for photosynthesis (MATTHIJS et al., 1996), but may favor carotenoid synthesis (KATSUDA et al., 2004; MA et al., 2018). Also, blue light is related to nutrient uptake and utilization, increasing nitrate reductase activity in *Chlamydomonas reinhardtii* and *Monoraphidium braunii* (APARICIO; QUILNONES, 1991; AZUARA; APARICIO, 1983), and promoting amino acid and ammonia uptake. in *Chlorella kessleri* (KAMIYA; SAITOH, 2002). In addition to these functions, blue light further promotes the breakdown of reserve carbohydrates by increasing the activity of eight carbohydrate metabolism-related enzymes (KAMIYA; SAITOH, 2002; RUYTERS, 1980, 1984). The molecular mechanisms that regulate these actions are not yet fully understood, although some studies indicate that phytochrome, a red light photoreceptor, may be involved as it may weakly absorb blue light (SAKURABA; YANAGISAWA; 2018). Some findings have revealed a blue light photoreceptor, cryptochrome, may interact with plant phytochrome transcription factors (PEDMALE et al., 2016). Still, the presence of phytochrome in *H. pluvialis* has not yet been reported. However, another blue light photoreceptor, phototropin (*phot*), was identified in *H. pluvialis* (Ma et al., 2018). This photoreceptor is involved in the regulation of photosynthetic functions in plants such as phototropism, chloroplast movement, and stomatal opening (KASAHARA et al.,

2004). In *C. reinhardtii*, *phot* is involved with the expression of glutamate-1-semialdehyde aminotransferase, phytoene desaturase, and light-collecting polypeptides (IM et al., 2006), as well as controlling the sexual cycle of this microalgae (HUANG; BECK, 2003).

Astaxanthin biosynthesis in *H. pluvialis* is a complex process (Fig 1.1), highly regulated under stress conditions that coincide with fatty acid synthesis (SAHA et al., 2013). Studies evaluate the effect of high light intensity on the expression of carotenogenic genes, but few assess the impact of blue or red light with intensity of 20 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ or less (KATSUDA et al., 2004; MA et al., 2018). In addition to light intensity, other stress conditions, such as nutrient deprivation, also appear to promote carotenogenic gene expression (GRÜNEWALD et al., 2000; GWAK et al., 2014; ZHANG et al., 2019). Nitrogen plays an essential role in microalgae autotrophic cultures. In nitrogen-deprived microalgae cultures, cells cease cell division and accumulate secondary metabolites such as lipids and carotenoids (LONGWORTH et al., 2016; TRAN et al., 2009). Thus, highly efficient systems are present in organisms to capture and use this vital resource (ERMILOVA et al., 2013). Commichau, Forchhammer, and Stülke (2006) have shown nitrogen uptake is linked to carbon flux and is regulated through the integration of signaling factors, with members of the PII protein family occupying the center of control of nitrogen uptake in bacteria. These proteins sense effector molecules, such as ATP and 2-oxoglutarate, in different ways, and can be covalently modified, thus integrating additional signals such as glutamine (ARCONDÉGUY et al., 2001; COMMICHAU; FORCHHAMMER; STÜLKE, 2006; HUERGO et al., 2013). Proteins regulated by PII can be ammonia channels, key metabolic enzymes, as well as transcription factors (FORCHHAMMER, 2004). In green microalgae, *Chlamydomonas reinhardtii* (ERMILOVA et al., 2013), *Chlorella variabilis* (EKATERINA, 2015) and *H. pluvialis* (MA et al., 2017), are the only species where PII has been reported, and there are few studies about the effect of blue and red lights on the number of PII transcripts (GWAK et al., 2014; Lee et al., 2018; Li et al., 2019).

FIGURE 1.1 – SYNTHESIS PATHWAY OF CAROTENOIDS



FONT: The author (2020)

LEGEND: De novo synthesis pathway of some carotenes (phytoene, lycopene, and β-carotene) and some xanthophylls (canthaxanthin, echinenone, cryptoxanthin, zeaxanthin, violaxanthin, 3-hydroxyechinenone, 3'-hydroxyechinenone, andonixanthin, andonirubin, and astaxanthin) in microalgae. Enzymes are represented within the rectangles (PHY: phytoene synthase, PDS: phytoene desaturase, ZDS: ξ-carotene desaturase, LYC-b: lycopene β-cyclase, BKT: β-carotene ketolase, CrtR-b: β-carotene hydroxylase). Bold arrows represent the preferred route in *H. pluvialis*. Scheme create using information from KEGG. GGPP: Geranylgeranyl pyrophosphate.

We hypothesized the blue and red light at 20 $\mu mol_{photons}$ m^{-2} s^{-1} of intensity, together with nitrogen deprivation, may affect the expression of the main genes involved in the synthesis of carotenoids, as well as the expression of genes related to metabolism nitrogen and carbon and the gene for the blue light photoreceptor, besides to inducing the accumulation of different compounds in the *H. pluvialis* biomass. In order to better understand the expression profile of the *phot* gene, we

analyzed the promoter region for light-responsive elements. Also, an analysis of the LOV domains of PHOT protein was also performed.

2 Material and method

2.1 Microalgae growth conditions

The microalgae *Haematococcus pluvialis* was obtained from the Canadian Center for the Culture of Microorganisms (strain 7072). The cultures were maintained in synthetic medium (mg L^{-1}: $NaNO_3$, 250; $CaCl_2$ $2H_2O$, 25; $MgSO_4$ $7H_2O$, 75; K_2HPO_4, 75; KH_2PO_4, 175; NaCl, 25; EDTA, 50; $FeSO_4$ $7H_2O$, 4.98; H_3BO_3, 11.3; $ZnSO_4$ $7H_2O$, 14.12; $MnSO_4$ $4H_2O$, 1,96; Na_2MoO_4 $2H_2O$, 1.92; $CuSO_4.5H_2O$, 2.52; $CaCl_2$ $6H_2O$, 1.44), for ten days until they reach to 5 10^5 cells mL^{-1} of density. The volume used for the inoculum was adjusted so that the final concentration of the culture reached 10 10^4 cells mL^{-1}. For biomass production, *H. pluvialis* cells were cultivated in 400 mL of synthetic medium. Nine Erlenmeyer bottles (neck: 38 mm; base: 72 mm; height: 174 mm) were illuminated by white LED lamps (20 $\mu mol_{photons}\, m^2\, s^{-1}$) for seven days. The cultures received uninterrupted atmospheric air injection at a flow rate of 0.8 vvm (volume of air per culture volume per minute). The temperature was maintained constant during the growth period at 22 °C. After seven days, the cells were harvested by centrifugation and washed twice to the removal of the nitrogen source and resuspended in nitrogen-free medium. The cultures were separated in two treatments and one control culture, and the light source of the cultures was changed to red light or blue light, using the same photon flux density (20 $\mu mol_{photons}\, m^{-2}\, s^{-1}$). Cultures were kept under these conditions for five days.

2.2 Biochemical analysis

To monitor the production of chlorophyll a and b, aliquots were collected at times 0, 48, 96, and 120 hours (onset of stress due to lack of nitrogen and variation of luminosity).10 mL aliquots of fresh cultures were recovered by centrifugation at 2600 x *g* for 10 minutes. The pellet was washed in distilled water and then treated with 4 mL of 90% acetone. The cells were kept in acetone 90% at 4 °C for 24 hours in the dark to increase the efficiency of chlorophyll extraction. After, the pellet was centrifuged at 2600 x g for 10 minutes. The extraction process was repeated until the total discoloration of the pellet. The concentrations of chlorophyll *a* and *b* were determined by spectrophotometry from the absorbance readings of the extracts at wavelengths of 630nm, 647nm and 664nm, chlorophyll *a*, chlorophyll *b*, and chlorophyll *c* respectively. All analyses were performed in triplicate. For the quantification of pigments, was used Equation 2 and 3 proposed by LORENZEN (1967):

$$Chl_a = [(11.85 \times A664) \quad (1.54 \times A647) \quad (0.08 \times A630)] \times \frac{V_{acetone}}{V_{culture} \times 1} \qquad (2)$$

$$Chl_b = [(21.03 \times A647) \quad (5.43 \times A664) \quad (2.66 \times A630)] \times \frac{V_{acetone}}{V_{culture} \times 1} \qquad (3)$$

where *A* corresponds to the absorbance at a specific wavelength, $V_{acetone}$ corresponds to the volume of acetone 90% (mL), used for chlorophyll extraction, $V_{culture}$ is the volume of culture sample (mL), and 1 is the optical path from the cuvette (cm).

Astaxanthin concentration was estimated as described by Zhao and colleagues (2018). Briefly, 10 mL of fresh *H. pluvialis* culture were recovered by centrifugation (2600 x g, 10 minutes) at times 0, 48, 96, e 120 hours (onset of stress due to lack of nitrogen and variation of luminosity). The pellet was washed with distilled water, frozen and then lyophilized for 24 hours. The lyophilized cells were treated with 5% (w/v) potassium hydroxide solution in 30% methanol (v/v) at 65 °C for 15 minutes to destroy the chlorophyll. Treated cells were centrifuged at 2600 x g for 10 minutes and washed three times with distilled water. The pellets were resuspended in 5 mL of dimethyl sulfoxide (DMSO) and kept on ultrasound (40 kW)

for 10 minutes. The extraction was carried out at 45 °C for 20 minutes in a water bath. When necessary, the extraction process was repeated until the total discoloration of the pellet. Total astaxanthin was determined by spectrophotometry by measuring the absorbance at 490 nm. All analyzes were performed in triplicate. Total astaxanthin (mg L^{-1}) was determined according to Equation 4.

$$Ast_{mg/L} = 4.5 \times A490 \times \frac{V_{DMSO}}{V_{culture}} \quad (4)$$

Where *A*490 is the absorbance of the extracts at 490 nm, V_{DMSO} corresponds to the volume of DMSO (mL), used for astaxanthin extraction, $V_{culture}$ is the volume of culture sample (mL).

Total lipids were extracted using a mixture of chloroform and methanol (2:1 v/v) and quantitated by gravimetric analysis according to Bligh and Dyer (1959). The determination of total carbohydrates was performed spectrophotometrically using the methodology proposed by Dubois and collaborators (1956). For quantification of the total proteins, 50 mg of lyophilized biomass was treated with 1 mL of Tris/HCl, pH 7.4, for 24 hours at 4 °C. The treated biosynthetic was centrifuged at 15,000 x *g* for 20 minutes at 4 ° C. The supernatant was reserved, and the pellet was resuspended in 1 mL of 0.1 N sodium hydroxide containing 0.5% β-mercaptoethanol (v / v). The pellet was held for one hour at room temperature with manual shaking every 15 minutes and then centrifuged at 15,000 x *g* for 20 minutes at 21°C. The supernatants were pooled together, and quantification was performed in a spectrophotometer following the Bradford (1976) assay. Lipids, carbohydrates, and proteins were determined on the last day of culture.

2.3 Gene expression

Aliquots of 10 mL were collected from the 0, 48, 96, and 120 hours of cultivation (onset of stress due to lack of nitrogen and variation of luminosity) and centrifuged. A saturated solution (25 mM sodium citrate, 10 mM EDTA, 70g ammonium sulfate/ 100 mL of solution) was added to the pellet to inhibit the activity of the RNA molecules. The samples were stored in a freezer at -80 °C until the extraction of RNA. Total RNA was extracted using the TRIzol™ reagent (catalog number: 15596018 Invitrogen) following the supplier's instructions. Nucleic acid concentrations were quantified spectrophotometrically at 260 nm. The purity of the RNA was determined by the ratio 260nm/280nm and by 1% agarose gel electrophoresis. cDNAs were synthesized with Power SYBR® GREEN RNA-to-CT™ 1-Step Kit (PN4391003 Applied Biosystems). The expression of genes related to the synthesis of carotenoids *psy* (NCBI Gene ID AF305430), *lyc* (NCBI Gene ID AY182008), *bkt* (NCBI Gene ID AY182008), and crtr - r (NCBI Gene ID AF162276), the gene *phot* (NCBI Gene ID KU698122.1), and of the *glb1* gene, (NCBI Gene ID KT696441.1), were quantified by RT-qPCR using Sybr® green as fluorescent dye. The amplification cycle followed the supplier's recommendations: 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute using StepOne Plus equipment (Thermo Fisher Scientific). Expression of each gene was normalized using two endogenous genes, actin (NCBI Gene ID DV203941) and 18S (NCBI Gene ID KC196726.1). Primers sequences are shown in supplementary material. The quantitative real-time PCR data were analyzed by $2^{-\Delta\Delta Ct}$ (LIVAK; SCHMITTGEN, 2001).

2.4 *Phot* gene promoter analysis and light–oxygen–voltage-sensitive domain assessment in Chlorophyta

Haematococcus pluvialis 1-kb upstream region of *phot* gene was scanned by PLACE (http://www.dna.affrc.go.jp/PLACE/) Higo et al. (1999) for putative elements responsive to light exposure. The protein structure was modeled by SWISS-

MODELWaterhouse et al. (2018), using AhRR/ARNT complex Sakurai et al. (2017) (PDB:5Y7Y) as template. LOV2 domain conservation in Chlorophyta was defined using all *phot* (from *H. pluvialis*) BLAST hits with E-Value lower than 0.001, excluding the ones within *Haematococcus* genus itself. DNA Logo was produced using WebLogo Crooks et al. (2004).

2.5 Statistic

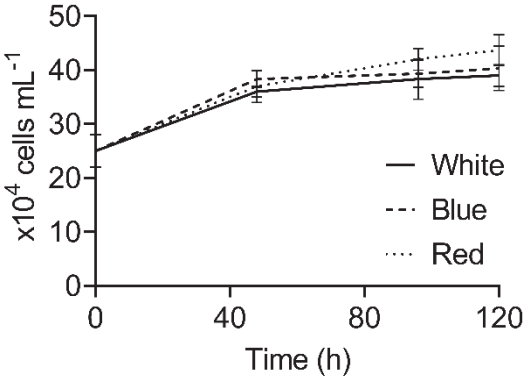
Cultures were kept in three biological replicates, and all analyzes were performed in triplicate. The data in the figures are represented as the average of triplicates and the bars are the standard deviation. For the hypothesis test, we used the AVOVA test followed by Tukey. The results were considered statistically different when the *p* value was less than 0.05.

3 Results and Discussion

3.1 Effect of red and blue LEDs on cell growth

Cultivation started at $25 \pm 3.5\ 10^4$ cells mL^{-1} and kept growing until 96 hours of cultivation (Fig 2.1). Red, blue and white light illumination did not significantly affect cell density. The cultures kept growing up to 48 hours of all conditions, entering the stationary phase only after 48 hours (Fig 2.1). The cell growth profile in the cultures illuminated by blue, red, and white light was similar, both reaching, respectively, $38 \pm 2\ 10^4$ cells mL^{-1}, $37 \pm 3\ 10^4$ cells mL^{-1}, and $36 \pm 1\ 10^4$ cells mL^{-1}, after 48 hours of culture. On the last day, the cellular density from cultures under blue light, reached to 44.5 ± 3.510^4 cells mL^{-1}, and at a rate of 0.08. Interestingly, an increase in cell numbers is observed within the first 48 hours which may mean that the cells assimilated the nitrate present in the medium before being removed.

FIGURE 2.1 – EFFECT OF BLUE, RED, AND WHITE LIGHT ON CELL CONCENTRATION



NOTE: Effects of blue, red, and white light on cell concentration in cultures of *Haematococcus pluvialis* after removes nitrogen from medium. Shown are mean values ± SD of three separately grown cultures

The cellular morphology of *H. pluvialis* is altered during carotenogenesis and, usually, the accumulation of astaxanthin is accompanied by loss of the flagella and thickening of the cell wall (SHAH et al. (2016). However, in the present study, cells maintained in blue, red and white light showed initial astaxanthin production in flagellate cells. *H. pluvialis* cells in blue light lost their flagella at 96 hours of growth (Data not shown), which coincides with the first peak of astaxanthin production. The cells kept in white and red light also lost the flagella with 96 hours of stress but maintained a gelatinous structure around the cells. Astaxanthin accumulation in flagellate cells has some advantages, as the thin wall of the macrozooids would facilitate the extraction of the pigment (BRINDA et al., 2004).

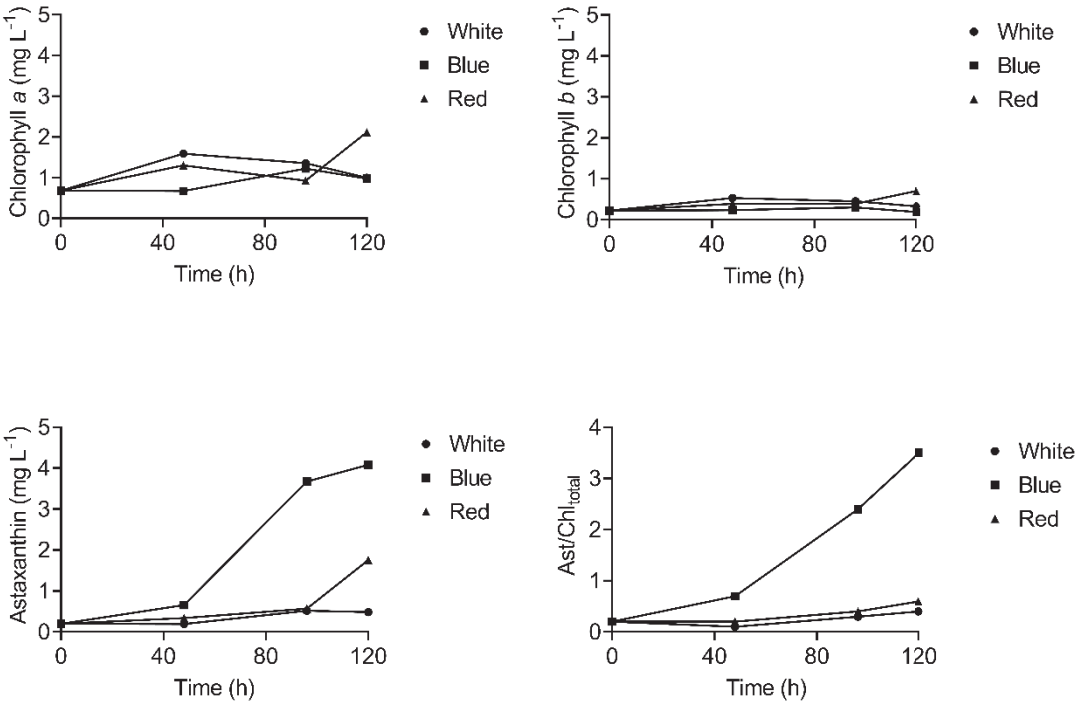
3.2 Effect of red and blue LEDs on pigments accumulation

The evaluation of the effects of red and blue lights on chlorophyll *a* and *b* production under nitrogen deprivation was performed every 48 hours. All cultures began with a concentration of 0.7 ± 0.008 mg L^{-1} of chlorophyll *a* and 0.2 ± 0.02 mg L^{-1} of chlorophyll *b* and, after five days, cultures under red light were

the only ones to maintain an increase in chlorophyll *a* and *b* concentration, reaching 2.1 ± 0.002 mg L^{-1} and 0.7 ± 0.003 mg L^{-1}, respectively (Fig 3.1). The highest yields were also found in cultures kept in red light, reaching to 0.3 mg L^{-1} day^{-1} of chlorophyll *a*, and 0.1 mg L^{-1} $day^{-1}$of chlorophyll *b*. Initially, the concentration of chlorophyll *a* and *b* in white cultures increased approximately 2.33 times and 2.38 times, respectively (Fig 3.1). However, after 48 hours, there was a decline in the production of these pigments. The blue light cultures showed an almost stable concentration over the five days of stress, showing a small increase after 96 hours, where the concentration of chlorophyll *a* and *b* reached to 1.2 ± 0.01 mg L^{-1} and to 0.2 ± 0.001 mg L^{-1}, respectively (Fig 3.1). Chlorophyll *b* is preferentially degraded during nitrogen deprivation, so the Chl_a/Chl_b ratio tends to increase during the deprivation period (SCIBILIA et al., 2015), a fact observed in blue cultures, where in the first 48 hours the Chl_a/Chl_b ratio is approximately 3, but by the end of cultivation this ratio increases to approximately 5 (Fig 3.1). On the other hand, in both red and white cultivations, it can be observed the Chl_a/Chl_b ratio remains at approximately 3 by the end of the five days (Fig 3.1).

During the stress period, astaxanthin concentration increased under all conditions, however, cells kept under white light did not show significant increase in astaxanthin synthesis in the first 48 (Fig 3.1). Unlike, there was an exponential increase in astaxanthin synthesis in blue light cultures for 72 hours, reaching a concentration 18 times higher than the first day of cultivation, 3.7 ± 0.009 mg L^{-1} (Fig 3.). Consequently, the Chl_{total}/Ast ratio has decreased since the first day of stress on blue light crops, rising from 4.6 on the first day and falling to 0.3 on the last day (Fig 3.1). Wavelengths that comprise the blue light, 440 – 500 nm, provide more energy than is required for photosynthesis, so blue light can generate reactive oxygen species (FU et al., 2013), and, to protect the photosynthetic apparatus, cells accumulate photoprotective pigments, such as carotenoids (BELTRAN, 2013). At the end of the five days of cultivation, it is possible to observe a 9-fold increase in astaxanthin concentration in red light cultures, compared to the first day, increasing from 0.2 ± 0.004 mg L^{-1} to 1.8 ± 0.003 mg L^{-1} (Fig 3.1). The peak production of astaxanthin is observed on the last day of cultivation under all conditions, with the highest concentration being found in blue light cultures, 4.1 ± 0.002 mg L^{-1}, representing a 20-fold increase over at the initial concentration.

FIGURE 3.1 – ACCUMULATION OF CHLOROPHYLL AND ASTAXANTHIN UNDER BLUE, RED, AND WHITE LIGHT



NOTE: Effects of blue, red, and white light on chlorophyll *a, b,* and astaxanthin concentration in cultures of *Haematococcus pluvialis* growing under nitrogen-free medium. Shown are mean values ± SD of three separately grown cultures.

Chlorophyll absorbs it in both blue and red light (MATTHIJS et al., 1996), but blue light is most interesting for photosynthesis. In this study, it is observed that even at low intensity (20 $\mu mol_{photons}$ m^{-2} s^{-1}), chlorophyll *a* and *b* reached higher concentrations in cultures maintained in red light, and when compared to cultures in blue light, not only the total chlorophyll concentration was higher, but the concentration astaxanthin was lower. In addition, the increase in Ast/Chl_{total} ratio observed in red light cultures is due to the increase of astaxanthin concentration, and not to the decrease of chlorophyll, since it is possible to verify that the cells maintain the chlorophyll synthesis concurrently with the synthesis of astaxanthin, corroborating with the study by HAGEN et al. (1993), in which the authors conclude that secondary carotenoid synthesis does not coincide with chlorophyll degradation. Chlorophyll synthesis is also found to be maintained under blue light for the first 48 hours, but the increased astaxanthin concentration may have led to the decay of photosynthetic

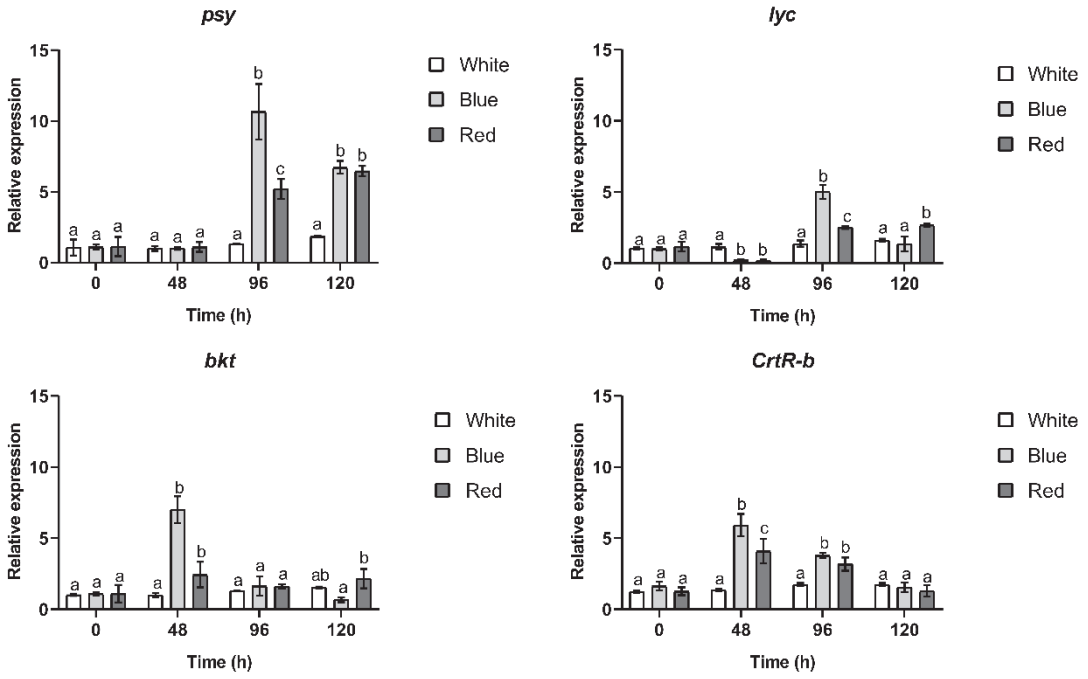
activity, as the shaded caused by astaxanthin may block absorption of blue light by chlorophyll (YONG; LEE, 1991).

3.3 Transcriptional patterns of carotenogenic genes induced by red and blue LEDs

Phytoene synthase (*phy*), lycopene cyclase (*lyc*), beta carotene ketolase (bkt) and beta carotene hydroxylase (*CrtR-b*), genes involved in carotenoid synthesis, are differentially expressed according to environmental changes. Red and blue light is known to induce cell division and carotenoid synthesis (XI et al., 2016; ZHU et al., 2018), thus, the expression of these genes was quantified during five days of cultivation under influence of blue, red or white light and in the absence of nitrogen (Fig. 4.1).

During the first 48 hours there was no variation in the *psy* gene expression, however, after 48 hours there was a significant increase in the expression of this gene in cultures under the influence of blue and red light, although, the abundance of transcripts in blue light was significant bigger than in the red light. The *psy* expression continued to increase in cultures under red and white light until 120 hours, while in blue light, expression decreased (Fig 4.1). Similar results were obtained by Ma and colleagues (2018), where the peak expression of the *psy* gene was observed after 48 hours of cultivation, but with the light intensity of 50 $\mu mol_{photons}\ m^{-2}\ s^{-1}$. The results of this study show the *psy* expression peak occurred at different times depending on the light. Note that there is a delay in the regulation of *psy* expression under the influence of red light and white light in relation to blue light (Fig 4.1).

FIGURE 4.1 – EFFECT OF BLUE, RED, AND WHITE LIGHT ON THE EXPRESSION OF *psy*, *lyc*, *bkt*, AND *CrtR-b* GENES



NOTE: Effect of blue, red, or white light on gene expression of phytoene synthase (*psy*), lycopene cyclase (*lyc*), β-carotene ketolase (*bkt*), and β-carotene hydroxylase (*CrtR-b*) in cultures of *Haematococcus pluvialis* growing in nitrogen-free medium. Shown are mean values ± SD of three separately grown cultures. *P* value < 0.05

The *lyc* gene codes for the enzyme lycopene beta-cyclase, responsible for the cyclization of the rings of the lycopene molecule, giving rise to β-carotene. At 48 hours of cultivation, there was a decrease in *lyc* expression in the cultures under red and blue light. Interestingly this decrease in white light was not observed (Fig. 4.1). Here, it was observed the highest expression level was found in blue light cultures at 96 hours of cultivation, but, on the last day of cultivation, the expression level of the *lyc* gene decreased in blue light, and increased in red and white light cultures.

Excess light can increase the concentration of plastoquinone (PQ) and other components of photosynthetic electron transport. PQ may target transcriptional activation of carotenogenic genes as it acts as a redox sensor (LI et al., 2008, 2010; STEINBRENNER; LINDEN, 2003). This could explain the peak expression of the *psy* and *lyc* genes in blue light that occurred within 48 hours, as blue light provides more energy than photosystem II uses for photosynthesis. Enzymes BKT and CRTR-b are involved at the end of the astaxanthin synthesis route, adding a ketone function and

an alcohol function in each ring. Our results indicate that the maximum of *bkt* transcripts was found in blue light crops with 48 hours of stress (Fig 4.1). After achieving seven-fold the expression obtained in control and three-fold the expression in red light, the amount of *bkt* transcripts, in blue light, decreases until complete 120 hours of cultivation (Fig 4.1). Under red light, the abundance of *bkt* transcripts increased in the first 48 hours, and after this period no significant variations were observed until completing 120 hours (Fig 4.1). On the other hand, little change in level of *bkt* transcripts was observed in cultures under white light during the entire stress period. Wang and collaborators (2012) evaluated *bkt* regulation mechanisms through promoter characterization. They observed intensity of 20 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ of white light did not cause significant changes in the transcripts. It is worth noting that the *psy* and *lyc* expression peak occur with 96 hours of stress, coinciding with the first astaxanthin synthesis peak (Fig 4.1). On the other side, peak expression of the *bkt* and *CrtR-b* genes occurred with 48 hours of stress, preceding about 48 hours before the first peak of astaxanthin production.

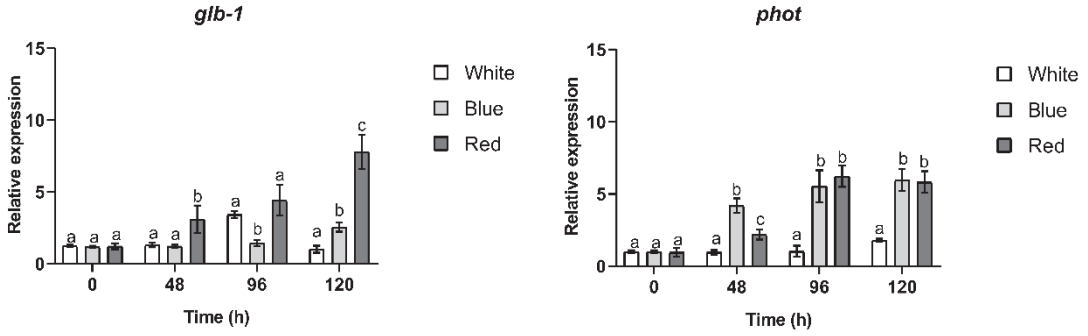
In this study, overregulation was observed in four carotenogenic genes, mainly in blue light cultures. Transcriptome analysis of brown *H. pluvialis* kept in blue or red light at an intensity of 50 $\mu mol_{photons}\ m^{2}\ s^{-1}$ revealed that blue light induced, more than red light, the expression of six genes involved in the astaxanthin synthesis route (LEE et al., 2018). Our data support the results obtained by Lee and collaborates and show that blue and red, even with low light intensity (20 $\mu mol_{photons}\ m^{2}\ s^{-1}$), have effects on the regulation of carotenogenic gene transcription, inducing astaxanthin synthesis.

3.4 Transcriptional patterns of *glb-1* and *phot* genes in red and blue LEDs

The *glb-b* gene is derived from a PII family signaling protein that is related to nitrogen and carbon metabolism (ARCONDÉGUY et al., 2001; HUERGO et al., 2013). The *glb-1* gene response in nitrogen-deprived cultures may vary according to the species studied (FERRARIO-MÉRY et al., 2005; FORCHHAMMER, 2004). In this

work, nitrogen removal in cultures illuminated by blue, red or white light has been shown to have a different effect on *glb-1* gene expression (Fig 5.1). The number of transcripts increases until the last day of cultivation, reaching an expression level seven times greater than control cultivation. In blue light, *glb-1* was also over-regulated, but this occurred only on the last day of cultivation. The *glb-1* transcript peak in the presence of white light occurred at 96 hours of cultivation, presenting a reduction in the transcript level on the last day of cultivation. Ma, Li and Lu (2017) evaluated the effect of nitrogen deprivation in *glb-1* expression on *H. pluvialis* cultures. The authors did not observe any variance within 24 hours, but a sharp increase was seen with 48 hours of stress. A similar profile can be observed in our results. However, only red light was able to induce the expression of *glb-1* with 48 hours of cultivation (Fig 5.1). Regarding blue light, it also affected the expression of *glb-1*, but only on the last day. Even so, blue light has some influence on *glb-1* transcription that may be related to nitrate reductase activity.

FIGURE 5.1 – EFFECT OF BLUE, RED, AND WHITE LIGHT ON THE EXPRESSION OF *glb-1* AND *phot* GENES



NOTE: Effect of blue, red, or white light on gene expression of PII (*glb-1l)* and phototropin (*phot*) in cultures of *Haematococcus pluvialis* growing in nitrogen-free medium. The results shown are mean values ± SD of three separately grown cultures. *P* value < 0.05

Pigment structure as well as chloroplast organization can affect the amount of blue and red light photons that chlorophyll is able to capture (SCHULZE et al., 2014). These wavelengths are involved in the regulation of some cellular processes, such as carotenoid synthesis and photosynthesis, and for regulation to occur, the cellular machinery uses not only the chlorophyll molecule, but also blue and red light

photoreceptors. Phototropin (*phot*) is one of the blue light receptors found in vascular and microalgae plants, but in vascular plants *phot1* and *phot2* are associated with responses that do not require changes in mRNA transcripts, such as, stomatal closure and chloroplast movement (KIANIANMOMENI; HALLMANN, 2014) . Conversely, *phot* induces the expression of genes related to photosynthesis and carotenoids, such as glutamate-1-semialdehyde aminotransferase and phytoene desaturase (IM et al., 2006). In this paper, the expression of *phot* in *H. pluvialis* during the period of stress due to nitrogen deprivation and illumination by different wavelengths was evaluated. The Figure 5.1 shows the *phot* expression profile in cultures maintained under three different conditions. Note that the *phot* gene was overregulated under all growing conditions, however, at 48 hours, the expression of *phot* in blue light outperformed that observed in red light cultures almost twice. The *phot* expression profile in blue light followed a logarithmic function, ie it obtained a rapid increase in the first 48 hours but leveled out after this period. While *phot* expression in red light increased exponentially up to 96 hours, however, after that period, it maintained an expression level without significant difference until completing 120 hours. The results obtained in cultures kept in red light are quite intriguing. Red light was expected to have little or no effect on *phot* gene expression, however, expression over the past few days was found to be statistically equal to expression observed in cultures under blue light. Ma and colleagues (2018), authors who isolated the *phot* gene from *H. pluvialis*, also observed red light induced phot expression, but the abundance of transcripts found in cultures maintained in blue light was significantly higher. However, Kasahara and collaborators (2004) also observed that red light is capable of inducing phototropin expression from moss *Physcomitrella patens* and suggested a model of phototropin induction by red light, where phytochrome induces phototropin expression.

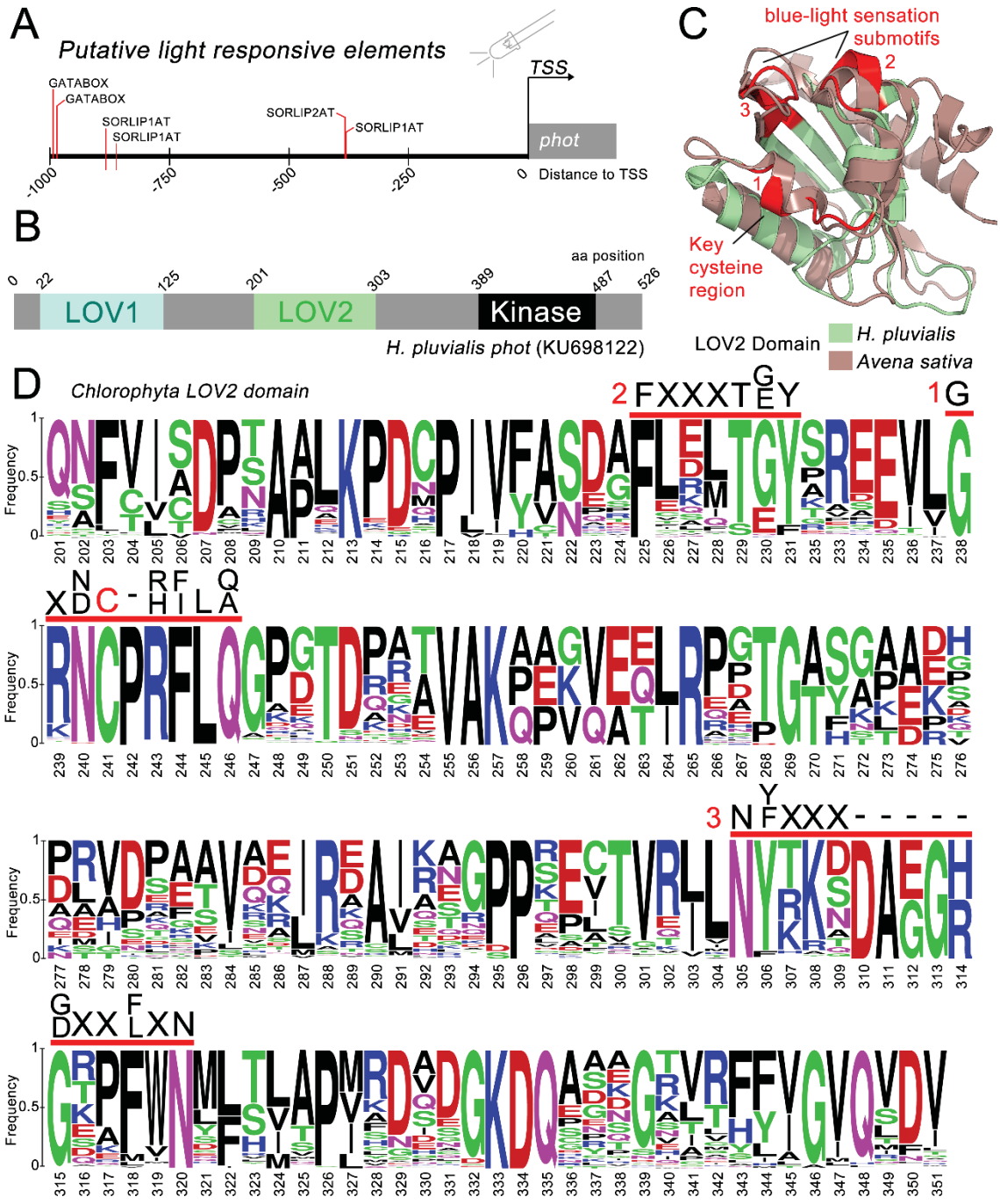
3.5 *Phot* gene promoter analysis and light–oxygen–voltage-sensitive domain assessment in Chlorophyta

To support our gene expression responsiveness to the blue and red light wavelengths, we performed prediction of proximal responsive elements in the *phot*

gene. Our data show that 8.7% of all are detected elements are associated with light responsiveness in previous literature, as SORLIP1AT, SORLIPA2AT (BAEK et al., 2016; HUDSON; QUAIL, 2003; PARK et al., 2013), and GATABOX (BRENNA; TALORA, 2019; DONALD; CASHMORE, 1990; LIU et al.; 2019; PUENTE et al.,1996) (Figure 6.1 A). The PHOT protein itself retains dual tandem LOV domains associated with a conserved kinase effector domain (Figure 6.1 B), also showing structural similarity with previously described functional LOV domains (HALAVATY; MOFFAT, 2007) (Figure 6.1 C), with key structural motifs predicted to be in functional 3D arrangement. The LOV2 domain (Figure 6.1D) is particularly conserved in whole Chlorophyta phylum, particularly for the two (2 and 3) important light-responsiveness motifs and the key motif around the photocycling-cysteine (GLANTZ et al., 2016) (1). In summary, those results support the conserved direct blue-light regulation of *phot* gene products in Chlorophyta phylum and the light regulation of its expression.

The observed blue-light responsiveness is partially explained by the function of the *phot* gene product, which is shown here to retain primary and tertiary structures in its product, particularly for the light-sensitive domain. Those findings seem to be relevant for the whole Chlorophyta phylum, corroborating to a conserved mechanism of light responsiveness and expectation of similar results obtained here for other green microalgae. For the right side of spectrum, red light sensitivity and *phot* gene regulation itself may be explained by the presence of multiple putative light-responsive elements in its promoter region, which could regulate and impact its expression during both the photoperiod and particular wavelength exposure.

FIGURE 6.1 – CHLOROPHYTA *phot* GENE AND KEY FUNCTIONAL ELEMENTS



LEGEND: Chlorophyta *phot* gene retains key functional elements.

(A) *H. pluvialis phot* gene upstream proximal promoter region contains six predicted light-responsive elements (PLACE (HIGO et al., 1999)) and

(B) its product contains two tandem LOV (Light–oxygen–voltage-sensitive) domains associated with a kinase effector domain.

(C) *H. pluvialis* modeled LOV2 domain is structurally similar to *Avena sativa* (HALAVATY; MOFFAT (2007) (PDB:2V0U) one, retaining key structural motifs (red marked) around the photocycling – cysteine and blue-light sensation relevant submotifs.

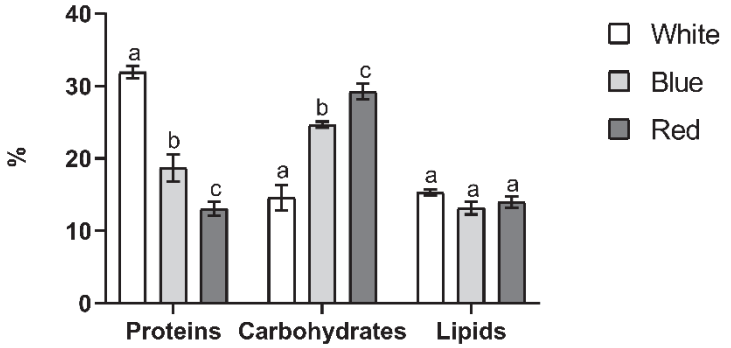
(D) DNA logo for Chlorophyta phylum LOV2 domain, highlighting the three conserved motifs for light sensing, key photocycling – cysteine in red (GLANTZ et al., 2016). Numbering is related to the first *H. pluvialis* amino acid from *phot* gene and follows protein alignment from the whole phylum.

3.6 Effect of red and blue LEDs on *H. pluvialis* biomass composition

Microalgae respond in different ways to environmental changes, such as nutrient deprivation and light. These adverse conditions can lead to the accumulation of carbohydrates, lipids, among others. The microalgae *H. pluvialis* responds to these adversities by investing mainly in carotenogenesis, but this response may influence the accumulation of other cellular compounds. Thus, the concentration of lipids, carbohydrates, and proteins was analyzed at the last day of culture to evaluate the effect of the wavelengths and astaxanthin accumulation on the synthesis modulation of these macromolecules (Fig. 7.1). The biomass obtained from blue light cultures reached 19% protein, 25% carbohydrate and 13% lipid content. Regarding productivity, the values reached 37 ± 3.8 mg L^{-1} day^{-1}, 49 ± 0.8 mg L^{-1} $day^{-1}$and 27 ±2.4 mg L^{-1} day^{-1}, proteins, carbohydrates, and lipids, respectively. It is worth noting that the maximum productivity of astaxanthin was obtained between days 2 and 4 of growth under blue light, reaching 1.5 ± 0.03 mg L^{-1} day^{-1}, a productivity nine times higher than control (Table 1.1). While the cultures keep under white light produced protein-rich biomass, reaching 32% and a productivity of 63 ± 1.7 mg L^{-1} day^{-1}. corroborating with the results from literature that shows *H. pluvialis* green biomass has protein levels ranging from 29% to 45%. On the other way, a carbohydrate-rich biomass was obtained from cultures kept under red light, reaching 29% content and productivity of 58 ± 2.1 mg L^{-1} day^{-1}. Although carbohydrate production reaches values higher than 30% in cultures where *H. pluvialis* cells have high astaxanthin content (BOUSSIBA; VONSHAK, 1991), the percentage of carbohydrates present in biomass produced under blue light did not exceed 25%. Two hypotheses may explain this finding. It is possible that at the beginning of stress, *H. pluvialis* cells will start to accumulate carbohydrates, but with the accumulation of astaxanthin and the photosynthetic rate decreased, the cells start to consume the reserve molecules. Our results show the biomass kept in red light was still brown, with lower astaxanthin concentration than the biomass kept in blue light. In addition, the carbohydrate content in biomass kept in blue light was higher, suggesting that carbohydrate consumption in biomass from red light either had not started or was occurring more slowly. Another hypothesis is that blue light induced reserve carbohydrate breakdown, as blue light may increase the activity of the pyruvate

kinase enzyme (KAMIYA; SAITOH, 2002; RUYTERS, 1980) and influence seven other carbohydrate-related enzymes degradation (RUYTERS, 1984).

FIGURE 7.1 – EFFECT OF BLUE, RED, AND WHITE LIGHT ON BIOCHEMICAL COMPOSITION BIOCHEMISTRY



NOTE: Effect of blue, red, and white light on composition biochemistry from biomass of *Haematococcus pluvialis* produced under nitrogen starvation. Shown are mean values ± SD of three separately grown cultures. *P* value < 0.05

Another cellular constituent that changes during carotenogenesis is the lipid. In this study, lipid production was statistically equal in all treatments, although some research shows that there is an increase in lipid content during carotenogenesis (SAHA et al., 2013). However, some researchers have also obtained lipid levels below 20% even in astaxanthin-rich biomass (BOUSSIBA; VONSHAK, 1991; COLUSSE et al. (2019).

Our results showed that blue light was excellent for inducing the accumulation of astaxanthin, but there was no induction in the accumulation of other pigments nor proteins, lipids, and carbohydrates. However, it is interesting to note that the protein content was higher in biomass grown under blue light compared to biomass grown under red light. On the other hand, the red light favored the accumulation of total carbohydrates and chlorophyll.

TABLE 1.1 – PRODUCTIVITY AND MAXIMUM PRODUCTIVITY OF COMPOUNDS OBTAINED FROM *Haematococcus pluvialis* BIOMASS PRODUCED UNDER NITROGEN STARVATION AND ILLUMINATION BY WHITE, BLUE, OR RED LIGHT.

mg L^{-1} day^{-1}	Light colors		
	White	Blue	Red
Proteins[1]	**63 ± 1.7[a]**	37 ± 3.8[b]	26 ± 1.9[c]
Carbohydrates[1]	29 ± 3.4[a]	49 ± 0.8[b]	**58 ± 2.1[c]**
Lipids[1]	31 ± 1.1[a]	27 ± 2.4[a]	27 ± 1.6[a]
Astaxanthin[2]	0.16 ± 0.01[a]	**1.5 ± 0.03[b]**	1.2 ± 0.04[c]
Chlorophyll *a*[2]	0.5 ± 0.04[a]	0.3 ± 0.05[b]	**1.2 ± 0.006[c]**
Chlorophyll *b*[2]	0.2 ± 0.004[a]	0.03 ± 0.006[b]	**0.3 ± 0.03[c]**

LEGEND: [1] Productivity found at last day of cultivation.
[2] Maximum productivity found in five days.
NOTE: Results are shown as mean ± SD (n=9). Different low letters superscript indicates significance difference ($p < 0.05$).

4 Conclusion

Our results showed carotenogenic genes expression is affected by blue and red light at the intensity of 20 $\mu mol_{photons}\ m^{-2}\ s^{-1}$. We observed a low level of transcripts of *glb-1* gene in cultures under blue light that may be related to carbohydrate consumption. Furthermore, no statistical difference in *phot* gene expression from cultures keep in blue or red light. The prediction of proximal responsive elements showed that light-responsive elements, as SORLIP1AT, SORLIPA2AT and GATABOX, correspond to 8.7% of all. Furthermore, *phot* protein retains dual tandem LOV domains associated with kinase effector domain, showing structural similarity to functional LOV domains described in the literature, keeping a notably conserved structure in whole Chlorophyta phylum. In addition, astaxanthin production in flagellated cells may be a consequence of blue or red light illumination with nitrogen deprivation, conferring an advantage on pigment extractability. Finally, our data show that biochemical composition is related to physiological change during

carotenogenesis and in response to the effects of blue light on enzymes related to carbohydrate metabolism.

Authors contributions

Beatriz Santos: Conceptualization, Methodology, Formal analysis, Investigation, and Writing – Original draft preparation. **Daniele P. Conceição, Monique P. Campos, and Matheus F. Passos:** Methodology, Resources, and Critical revision of the article. **Diego de O. Corrêa:** Conceptualization, Methodology, Resources, and Critical revision of the article. **Douglas Adamoski:** Writing – Original draft preparation and Critical revision of the article. **André B. Mariano** Resources. **José V. C. Vargas, and Vanessa M. Kava:** Supervision, Funding acquisition, and Critical revision of the article. All the authors read and approved the final manuscript.

Declaration of Competing Interest

None

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Supplementary data

TABLE S1 – PRIMER SEQUENCE

Primer	Sequence (5' – 3')	Ref.
Phytoene synthase (*psy*) - F	CGATACCAGACCTTCGACG	Gao et al. (2012)
Phytoene synthase (psy) - R	TGCCTTATAGACCACATCCAT	
Licopene β-cyclase (*lyc*) - F	TGGAGCTGCTGCTGTCCCT	Gao et al. (2012)
Licopene β-cyclase (*lyc*) - R	GAAGAAGAGCGTGATGCCGA	
β-carotene ketolase (*bkt*) - F	CAATCTTGTCAGCATTCCGC	Gao et al. (2012)
β-carotene ketolase (*bkt*) - R	CAGGAAGCTCATCACATCAGAT	
β-carotene hydroxylase (*CrtR-b*) - F	ACACCTCGCACTGGACCCT	Gao et al. (2012)
β-carotene hydroxylase (*CrtR-b*) - R	GTATAGCGTGATGCCCAGCC	
Phototropin (*phot*) - F	ACTTCTGCATCAGCGACC	Ma et al. (2018)
Phototropin (*phot*) - R	TTGGAGGAAGCGGCAGTT	
glb-1 - F	CGCCTGGCATTTGTCATTG	Ma et al. (2017)
glb-1 - R	AAACTCAGTGCCAGCATAGCG	
18S – R	CAAAGCAAGCCTACGCTCT	
18S – F	ATACGAATGCCCCCGACT	
Actin - F	AGCGGGAGATAGTGCGGGACA	Wen et al. (2015)
Actin - R	ATGCCCACCGCCTCCATGC	

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CAPÍTULO 2 – LIPIDS, PROTEINS AND PIGMENTS PRODUCTION IN MIXOTROPHIC CULTURES OF *Haematococcus pluvialis* AND *Tetradesmus obliquus*

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Enhancement of lipids and astaxanthin in mixotrophic cultures of microalgae

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Abstract

In the present study, the effect of different concentrations of glycerol on cellular response of *Tetradesmus obliquus* and *Haematococcus pluvialis* cultivated under mixotrophic conditions was evaluated. The addition of glycerol stimulated the accumulation of lipids and carbohydrates, both in *T. obliquus* and *H. pluvialis*. The 5 g $L^{-1}$and 10 g L^{-1} concentrations of glycerol were provided the highest lipid yields in *T. obliquus*. While the addition of 1 g L^{-1} glycerol promoted a 3-fold increase in the lipid content of *H. pluvialis*. The carotenoid concentration increased from 1.7 mg L^{-1} (control) to 3.5 mg L^{-1} (1 g L^{-1} of glycerol) in *H. pluvialis* and registered an increase of 3.5 times in astaxanthin concentration in relation to the control. Glycerol is a by-product of biodiesel synthesis, being a low-cost source of organic carbon. The increase in the levels of lipids, carbohydrates and astaxanthin indicate that the use of glycerol may be a viable alternative for cultivations of *T. obliquus* and *H. pluvialis*.

Keywords: *Tetradesmus obliquus*; *Haematococcus pluvialis*; Biomass composition; Organic carbon; Mixotrophic growth.

1. Introduction

Microalgae biomass is rich in several compounds of commercial interest, stimulating the interest of industry sectors and researchers. The rapid growth and the possibility of combining biomass production with the treatment of effluents and emissions make them even more attractive in a context that values sustainability (CORREA et al., 2017). However, staggering might present an obstacle in the

production of biomass. In this scenario, the concept of biorefinery comes as a measure of adding value to algae production.

Tetradesmus obliquus (Turpin) and *Haematococcus pluvialis* (Flotow) are two species of freshwater Chlorophyceae that have been extensively studied. *T. obliquus* (Homotypic synonym: *Scenedesmus obliquus* and *Acutodesmus obliquus*) has a high growth rate and the ability to accumulate high concentrations of fatty acids and pigments under different growth conditions (CHEN et al., 2019; CHOI et al., 2019; SHEN et al., 2019), in addition, this microalgae is a potential producer of biohydrogen (CORREA et al., 2017; DIAS et al., 2019). *H. pluvialis* is a unicellular green alga considered one of the main natural sources of astaxanthin. Stress conditions, such as high light incidence and nutrient starvation, stimulate the accumulation of astaxanthin in *H pluvialis*, reaching up to 5% of its dry weight (KIM et al., 2020). Astaxanthin is a secondary carotenoid with high antioxidant activity and anti-inflammatory properties (DAVINELLI et al., 2018). In addition, studies show that astaxanthin has anti-angiogenic activity, anti-cancer activity, among other benefits to human health (SATHASIVAM; KI, 2018).

Micro-algae are photosynthetic organisms, but some species can grow in total absence of light using an organic substrate. Glucose, acetate, and glycerol are the most used organic source. The oxidation of these compounds occurs at different locations within the cell, so that one pathway can be favored over the other. Therefore, it is necessary to take into consideration the final product. Cultures under heterotrophic conditions have been seen as a good opportunity to increase the production of carbohydrates, proteins and lipids (PEREZ-GARCIA; YOAV, 2015). However, some compounds, such as carotenoids, for example, are produced in higher concentrations only in the presence of light (KONG et al., 2020). In this way, cultivation grown under mixotrophic conditions can be an attractive alternative.

Using light combined with an organic carbon source (mixotrophic condition) can increase the production of compounds of interest, without compromising the synthesis of compounds produced under lighting. Studies testing glycerol in mixotrophic cultures have shown that it is possible to enhance the productivity of biomass, and the accumulation of lipids and carbohydrates (DITTAMART et al., 2014; KONG et al., 2013). A mechanism that promotes the increase of lipids and carbohydrates in cultures fed with glycerol, and kept in the light, was proposed by Xu

and collaborators (2019). The assimilation of glycerol by algal cells causes an increase in the concentration of glyceraldehyde 3-phosphate, which upon entering the glycolysis pathway, generating the precursor to carbohydrates. Besides, glyceraldehyde 3-phosphate might produce monoacylglycerides, diacylglycerides, or triacylglycerides, stimulating fatty acid cycle (XU et al., 2019). Microalgae have genes responsible for the assimilation of glycerol, however, there are two possible ways in which glycerol can be metabolized. One is the conversion of glycerol to dihydroxyacetone, by the activity of glycerol dehydrogenase. The other way converts it to glycerol-3-phosphate by glycerol kinase. Some studies indicate that the route of use of glycerol in microalgae can be through the G-3-P, using the enzymes glycerol kinase and G-3-P dehydrogenase generating dihydroxyacetone phosphate. However, Zhang and collaborators (2020) suggest that glycerol can be assimilated independently of the G-3-P route in *H. pluvialis*. Despite the work about growth and biomass production using glycerol, there is no information in the literature about these genes in *H. pluvialis* or *T. obliquus*.

The condensation of glyceraldehyde 3-phosphate and pyruvate produces isopentenyl pyrophosphate (IPP) in the chloroplast via the non-mevalonate route (LICHTENTHALER, 1999). Since IPP is a precursor to carotenoids, Zhang and colleagues (2020) suggest that the addition of exogenous glycerol may cause an increase in carotenoids in *H. pluvialis*. Considering that the synthesis of IPP in *T. obliquus (S. obliquus*) occurs by the non-mevalonate pathway (SCHWENDER et al., 1996), it is possible that there is an increase in the production of carotenoids in cultures of *T. obliquus* in the presence of glycerol. Other microalgae species had increased carotenoid production in crops with the addition of glycerol. The carotenoids production was higher in cultures under mixotrophic conditions in *Nannochloropsis gaditana* and *Dunaliella tertiolecta* than cultivation without adding any source of organic carbon (LIANG et al., 2019; MENEGOL et al., 2019).

Some studies have shown that protein synthesis can increase in crops fed with glycerol. Under mixotrophic conditions, *Arthrospira platensis* produced higher protein content in different concentrations of glycerol (MARKOU et al., 2019; MORAIS et al., 2019). In mixotrophic cultures with different carbon sources, the highest protein content in *Chlorella vulgaris* was get with 1% glycerol (LIANG et al., 2009). Although it is not clear how the addition of glycerol can increase the

production of proteins, it is known that proteins are related to cell division and formation of nucleic acids. Thus, increased cellular concentration can result in an increase in proteins.

In the present work, we performed an *in silico* analysis of the glycerol kinase gene and evaluated how glycerol affects the production of lipids, carbohydrates, proteins, chlorophyll, and carotenoids in cultures of *Tetradesmus obliquus* and *Haematococcus pluvialis* under mixotrophic conditions.

2. Material and Method

2.1 Experimental conditions and culture analysis

The microalgae *Tetradesmus obliquus* obtained from the cultivation bank in the Center of Research and Development for Self-Sustainable Energy (NPDEAS) was grown in CHU medium (ABNT, 2011) modified according to Correa and colleagues (2017). *Haematococcus pluvialis*, obtained from the Canadian Center for the Culture of Microorganisms (strain 7072), was cultivated in synthetic medium (mg L^{-1}: $NaNO_3$, 250; $CaCl_2$ $2H_2O$, 25; $MgSO_4$ $7H_2O$, 75; K_2HPO_4, 75; KH_2PO_4, 175; NaCl, 25; EDTA, 50; $FeSO_4$ $7H_2O$, 4.98; H_3BO_3, 11.3; $ZnSO_4$ $7H_2O$, 14.12; $MnSO_4$ $4H_2O$, 1,96; Na_2MoO_4 $2H_2O$, 1.92; $CuSO_4.5H_2O$, 2.52; $CaCl_2$ $6H_2O$, 1.44). The tests were performed in duplicates using 2 L Erlenmeyers flasks filled using 1.6 L of the culture and keep at 22 ºC and air flow of 1.5 L min^{-1}. The cultures were illuminated at intensity of 100 µmol s^{-1} m^{-2}. Glycerol was added in concentrations of 1, 5 and 10 g L^{-1}, according to Andruleviciute and collaborators (2014) in the first day of cultivation. Initial inoculum concentration was 45 ± 1.9 mg L^{-1} for *T. obliquus* and 16 ± 1.1 mg L^{-1} for *H. pluvialis*. The experiment lasted 10 days. The biochemistry composition was analyzing on the last day of cultivation.

2.2 Productivity and biomass composition

Dry biomass concentration was determined by a gravimetric method, where 10 mL of the culture was filtered on a glass fiber membrane of porosity 0.7 μm and pre-determined mass. The filtration occurred in a filtration system coupled to a vacuum pump. The membrane was dried at 60 ºC for 24 hours. The productivity was calculation using the Equation (1).

$$B_p = \frac{B_{final} \quad B_{initial}}{t} \tag{1}$$

Where B_p is productivity in mg L^{-1} day^{-1}, B_{final} and $B_{initial}$ is the biomass on the 10^{th} day and the first day of cultivation, respectively, in mg L^{-1}, and t is the time of cultivation in day.

Total carbohydrates were measured using phenol and sulfur acid (DUBOIS et al. (1956). Initially, 10 mg of biomass was treated with 1 mL sulfur acid 80% and keep in ice for 4 hours. After 16 hours, 9 mL of distilled water was added and mixed. For extracts obtention, 1.5 mL was centrifuged for 10 minutes at 10000 rpm. Subsequently, 50 μL of extract was dilute in 450 μL and mixture with 500 μL of phenol 5% and 2.5 mL of sulfur acid. The samples were stirring and allowing to stand until room temperature and, then, the absorbance was determined at λ = 490 nm. Standard curve was preparing with a glucose solution 1 mg mL^{-1}, to obtaining the line equation (Equation 2). All tubes were previously sanitized with 10% acid solution to avoid contamination in the analysis.

$$y = 0.0203\,x \quad (R^2 = 0.9939,\ p < 0.05) \tag{2}$$

Lipids was extracted using a mixture of solvents and determined by gravimetry according to Folch, Lees, and Stanley (1956). The method consists in the addition of 1.5 mL of chloroform : methanol (2 : 1, v : v) in 50 mg of biomass with posterior cell disruption in ultrasonic machine (40 kHz) for three cycles of 30 minutes. At each cycle, the samples were centrifuged to collected the supernatant. At the end,

the supernatants were put together. The extracts were kept at 60 until totally evaporating of the solvent. The Equation 3 was used for quantification.

$$L = \left(\frac{L_{final} - L_{initial}}{B}\right) \times 100 \quad (3)$$

Where L is total lipids in %, L_{final} and $L_{initial}$ are flasks mass with lipids and without lipids, respectively, in mg; and B is the microalga mass utilized for lipids extraction.

The water-soluble proteins quantification was performed using of the Lowry assay (LOWRY et al., 1951). To obtain the protein extracts, 50 mg of biomass was treated with 4 mL of sodium hydroxide 1 mol L^{-1} in a water bath at 100 °C for one hour. After centrifugation, the supernatant was collected and reserved. For protein quantification, 0.05 mL of the protein extract was transferred to a test tube and homogenized with 5 mL of reagent D (reagent D is a mixture of three solutions in the proportion 100:1:1 , v:v:v, a:b:c, where the solution *a* corresponds to 100 mL of 2% sodium carbonate and 900 mL of sodium hydroxide 0.1 mol L^{-1}; solution *b* corresponds to 0.5% copper sulfate pentahydrate; solution *c* corresponds to sodium tartrate and potassium 1%). Subsequently, the tubes were kept at room temperature for 10 minutes. Then, 0.5 mL of 2-fold dilute Folin-Ciocalteau solution was added. The solutions were homogenized and kept at room temperature for 30 minutes. The reads were performed at spectrophotometer at λ = 750 nm. To produce the standard curve and obtain the linear equation (Equation 4), was used the albumin solution (0.4 g L^{-1}).

$$y = 0.0014 \times X + 0.0083 \quad (R^2 = 0.9909,\ p < 0.05)$$

For Chlorophyll *a* and *b* were measure, 3 mL of ethanol was added in 5 mg of dry biomass. The mixture was treated at 60 °C by 40 minutes, then the mixture was cooled in ice by 15 minutes. After, the supernatant was collected by centrifugation. The extraction process was repeated until the total discoloration of the pellet. The concentrations of chlorophyll *a* and *b,* and carotenoids were determined by spectrophotometry from the absorbance readings of the extracts at wavelengths of 665nm, 649nm and 470nm, respectively. All analyses were performed in triplicate.

For the quantification of pigments, was used Equation 5 to 7. The results are shown in mg L^{-1}.

$$Chl_a = 12.7 \times OD_{663} \_ 2.69 \times OD_{649} \quad (5)$$

$$Chl_b = 22.9 \times OD_{649} \_ 4.68 \times OD_{663} \quad (6)$$

$$Car_{total} = 4.7 \times OD_{440} \_ 0.27 \times (Chl_a + Chl_b) \quad (7)$$

Astaxanthin was extracted with dimethyl sulfoxide (DMSO) according to Zhao and colleagues (2018), where 10 mL of sample was recovered by centrifugation by 10 minutes at 2600 x g. The pellet was washed twice and dry. The biomass was treated with 5% (w/v) potassium hydroxide solution in 30% methanol (v/v) at 65 °C for 15 minutes to destroy the chlorophyll. Treated cells were centrifuged at 2600 x g for 10 minutes and washed three times with distilled water. Then, 5 mL of DMSO was added to pellet and kept in ultrasound (40 kHz) for 10 minutes. For extraction, the samples were kept at 45 °C for 20 minutes in water bath. The extraction process was repeated until the total discoloration of the pellet. Total astaxanthin was determined by spectrophotometry by measuring the absorbance at 490 nm. All analyzes were performed in triplicate. Total astaxanthin (mg L^{-1}) was determined according to Equation 8.

$$Ast = 4.5 \times OD_{490} \times \frac{V_{DMSO}}{V_{culture}} \quad (8)$$

2,3 Analysis *in silico*

The search for genes that code for glycerol-3-phosphate dehydrogenase, glycerol kinase, and glycerol-3-phosphate acyltransferase were performed using the *Dunaliella salina* (GenBank accession number no AAX56341), *Thalassiosira pseudonone* peptide sequence (GenBank accession no. XP_002295903.1), and *Scenedesmus* sp. (GenBank accession no KAF6266197.1)

as a query. The search for genes was performed with the Bioedit program, version 7.0.5.3 (HALL, 1999), using the local BLAST tool. The percentage of identity of the amino acids was determined with the MegAlign program of the Lasergene program, version 7. The presence of conserved domains was detected by the tool Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The three-dimensional structure of the glycerol kinase, glycerol-3-phosphate dehydrogenase, and glycerol 3-phosphate acyltransferase proteins was predicted using SWISS-MODEL workspace (WATERHOUSE et al., 2018) and UCSF Chimera version 1.14 (PETTERSEN et al., 2004).

2.4 Statistical analysis

All analysis were carried out in triplicate. The results are average of triplicates with standard deviation. Significant differences were calculated using the one-way analysis of variance (ANOVA) at 95% confidence interval and Tukey's test ($p < 0.05$).

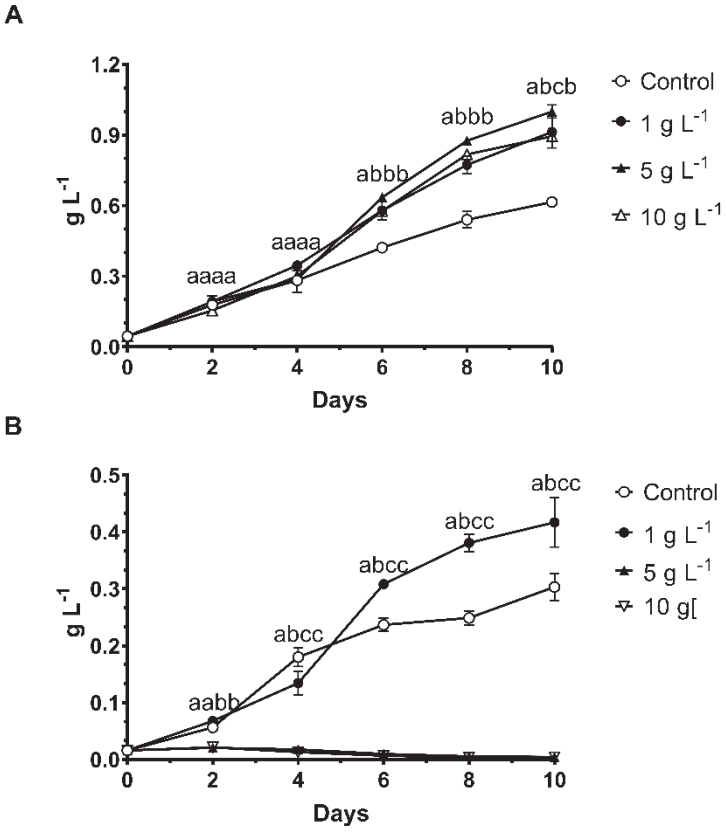
3 Results and discussion

3.1 Productivity and biomass composition

The production of biomass in cultures of *T. obliquus* fed with glycerol produced about 1.5 times more biomass than the control. Using 5 g L^{-1} of glycerol provided a 22-fold increase in biomass yield, reaching to 0.9 ± 0.03 g L^{-1} of dry biomass. It is observed that until the fourth day there is no difference in the production of biomass between the control and the treatments and, until the eighth day of growth, there is no difference in the production of biomass between the treatments (Fig. 1.2 A). Interestingly, biomass production in cultures fed with 1 g L^{-1} or 10 g L^{-1} of glycerol was similar (Fig. 1.2 A). Xu and colleagues (2019) observed a

similar result. The researchers evaluated waste supplemented with 2.5 g L^{-1}, 5 g L^{-1}, 10 g L^{-1}, and 20 g L^{-1} of glycerol. While the two lowest concentrations provided greater biomass production, cultures fed with 10 g L^{-1}, and 20 g L^{-1} of glycerol ended up with the similar biomass production as the control. Choi and Yu (2015) observed a similar growth profile, where the addition of 2 g L^{-1} and 10 g L^{-1} glycerol provided similar yields, while the highest concentration of dry biomass was obtained in cultures with 5 g L^{-1} glycerol. On the other hand, in this study, *H. pluvialis* was not able to grow with the addition of 5 g L^{-1} and 10 g L^{-1} of glycerol. Growth of *H. pluvialis* in mixotrophic cultures with these concentrations of glycerol has been reported (ANDRULEVICIUTE et al., 2014), however, it is worth noting that the sodium nitrate concentration used in the cultures was 1.5 g L^{-1}, so that the carbon/nitrogen (C/N) ratio was different from that used in our study. Despite this, cultures with 1 g L^{-1} of glycerol have sustained higher growth than control. In the first four days, the control culture grew at a faster rate than the treatment with 1 g L^{-1} of glycerol. However, this situation changes from the sixth day of cultivation. After 10 days of cultivation, the biomass production in the treatment with 1 g L^{-1} of glycerol increased 26-fold, against a 19-fold increase in the control culture, reaching to 0.417 ± 43 g L^{-1} and 0.303 ± 24 g L^{-1}, respectively (1.2 B). Some studies shown glycerol can be toxic in high concentrations. Increasing the concentration of organic carbon in microalgae cultures may increase the need for ATP to assimilate organic carbon and limit the reduction of carbon obtained in photosynthesis, resulting in growth inhibition (HEIFETZ et al., 2000; SILABAN et al., 2014). Besides, high initial concentrations of organic carbon, as glucose and glycerol, can lead to a reduction in the availability of water for the cells, even if temporarily (BOUYAM et al., 2017; MENEGOL et al., 2019). In mixotrophic cultures of *Chlorella vulgaris*, the authors observed 5% and 10% of glycerol inhibited cell growth (LIANG et al. (2009).

FIGURE 1.2 – EFFECTS OF GLYCEROL ON THE BIOMASS PRODUCTION



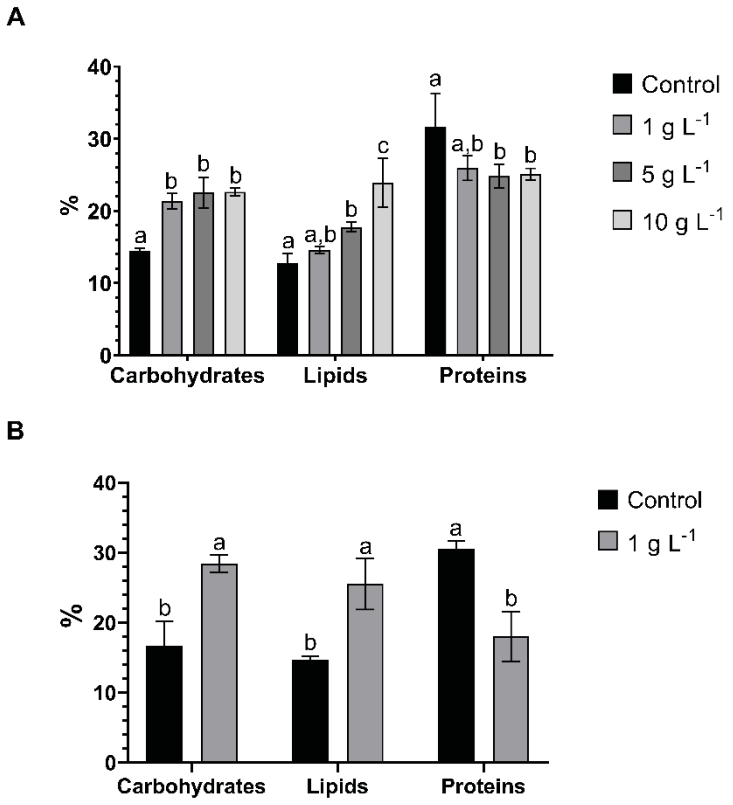
LEGEND: Effects of glycerol on the biomass production of (A) *Tetradesmus obliquus* and (B) *Haematococcus pluvialis,* kept under mixotrophic conditions. The results are shown as mean ± SD (n = 3). Same lowercase letters indicate that there was no significant difference ($p < 0.05$) between cultures (control, 1 g L^{-1}, 5 g L^{-1}, 10 g L^{-1}, respectively).

Our results, in agreement with literature, indicate that the highest concentrations of dry biomass of *T. obliquus* and *H. pluvialis* are obtained with lower concentrations of glycerol. In addition, *H. pluvialis* cells prefer, based on our results, a lower C/N, with growth completely inhibited when using higher ratios.

The addition of glycerol in the cultivars of *T. obliquus* induced the accumulation of carbohydrates and lipids but did not affect the synthesis of soluble proteins (Fig. 2.2 A). The carbohydrate content in the control culture of *T. obliquus* reached up 14.4 ± 0.4%. On the other hand, the carbohydrate content obtained in biomass produced with glycerol was 1.5 ± 0.05 times higher than that of the control, however, the glycerol concentration did not influence the accumulation of carbohydrates. The content obtained were 21.3 ± 1.1%, 22.5 ± 2.1%, and 22.6 0.5% of total carbohydrates in biomass produced with 1 g L^{-1}, 5 g L^{-1}, and 10 g L^{-1} of

glycerol, respectively (Fig 2.2 A). In the same way, there was no difference ($p < 0.05$) between the productivities obtained in cultivations fed with glycerol, however, all were greater than the productivity obtained in the control (Table 1.1). Concentrations above 1 g L^{-1} of glycerol inhibited the production of biomass by microalgae *H. pluvialis*. In this way, Figure 2.2 B shows only the data on the biochemical composition of the control culture and the culture treated with 1 g L^{-1} de glycerol.

FIGURE 2.2 – EFFECT OF DIFFERENT GLYCEROL CONCENTRATIONS ON BIOCHEMICAL COMPOSITION



LEGEND: Effect of different glycerol concentrations on biochemical composition in (A) *Tetradesmus obliquus* and (B) *Haematococcus pluvialis* under mixotrophic conditions. Results shown for control (no glycerol), 1 g L^{-1}, 5 g L^{-1}, and 10 g L^{-1} of glycerol for *T. obliquus* and control (no glycerol) and 1 g L^{-1} of glycerol for *H. pluvialis*. The results are shown as the mean ± SD (n = 3). Same lowercase letters within the same type of composition (carbohydrates, lipids, or proteins), represents that there is no significant difference ($p < 0.05$).

As observed in the cultures of *T. obliquus*, the addition of glycerol affected the biochemical composition of *H. pluvialis*. Treatment with 1 g L^{-1} glycerol generated a biomass rich in lipids and carbohydrates and poor in protein. The

carbohydrate content in *H. pluvialis* was 1.7 times higher in biomass produced with glycerol than that obtained in the control culture, with 28.4 ± 1.2 and 16.7 ± 3.5, respectively. Other species grown in the presence of glycerol also showed an increase in the accumulation of carbohydrates in relation to autotrophic cultivation. Productivities corroborate with data on carbohydrate levels, reaching 5.1 ± 1.1 mg L^{-1} day^{-1} and 11.8 ± 0.6 mg L^{-1} day^{-1} in control cultures and with the addition of glycerol, respectively.

Unlike the production of carbohydrates, the production of lipids varied among cultures treated with glycerol, in the *T. obliquus* cultures. The highest lipid content was found in the biomass produced with 10 g L^{-1} of glycerol, with 24.9 ± 4.2%. about twice the content obtained in the control (12.1 ± 1.5%) (Fig. 2.2 A). The lipid content obtained in the *T. obliquus* biomass grown with 5 g L^{-1} glycerol is approximately 1.5 times higher than the control, reaching 17.8 ± 1% and statistically equal to the lipid content found in the biomass of the culture fed with 1 g L^{-1} glycerol. On the contrary, about productivities, the difference observed between cultures treated with 5 g L^{-1} and 10 g L^{-1} glycerol was insignificant ($p < 0.05$).

In *H. pluvialis* cultures, after 10 days of cultivation, the addition of 1 g L^{-1} of glycerol increased the lipid content by approximately 1.7 times, reaching to 25 ± 3.6%, while the content observed in the control culture was 15 ± 0.6% (Fig. 2.2 B). Productivity was also significantly higher in glycerol cultures, producing 2.4 times as much per liter, per day compared to control (Table 2.2). Zhang and colleagues (2020) evaluated the effect of 0.5 mL L^{-1} and 1 mL L^{-1} of glycerol in mixotrophic cultures of *H. pluvialis*. The authors also observed a significant increase in lipids accumulation in cultures treated with glycerol and hypothesize that glycerol is converted into glyceric acid in *H. pluvialis*, culminating in an increase in Acetyl-CoA, a precursor to fatty acid synthesis. Other researchers have not observed a significant increase in the accumulation of lipids to the detriment of the concentration of glycerol used, even keeping the cultures in the stationary phase for longer (XU et al., 2019). A plausible answer would be the C/N nitrogen ratio used in our study, which favored an increase in the accumulation of lipids in cultures treated with higher concentrations of glycerol. To assess this hypothesis, other concentrations of glycerol and nitrogen must be tested. The effect of different concentrations of glycerol on the accumulation of lipids in other species has been reported. Mixotrophic cultures of *Chlorella vulgaris* only

produced a biomass with a significant increase in lipid content, compare to control, in cultures treated with 10 g L^{-1} of glycerol. Still, the difference is insignificant when compare between mixotrophic cultures (KONG et al. (2013). Once glycerol enters the cell, this molecule can be converted into dihydroxyacetone (DHA) or glycerol-3-phosphate (G-3-P), by the action of the enzymes dehydrogenase or glycerol kinase, respectively (XUE et al., 2017). Dihydroxyacetone phosphate (DHAP) can be generated from DHA, by the action of the enzyme dihydroxyacetone kinase, or from G-3-P, by the action of FAD-dependent glycerol-3-phosphate. DHAP can be precursors to intermediates of Acetyl-CoA, that is a precursor to the synthesis of fatty acids (SILVA; MACK; CONTIERO, 2008), which could also explain the increase in lipids in *T. obliquus* and *H. pluvialis* cultures fed with glycerol.

Our results showed the synthesis of soluble proteins is affected by the addition of glycerol. Fig 2.2 A shows that the highest protein levels in *T. obliquus* were found in control and in culture treated with 1 g L^{-1} glycerol, with 31.7 ± 4.7% e 26 ± 1.7%, respectively. Addition of 5 g L^{-1} and 10 g L^{-1} of glycerol produced an inhibitory effect on the synthesis of soluble proteins, reaching 24.8 ± 1.6% and 25.1 0.7 %, respectively. In *H. pluvialis* cultivation, the protein content decreases significantly ($p < 0.05$) under mixotrophic condition. Reaching about 18 ± 3.5%, protein production was 1.7 times lower in glycerol cultures compared to control (2.2 B). This cellular response has already been reported by other researchers, where the addition of glycerol induces the accumulation of carbohydrates and lipids while there is a reduction in the accumulation of proteins (EL-FATAH ABOMOHRA et al., 2018; XU et al., 2019). Xu and collaborators (2019) found lower levels of proteins in the biomass of *S obliquus* treated with glycerol, with the lowest content being reached in cultures with 10 g L^{-1} and 20 g L^{-1} glycerol. In general, nitrogen deprivation induces the accumulation of carbohydrates and lipids and the cells stop the division. Thus, one explanation for low proteins concentration may be a possible limitation of nutrients caused by rapid cell growth in glycerol culture.

TABLE 1.2 – PRODUCTIVITIES OBTAINED IN *Tetradesmus obliquus* CULTIVATION UNDER AUTOTROPHIC AND MIXOTROPHIC CONDITIONS.

Productivity (mg L^{-1} day^{-1})	Glycerol			
	Control	**1 g L^{-1}**	**5 g L^{-1}**	**10 g L^{-1}**
Biomass	57 ± 0.0[a]9	87 ± 6.8[b]	95 ± 2.8[c]	85 ± 0.9[b]
Carbohydrates	8.9 ± 0.2[a]	20.1 ± 0.9[b]	22.5 ± 2.1[b]	20.3 ± 0.5[b]
Lipids	7.9 ± 0.8[a]	13.1 ± 0.5[b]	18 ± 0.8[c]	21.3 ± 3.1[c]
Proteins	19 ± 2.9[a]	24 ± 1.6[b]	25 ± 1.6[b]	22 ± 0.7[ab]
Chlorophylls	1.5 ± 0.003[a]	0.7 ± 0.01[b]	0.6 ± 0.01[b]	0.5 ± 0.03[b]
Carotenoids	0.09 ± 0.004[a]	0.08 ± 0.01[b]	0.06 ± 0.01[c]	0.06 ± 0.01[d]
Astaxanthin	0.006 ± 0.001[a]	0.01 ± 0.001[a]	0.01 ± 0.002[a]	0.02 ± 0.003[b]

NOTE: Productivities obtained in *Tetradesmus obliquus* cultivation under autotrophic (control) and mixotrophic (glycerol addition) conditions. The results shown are averages ± SD (n=3). Same superscript lowercase letters in the row indicate that there is no significant difference ($p < 0.05$).

TABLE 2.2 – PRODUCTIVITIES OBTAINED IN *Haematococcus pluvialis* CULTIVATION UNDER AUTOTROPHIC AND MIXOTROPHIC CONDITIONS.

Productivity (mg L^{-1} day^{-1})	Glycerol			
	Control	**1 g L^{-1}**	**5 g L^{-1}**	**10 g L^{-1}**
Biomass	1.9 ± 0.2	2.6 ± 0.2	*nd*	*nd*
Carbohydrates	5.1 ± 1.1	11.8 ± 0.5	*nd*	*nd*
Lipids	4.5 ± 0.1	11 ± 1.6	*nd*	*nd*
Proteins	12.7 ± 0.5	7.5 ± 1.5	*nd*	*nd*
Chlorophylls	0.29 ± 0.06	0.24 ± 0.006	*nd*	*nd*
Carotenoids	0.2 ± 0.01	0.4 ± 0.01	*nd*	*nd*
Astaxanthin	0.07 ± 0.02	0.3 ± 0.009	*nd*	*nd*

NOTE: Productivities obtained in *Haematococcus pluvialis* cultivation under autotrophic (control) and mixotrophic (glycerol addition) conditions. The results shown are averages ± SD (n=3). Same superscript lowercase letters in the row indicate that there is no significant difference ($p < 0.05$). Biochemical analysis for mixotrophic cultures of *H. pluvialis* with the addition of 5 g L^{-1} and 10 g L^{-1} were not determined (*nd*), due to growth inhibition.

Our results corroborate with data in the literature, indicating that the addition of glycerol can stimulate the accumulation of lipids in *T. obliquus* and *H. pluvialis*. In addition to increasing the lipid content, treatment with glycerol stimulated the accumulation of carbohydrates, results that corroborate with the increase in biomass production. Glycerol might stimulate the fatty acid synthesis route, however, in

addition, this molecule might induce the conversion of proteins into α-ketoglutarate resulting in the accumulation of lipids and low protein concentration (XU et al., 2019).

3.2 Accumulation of pigments

The addition of glycerol triggered different cellular responses to the synthesis of pigments in the two microalgae species cultivated in this study. The results show that the increase in glycerol concentration affected the synthesis of total chlorophylls in *T. obliquus*. The highest concentration of total chlorophyll was obtained in the control cultures, reaching 15 ± 0.03 mg L^{-1}. On the other hand, there is a 50% decrease in the accumulation of chlorophyll in the cultures with 1 g L^{-1}, 5 g L^{-1}, and 10 g L^{-1} of glycerol. achievement 7.3 ± 0.07 mg L^{-1}, 6.4 ± 0.05 mg L^{-1}, and 4.7 ± 0.3 mg L^{-1}, respectively (Fig. 3.2 A). After 10 days, the concentration of carotenoids also decreased in cultures with the addition of glycerol, indicating that the presence of glycerol causes a negative effect on the accumulation of chlorophyll and carotenoids in *T. obliquus* cultures. There is no effect on the accumulation of carotenoids in the control cultures and cultures treated with 1 g L^{-1} of glycerol, where the concentration achieved 0.86 mg L^{-1} and 0.83 mg L^{-1} of carotenoid, respectively. However, cultures treated with 5 g L^{-1} and 10 g L^{-1} glycerol produced a significantly lower concentration of carotenoids, reaching to 0.56 mg L^{-1} and 0.54 mg L^{-1}, respectively. The same response can be observed in the cultures of *H. pluvialis* (Fig 3.2B). There was a 16% decrease in the total chlorophyll concentration in relation to the control, dropping from 2.9 mg L^{-1} to 2.4 mg L^{-1}. According to Yang, Hua, and Shimizu (2000), it is more difficult to capture light energy and convert it into ATP when compared to the capture of energy from an organic carbon source. This cellular response results in the preference for organic carbon, causing a disturbance in the photosynthetic apparatus. Consequently, there is a reduction in chlorophyll and carotenoids. Liu and colleagues (2009) reported that the presence of glycerol can alter the structure of chloroplasts, reducing photosynthetic efficiency. Similar results have been reported for mixotrophic cultures of *Arthrospira* sp. The authors observed that the chlorophyll and carotenoids concentration decreased as the glycerol

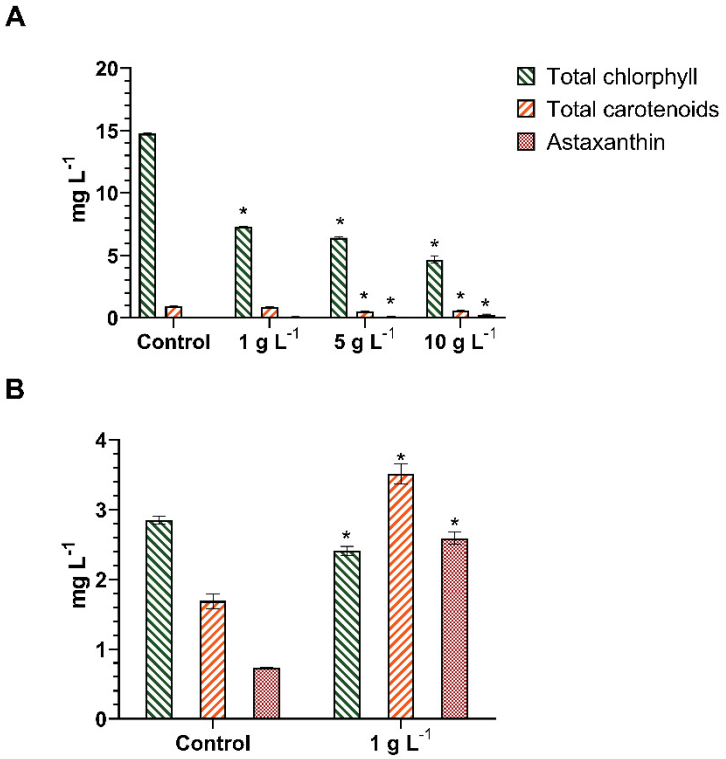
concentration in the culture medium increased (MORAIS et al., 2019). However, despite the reduction in the concentration of carotenoids in *T. obliquus* mixotrophic cultures, it is observed the proportion astaxanthin increased, as can see in the reducing in the carotenoids/astaxanthin ratio in the Table 3.2. The increase in the proportion of astaxanthin in mixotrophic cultures of *T. obliquus* represents a response strategy by microalgae in the presence of glycerol, as has already been observed in cultures with the addition of acetate (Mansouri; Hajizadeh, 2018). Further study can provide more complete information regarding the accumulation of astaxanthin in cultures of *T. obliquus* in the presence of glycerol.

TABLE 3.2 – VALUES OF THE CHLOROPHYLL/CAROTENOIDS (CHL/CAR) AND CAROTENOIDS/ASTAXANTHIN (CAR/AST) RATIOS OBTAINED IN AUTOTROPHIC AND MIXOTROPHIC CULTURES

Treatments	*T. obliquus*		*H. pluvialis*	
	Chl/Car	Car/Ast	Chl/Car	Car/Ast
Control	17.1 ± 0.7[a]	15.3 ± 1.8[a]	1.7 ± 0.1[a]	2.3 ± 0.2[a]
1 g L^{-1}	8.8 ± 0.7[b]	9.7 ± 1.3[b]	0.7 ± 0.02[b]	1.4 ± 0.1[b]
5 g L^{-1}	11.6 ± 2[b]	6.7 ± 0.4[c]	*nd*	*nd*
10 g L^{-1}	8.7 ± 1.6[b]	2.3 ± 0.5[d]	*nd*	*nd*

NOTE: Values of the chlorophyll/carotenoids (chl/car) and carotenoids/astaxanthin (car/ast) ratios obtained in autotrophic (control) and mixotrophic (1 g L^{-1}, 5 g L^{-1}, and 10 g L^{-1}) cultures of *Tetradesmus obliquus* and *Haematococcus pluvialis*. the results are shown as the mean ± sd (n = 3). equal letters in the column represent that there is no significant difference ($p < 0.05$). values for mixotrophic cultures of *H. pluvialis* with the addition of 5 g L^{-1} and 10 g L^{-1} were not determined (*nd*), due to growth inhibition.

FIGURE 3.2 – EFFECT OF DIFFERENT GLYCEROL CONCENTRATIONS ON PIGMENT ACCUMULATION



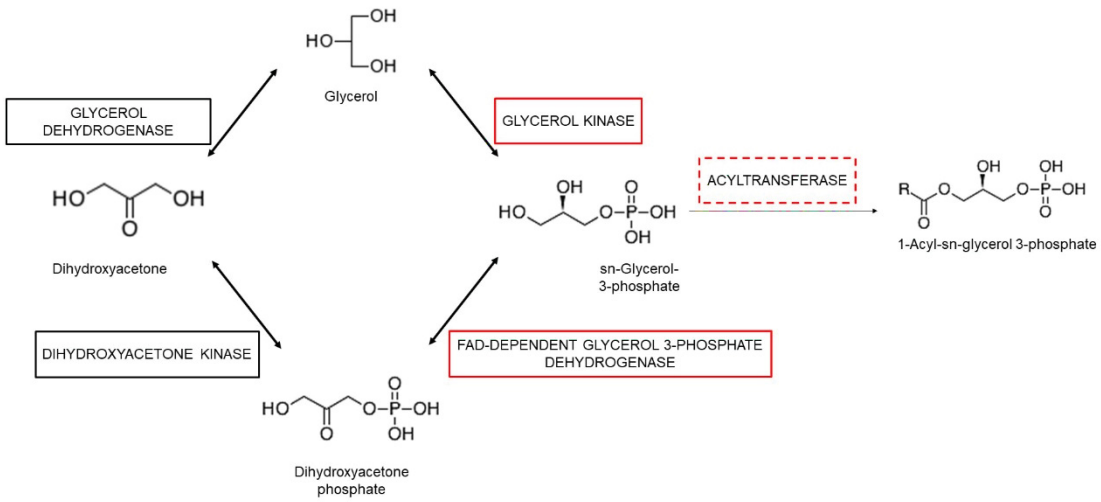
NOTE: Effect of different glycerol concentrations on biochemical composition in A) *Tetradesmus obliquus* and B) *Haematococcus pluvialis* under mixotrophic growth. The results are shown as the mean ± SD (n = 3). Equal letters represent that there is no significant difference ($p < 0.05$).

While a decrease in the concentration of carotenoids was observed in the mixotrophic cultures of *T. obliquus*, in the mixotrophic cultures of *H. pluvialis* doubled compared to the control, increasing from 1.7 ± 0.1 mg L^{-1} to 3.5 ± 0,1 mg L^{-1}. Likewise, the concentration of astaxanthin increased significantly, reaching 2.6 ± 0.1 mg L^{-1}, an increase of 70% compared to the control. Zhang and colleagues (2020) obtained similar results using 0.5 mL L^{-1} and 1 mL L^{-1} of glycerol in *H. pluvialis* cultures. The authors observed that the addition of 1 mL L^{-1} of glycerol resulted in an increase of 27% in the content of astaxanthin after 11 days of cultivation. In addition, Zhang and colleagues (2020) used light intensity of 150 μmol_{photon} m^{-2} s^{-1}, while we used 100 μmol_{photon} m^{-2} s^{-1}. Our results corroborate with the literature data and reinforce the hypothesis that glycerol can induce the accumulation of astaxanthin in *H. pluvialis* and, to a lesser extent, in *T. obliquus*. Using light intensity below 150 μmol_{photon} m^{-2} s^{-1}.

3.3 Analysis *in silico*

Glycerol can enter the cells of some microalgae species by simple diffusion, and, once inside the cells, glycerol can be assimilated in two different ways. The first involves a glycerol kinase enzyme that converts glycerol to glycerol-3-phosphate. In the second way, glycerol is converted into dihydroxyacetone by the action of the enzyme glycerol dehydrogenase. Glycerol-3-phosphate and dihydroxyacetone, in turn, can be converted to dihydroxyacetone phosphate by the action of the enzymes glycerol-3-phosphate dehydrogenase and dihydroxyacetone kinase, respectively (Fig. 5.2). The literature brings several studies that evaluate the possibility of using glycerol in mixotrophic and heterotrophic cultures of microalgae. However, there is little information regarding the characterization of genes and proteins related to an assimilation of exogenous glycerol.

FIGURE 4.2 – POSSIBLE WAYS OF ASSIMILATION OF GLYCEROL INTO MICRO-ALGAE



LEGEND: The names inside the rectangles represent the enzymes present in a reaction step. The name inside the drawn rectangle represents a class of enzyme. The enzymes inside the red rectangles are the ones studied in this work.

In this work, the search for homology revealed two putative protein sequences in *H. pluvialis* one for glycerol kinase (Fig. 5), and one for glycerol-3-phosphate dehydrogenase (Fig. 6). On the other hand, one putative sequence for glycerol-3-phosphate O-acyltransferase was found in *T. obliquus* (Fig. 7).

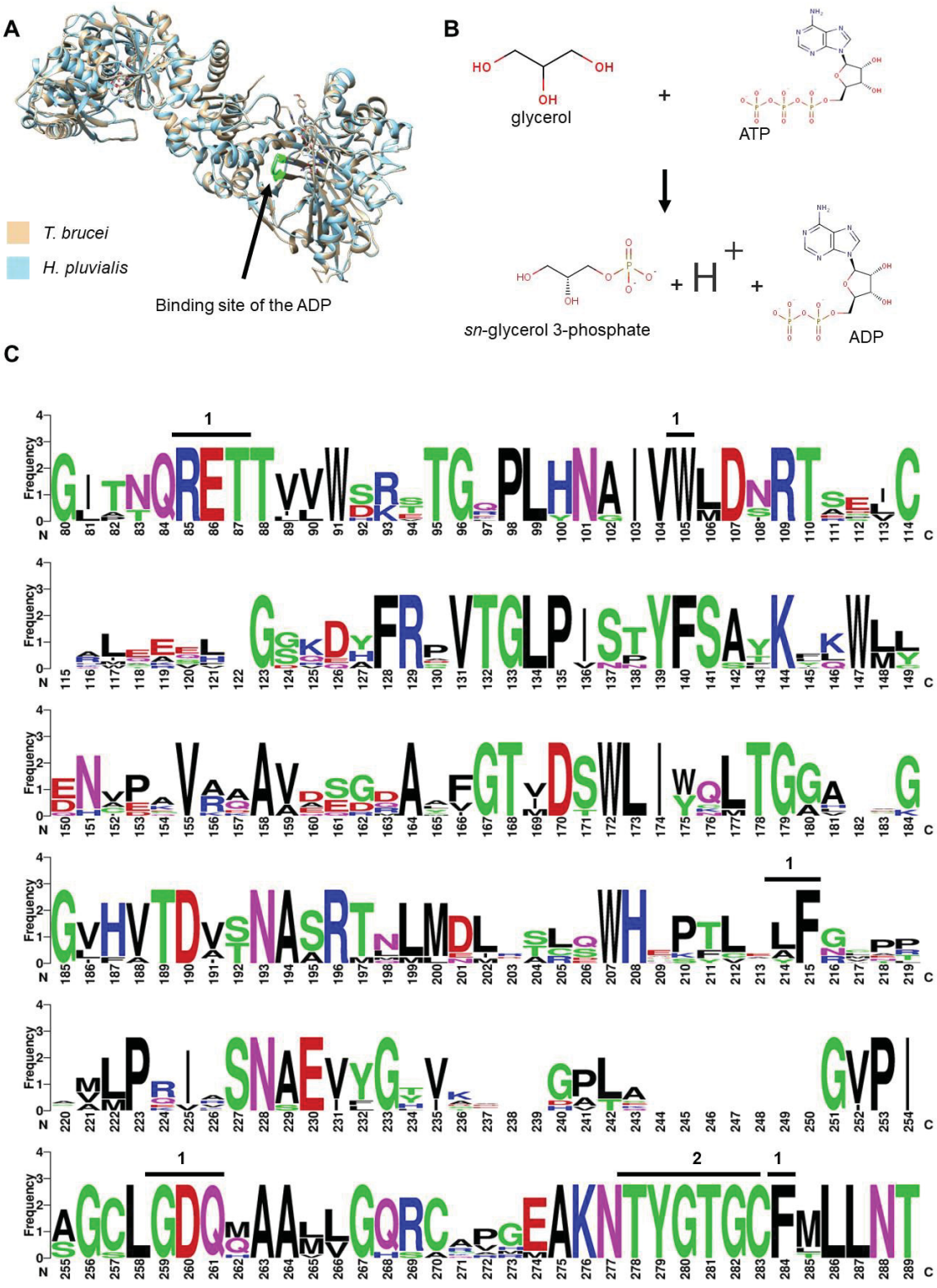
The search for domains found a domain of glycerol kinase (glpk), belonging to a large family of sugar kinase. The glpk domain found in *H. pluvialis* has 504 residues with molecular mass of 64.7 kDa. The alignment of the glk-Hp domain with sequences from other species of Chlorophyta indicates that there are 116 identical and 117 similar residues. In addition, the putative glpk-Hp retains conserved glycerol-binding sites and ADP-binding residues (1 and 2 highlighted in Fig. 6.2) (RIGDEN et al., 2000).

The glycerol-3-phosphate dehydrogenase (gpsA) domain was also found in *H. pluvialis*. The sequence has approximately 433 amino acids with molecular mass of 45.5 kDa and the conserved GXGXXG motif (X can be any amino acid). According to Otto, Argos, and Rossmann (1980), this motif is related to the coenzyme binding site NAD +. The alignment of the G3PD domain sequence with other species of Chlorophyta and prokaryotes, showed greater similarity with the prokaryotes sequences, with identical and 99 similar residues. However, the highest percentage of identity was observed in the alignment of G3PD-Hp with prokaryotes, with 78% identity and 337 identical residues.

In *T. obliquus*, one sequence with glycerol-3-phosphate acyltransferase domain (G3PAT) was found. This sequence has 1003 residues and its domain has 233 and molecular mass is 107 kDa. After alignment, it was observed 55 residues of G3PTA are highly conserved in Chlorophyta, including *T. obliquus*.

The three-dimensional structure of the proteins were modeled using *Trypanosoma brucei*, *Coxiella burnetii*, and *Cucurbita moscata* to predict the structures of glycerol kinase (Fig. 5.2A), glycerol-3-phosphate dehydrogenase (Fig 6.2A), and glycerol 3-phosphato acyltransferase (Fig. 7.2A), respectively.

FIGURE 5.2 – PROTEIN GLYCEROL KINASE

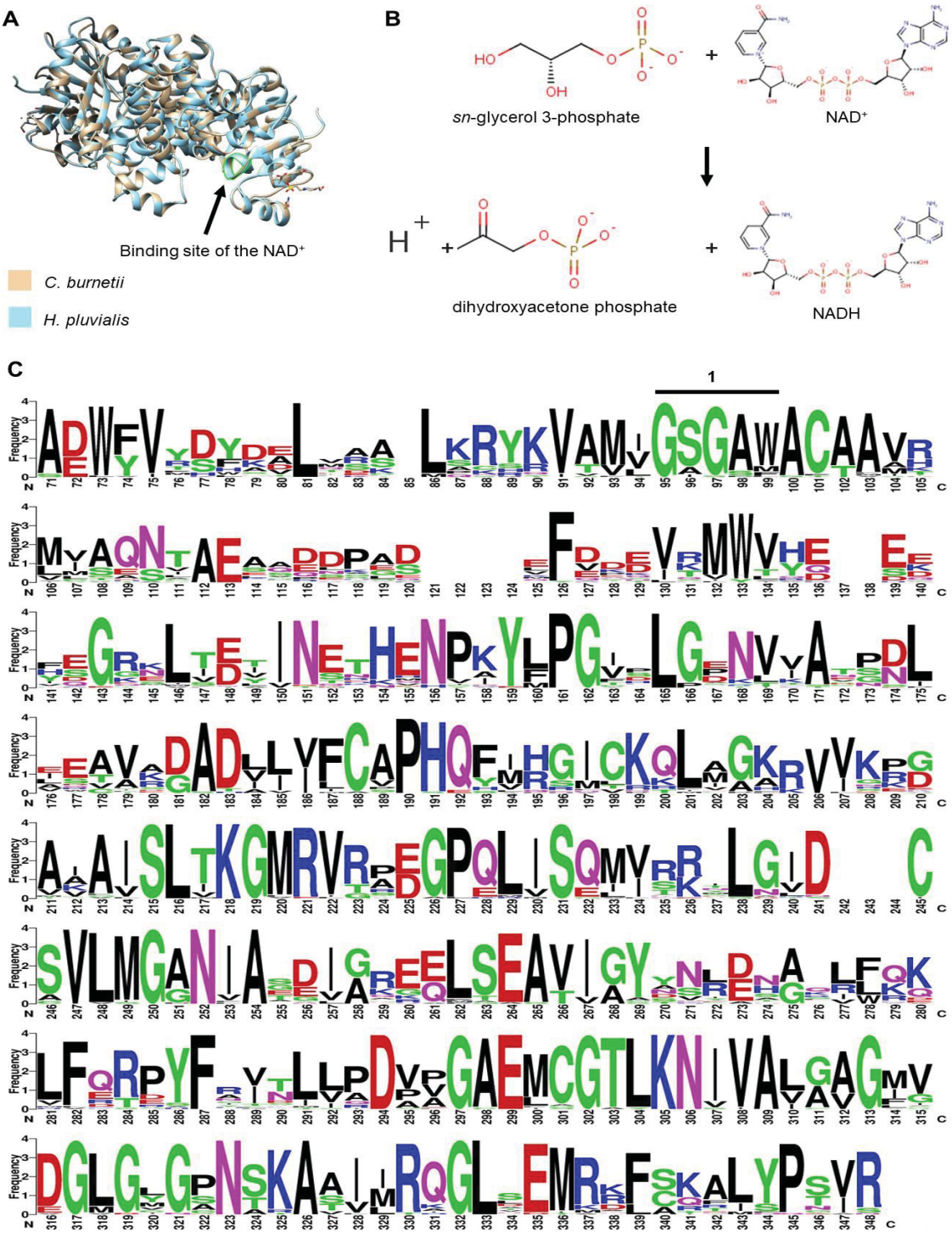


LEGEND: A) Three-dimensional model of the protein glycerol kinase of *Haematococcus pluvialis* is structurally similar to *Trypanosoma brucei*.

B) Reaction catalyzed by the enzyme glycerol kinase.

C) DNA logo for the glycerol kinase domain of the phylum Chlorophyta, highlighting the binding-glycerol (1) and binding-ADP (2) residues (RIGDEN et al., 2000).

FIGURE 6.2 – PROTEIN NAD⁺-DEPENDENT GLYCEROL-3-PHOSPHATE DEHYDROGENASE

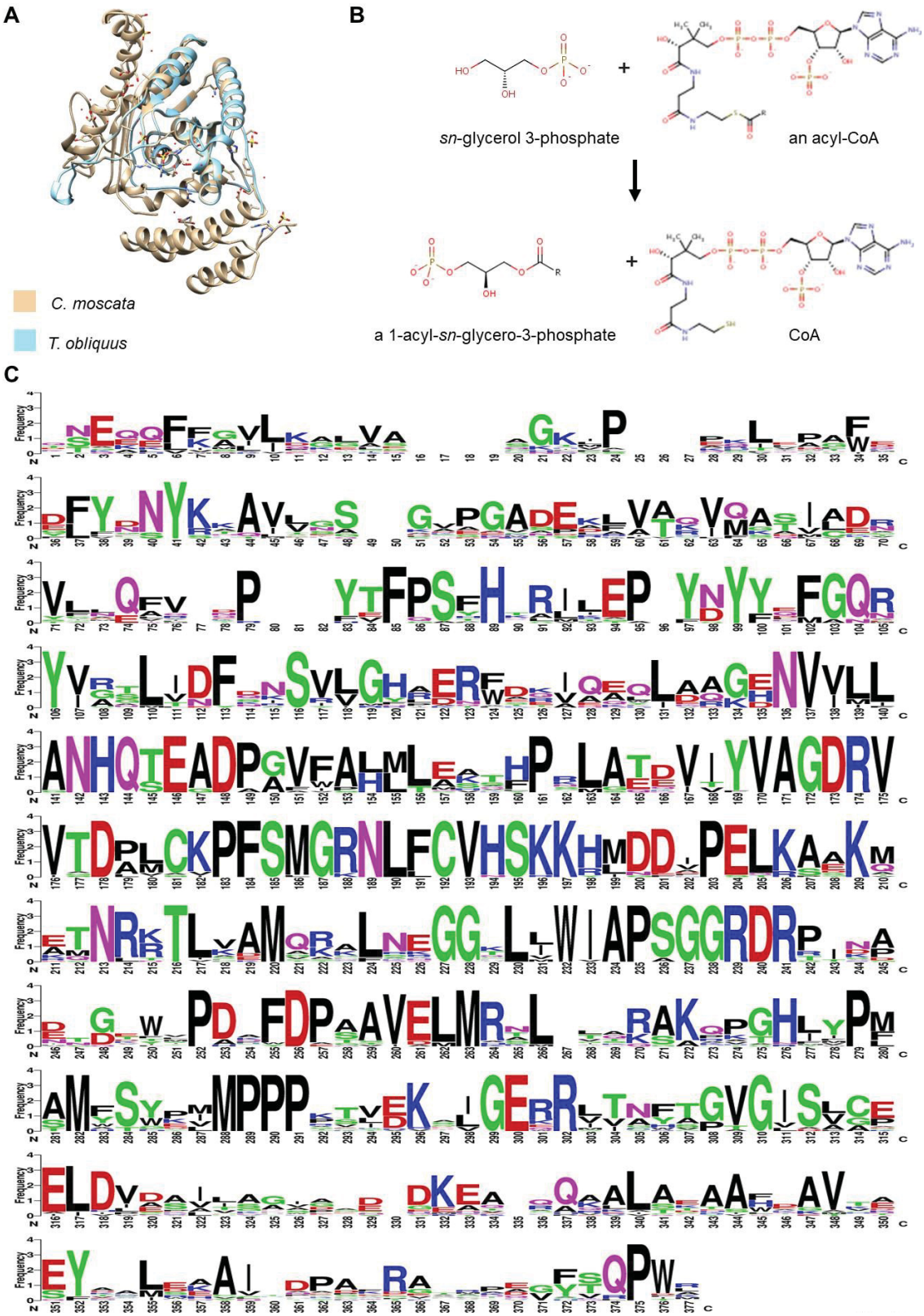


LEGEND: A) The three-dimensional model of the protein NAD-dependent glycerol-3-phosphate dehydrogenase from *Haematococcus pluvialis* is structurally similar to *Coxiella burnetii*.

B) Reaction catalyzed by the enzyme NAD-dependent glycerol-3-phosphate dehydrogenase.

C) DNA logo for the NAD-dependent glycerol-3-phosphate dehydrogenase (GPDH) domain of the phylum Chlorophyta, highlighting the NAD binding sites.

FIGURE 7.2 – PROTEIN GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE



LEGEND: A) The three-dimensional model of the protein glycerol-3-phosphate acyltransferase from *Haematococcus pluvialis* is structurally similar to *Cucurbita moscata*.

B) The reaction catalyzed by the enzyme glycerol-3-phosphate acyltransferase.

C) DNA logo for the glycerol-3-phosphate acyltransferase (GPAT) domain of the phylum Chlorophyta.

4. Conclusion

Our results show the addition of glycerol with an organic carbon source in *T. obliquus* and *H. pluvialis* cultures can be an interesting strategy to improve the content of some compounds. The highest productivity of lipids and carbohydrates were obtained in cultivars with addition of glycerol, with 5 g L^{-1} and 10 g L^{-1} of glycerol providing higher productivity of these compounds in cultivations of *T. obliquus*. On the other hand, high concentrations of glycerol (5 g L^{-1} and 10 g L^{-1}) inhibited the growth of *H. pluvialis*. However, the addition of 1 g L^{-1} glycerol resulted in a 3-fold increase in lipid content and almost doubled the carbohydrate content. The chlorophyll concentration significantly decreases in all cultures treated with glycerol, however, biomass production was significantly higher in mixotrophic cultures, indicating that the addition of a relatively cheap source of carbon may be used as an alternative to aerating cultures. In addition, the use of glycerol significantly increased the concentration of astaxanthin in cultures of *H. pluvialis*, reinforcing the idea of using glycerol as an alternative to the production of compounds of interest.

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CAPÍTULO 3 – MICROALGAE EXTRACT BIOACTIVITY: FROM PLANT BIOSTIMULANT TO ANTILEISHMANIA PROPERTIES

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Microalgae extract bioactivity: from plant biostimulant to antileishmania properties

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Abstract

Microalgae biomass has numerous biotechnological applications, among which it is possible to highlight the antimicrobial and plant biostimulant activities. In this study, extracts obtained using water and acetone were evaluated for antimicrobial and biostimulant activity. Antimicrobial activity was tested against Vancomicym-resistant Enterococci (VRE) (Gram-positive) and *Acinetobacter baumannii* (Gram-negative). Unfortunately, extracts from two microalgae species with final concentration of 6.7 mg mL^{-1} and 0.1 µg mL^{-1} did not inhibit the growth of bacterias. Regarding the biostimulant activity, the aqueous extracts of *H. pluvialis* and *T. obliquus* at 1 mg mL^{-1} showed auxin activity in cucumber cotyledons, with the best result obtained with *T. obliquus* extract. Cytokinin activity was performed with cucumber cotyledons, where only *T. obliquus* extract showed promising results, increasing the cotyledon mass to 319 mg. Our results are promising, however, new extraction strategies must be evaluated to obtain bioactive compounds, especially for *H. pluvialis*. In addition, the use of higher concentrations of the extracts should be considered.

Keywords: *Tetradesmus obliquus*; *Haematococcus pluvialis*; Biomass composition; Organic carbon; Mixotrophic growth.

1 Introduction

Microalgae represent a group diversity. These microorganisms can be found in unicellular form, forming colonies, or filaments. Some species are prokaryotes, so that different compounds can be obtained from these microorganisms.

The obtaining of microalgae biomass products has been evaluated in several sectors, as food, agricultural, cosmetic, pharmaceutical, energy and research. Most studies are focused on the synthesis and accumulation of lipids for biodiesel, but with the understanding of the concept of biorefinery, many researchers seek to combine the production and application of different compounds from microalgae biomass.

Several secondary metabolites with high added value have been identified. The main ones are carotenoids (astaxanthin and lutein), polyunsaturated fatty acids (eicosapentanoic and docosahexaenoic acids), and structural polysaccharides. These molecules have antioxidant activity, plant biostimulant, antileishmania, antiviral, antimicrobial, antitumor activities, among others. To evaluate the activities of different molecules, solvents with different polarities are used, in an attempt to isolate the compound of interest.

In the next paragraphs, works with antimicrobial activity, antileishmania and plant biostimulant from microalgae extracts will be reviewed.

1.1 Bioestimulant

One of humanity contemporary challenges is ally the environmental conservation with the development of new technologies. In this view, agriculture at the center of this discussion. The use of substances able to promote plant growth

have resulted in increased productivity and enhanced quality in several cultures (Castro, Vieira, 2001). In addition, more quality vegetables and fruits from a healthy harvest, with low impact on the environment, have been the preference of consumers currently Losasso et al. (2012). Usually, synthetic fertilizers from non-renewable sources are used. As a consequence, its use is associated with environmental problems, such as acidification and increased soil salinity, infertility and generation of greenhouse gases. In addition, eutrophication of water resources is a real problem due to the presence of large amounts of nitrogen, phosphorus and potassium Chen, Y. hua et al. (2017). New technologies that prioritize the use of natural sources have gained influence, being considered a profitable and sustainable alternative. Biostimulants are compounds that increase the absorption of nutrients and minerals and expand the tolerance to abiotic stress in plants (JARDIN, 2015). In addition, it promotes the growth and quality of food crops (JARDIN, 2015).

Extracts obtained from microalgae biomass, or biomass itself, have received special attention. These extracts contain many groups of bioactive compounds, such as polysaccharides, pigments, polyphenols, polyunsaturated fatty acids, proteins, vitamins, and growth-promoting compounds, such as cytokinins, auxinics, gibberins, and ethylene (MICHALAK et al., 2017).

Auxins are molecules that stimulate cell growth, sprouting and root initiation. Auxins in conjunction with cytokinin promote the growth of shoots, roots and fruits, in addition to converting stem to flower. Cytokinins are molecules that stimulate cell division and the formation of shoots. They are also related to delayed tissue senescence and are responsible for mediating the transport of auxin throughout the plant, in addition to affecting intermodal length and leaf growth (BHATTACHARYA, 2019). In view of the importance of auxins and cytokinins, there is a search for compounds with auxin-effect and cytokinin-effect activity. While compounds such as cytokinin and auxin promote growth by acting as plant hormones, other compounds can stimulate the synthesis of endogenous hormones in plants treated with extracts.

Auxin-like effect and cytokinin-like effect of microalgae extracts have been reported in the literature. Navarro-López and colleagues (2020) obtained extracts from hydrolyzed and non-hydrolyzed biomass, as and without cell disruption, with and without centrifugation, from *Scenedesmus obliquus*. The authors observed that the best auxin-like activity was found with 0.5 g L^{-1} of biomass extract with the

combination of cell disruption, hydrolysis and centrifugation. As for the cytokinin-like effect, the best result was obtained in the treatment with extracts obtained without cell rupture, with hydrolysis and centrifugation. Extracts from different species of Chlorophyta and Cyanophyta were evaluated for auxin-effect and cytokinin-effect activity. The results show that the extracts of the different microalgae species have similar cytokinin activities, however, little auxin-effect activity (STIRK et al., 2002). Tomato plants treated with aqueous extracts of the microalgae *Acutodesmus dimorphus* had an increase compared to untreated tomato plants. In addition, the authors note that only 50 g of extract was sufficient to increase the productivity (GARCIA-GONZALEZ; SOMMERFELD, 2016).

1.2 Antimicrobial activity

Ghasemi and colleagues (2007) used water, methanol and hexane to extract bioactive compounds from *Chroococcus dispersus*, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris*. The authors observed that, while the water and methanol extracts demonstrated to have antimicrobial activity, the hexane extract had no effect on the growth of fungi and bacteria (GHASEMI et al., 2007). Ethanol, methanol, butanol, acetone, DMSO and aqueous extracts of *Scenedesmus subspicatus* had different responses to the growth of *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Escherichia coli* (DANTAS et al., 2019). Only the extracts in DMSO have demonstrated antimicrobial activity against all three bacteria species. Extracts in acetone and water had antimicrobial activity against *B. subtilis* and *E. coli*, respectively. In addition, the highest antioxidant activity was obtained in the aqueous extract (DANTAS et al., 2019). Patil and collaborators (2019) obtained extracts from the microalgae *Scenedesmus bajacalifornicus* using chloroform, ethyl acetate, methanol, ethanol and water. The authors evaluated the extracts for anti-inflammatory, anti-diabetes, anti-microbial and antioxidant activity. The responses differed between the extracts, but the aqueous extract had the highest number of bioactive compounds (PATIL; KALIWAL, 2019). Some studies have been carried out in the search for new techniques for obtaining the extracts in order to reduce the

amount of solvents and the time used. One of these strategies is the use of pressurized liquid extraction (PLE). PLE extracts, using ethanol and or hexane, from the green and red biomass of the microalgae *Haematococcus pluvialis* were tested for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*. The results showed that the main compounds with antimicrobial activity are butanoic acid and methyl lactate and were found in the extracts using ethanol as a solvent (SANTOYO et al., 2009).

Epidemics of plant diseases mainly in situations where there are no barriers to control the spread of the pathogen and when there is loss of genetic diversity, as can be seen in monocultures. There are several agrochemicals with bactericidal, fungicidal, and insecticidal action, however these cause environmental problems and may pose a health hazard. Some studies show the potential of microalgae in agriculture, both indicating biostimulant activity and fungicidal activity against phytopathogen. Ördög and colleagues (2004) obtained aqueous extracts from different species of microalgae (Cyanobacteria and Chlorophyte) and evaluated them for biological activity against different species of filamentous fungi and bacteria, including phytopathogens. Extracts from two Chlorophyte, *Desmococcus olivaceus* and *Scenedesmus* sp., had bactericidal activity against *Pseudomonas syringae* and fungicidal activity against *Alternaria* sp., respectively (ÖRDÖG et al., 2004). Marine algae are known to be natural sources of bioactive compounds. Chanthini, Kumar, and Kingsley (2012) obtained extracts from six species of green and red seaweed with different solvents (methanol, ethyl acetate, and hexane) and tested them for fungicidal activity against *Alternaria solani*. The best results found were obtained from the extracts of *Enteromorpha flexuosa* and *Chaetomorpha antennina*. In addition, for some seaweed, only the extracts obtained with hexane had fungicidal results. The authors observed that the fungicidal activity of green algae extracts was better than that of red algae.

1.3 Antileishmania activity

Leishmania infantum is a protozoan transmitted by phlebotomine sandflies, with dogs as the main reservoirs in urban areas (OLIVEIRA et al., 2019). This disease causes canine leishmaniasis and can infect humans in cutaneous and visceral forms (PEREIRA et al., 2015). When it reaches the skin, leishmaniosis affects the macrophages present in the skin. However, a more severe form can affect the mononuclear phagocyte system of the intestine, lymph nodes, bone marrow, spleen and liver (STEVERDING, 2017). Currently, chemotherapy is the treatment used as a way to control leishmaniasis. However, the drugs used are administered for a long period, causing collateral effects and parasite resistance (YAMTHE et al., 2017). In addition, some drugs, such as pentamidine and amphotericin B are being hepatotoxic, nephrotoxic and cardiotoxic leading many to abandon treatment (MURRAY et al. (2005). Therefore, there is an urgent need for new improved therapies.

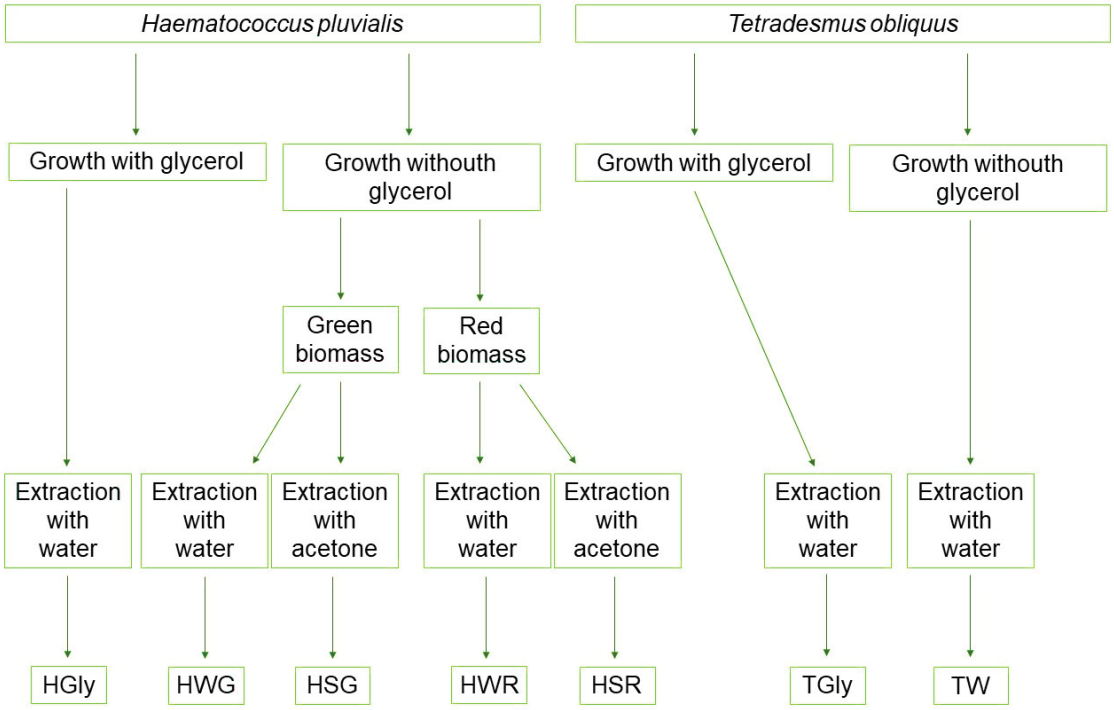
The literature data bring results of antileishmania activity of marine alga extracts. Extracts of five species of marine macroalgae from the Brazilian coast were obtained with solvents of low and medium polarity and tested for anti-leishmania activity. In vitro and in vivo tests were performed and satisfactory results were obtained from the extracts of *Osmundaria obtusiloba* (LIRA et al., 2016). Antileishmania activity of organic macroalgae extracts has been reported in the literature, but few studies have evaluated the potential of microalgae biomass extracts. Extracts of *Nannochloropsis* sp., *Picochlorum* sp., *Desmochloris* sp., and *Nannochloris* sp. were obtained by Pereira and colaborators (2015) using methanol as a solvent. Antileishmania activity was performed using *L. infantum* promastigote cells. After 48 hours, *Nannochloris* sp. extract was able to significantly reduce the cell viability of the promastigotes, reaching 62% compared to untreated cells (Pereira et al., 2015). These results show that further studies should be carried out using microalgae extracts.

The aim of this study was to evaluate the plant biostimulant activity, antimicrobial, and anti-leishmania of extracts obtained by aqueous or acetone extraction from the microalgae *Haematococcus pluvialis* and *Tetradesmus obliquus*.

2. Material and Method

2.1 Preparation of microalgal extracts

FIGURE 1.3 – ILLUSTRATIVE SCHEME OF OBTAINING THE EXTRACTS OF THE MICRO-ALGAE *H. pluvialis* AND *T. obliquus*



FONT: THE AUTHOR (2020)

To obtain the extracts, dry biomass of *H. pluvilais* and *T. obliquus* produced under different conditions was used. *H. pluvialis* was grown in synthetic medium (mg L^{-1}: $NaNO_3$, 250; $CaCl_2$ $2H_2O$, 25; $MgSO_4$ $7H_2O$, 75; K_2HPO_4, 75; KH_2PO_4, 175; NaCl, 25; EDTA, 50; $FeSO_4$ $7H_2O$, 4.98; H_3BO_3, 11.3; $ZnSO_4$ $7H_2O$, 14.12; $MnSO_4$ $4H_2O$, 1,96; Na_2MoO_4 $2H_2O$, 1.92; $CuSO_4.5H_2O$, 2.52; $CaCl_2$ $6H_2O$, 1.44) and *T. obliquus* in CHU medium (ABNT, 2011) in 2 L Erlenmeyers flasks with 1.6 L working volume. The cultures were illuminated by 100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ with or without the addition of glycerol (1 g L^{-1} for *H. pluvialis* and 5 g L^{-1} for *T. obliquus*) for 10 days. In order to induce the synthesis of astaxanthin in *H. pluvialis* maintained without glycerol, the cultures were centrifuged and resuspended in a nitrogen-free medium and kept at an illumination of 200 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ for 10 days. Two solvents were utilized to extract compounds with different polarities. Thus, 10 mL of

water or acetone were added in 1 gram of dry biomass and kept at room temperature under stirring for 2 hours, intercalate for a period at 4 °C overnight. After extraction, the solvents were removed at low pressure on a rotary evaporator. The extracts were lyophilized and then solubilized in water or acetone at a concentration of 1 mg mL^{-1}. Figure 1 illustrates schematically the obtention the extracts and identifying each one.

2.2 Quantification of carbohydrates and proteins of extracts

Carbohydrates and proteins present in the extracts were measured by spectrophotometry. To determine total carbohydrates, the phenol sulfuric method was used (DUBOIS et al., 1956), using glucose to produce the standard curve. Proteins were quantified using the Bradford assay (BRADFORD, 1976) and BSA was used to generate the standard curve.

2.3 Biostimulant potential of the microalgae extracts

2.3.1 Rooting induction for auxin-like effect.

To assess the auxin-like effect, commercial seeds of *Cucumber sativus* were added. The tests were performed according to the methodology proposed by (Zhao and colleagues, 1992). Initially, the cucumber seeds were germinated in plates containing 7% agar-water. The plates were kept at 25 °C in the dark for 3 days. After the germination period, the cotyledons were excised and transferred to 60 mm Petri dishes containing 3 mL of extracts or indole acetic acid, used to make the standard curve, or water for negative control. Each plate received 10 cotyledons and were kept at 25 °C for five days. The average number of roots for each treatment was compared with the standard curve.

2.3.2 Expansion test for cytokinin-like effect

To assess the cytokinin-like effect, commercial seeds of *Cucumber sativus* were utilized. The tests were performed according to the methodology proposed by Zhao and colleagues (1992). The cucumber seeds were germinated as described in item 2.3.1. After the germination period, the cotyledons were excised and transferred to 60 mm Petri dishes containing 3 mL of microalgal extracts (1 mg mL^{-1}) or zeatin, used to make the standard curve, or water for negative control. Each plate received 10 cotyledons and were kept at 25 °C for three days. Cotyledon mass was measured before and after the incubation period. The average value of the cotyledons was compared with the standard curve.

2.4 Antimicrobial activity of extracts

Antimicrobial activity from *H. pluvialis* and *T. pluvialis* extracts was evaluated against two bacteria species, one Gram-positive (Vancomycin Resistant Enterococcus – VBE) and Gram-negative (*Acinetobacter baumannii*). For this purpose, the recommended broth microdilution in microplate method was used to determine the minimum inhibitory concentration (MIC). VBE and *A. baumannii* grew in LB medium at 37 °C overnight. The cultures were diluted in saline and adjusted to 0.5 on the McFarland scale and diluted in LB medium to achieve a concentration of 10^6 CFU mL^{-1}. 50 µL of LB medium was added from 1^{st} to 12^{th} well, then 100 µL of extracts or antibiotics was added to 2^{nd} well. From 2^{nd} well, a serial double dilution was performed until reaching 11^{th} well, always transferring 50 µL to the next well. Upon reaching 11^{th} well, 50 µL was discarded, as 12^{th} well was used as growth control with the addition of microorganisms, but without the addition of extract or antibiotic. 1^{st} well was used as a control medium, without the addition of microorganisms. The final concentrations of extracts vary from 6.7 mg mL^{-1} to 0.11 µg mL^{-1}. After addition of extracts or antibiotics, 50 µL of bacteria was added from 2^{nd} to 12^{th} well. After 18 hours, 30 µL of resazurin (150 µg mL^{-1}) was added to every well

and the microplate was incubated for one hour at 37 °C. Change in color from purple to pink, indicates that there was no inhibition of bacterial growth.

2.5 Antileishmania activity of extracts

For the performance of the tests, the promastigote form of *Leishmania infantum* provided by the Carlos Chagas Institute - Paraná was used. The promastigote forms were grown in Schneider medium at 25 °C and 5% CO_2. The tests were carried out in a 96-well microplate, in which 180 µL of medium containing the parasites were added in each well at a concentration of 4.5 10^7 cells. 20 µL of extracts or Amphotericin B with different concentrations were added to the wells, with the exception of the control wells. The plates were kept at 25 °C with 5% CO_2 for 24 hours. After incubation, 50 µl of MTT solution (10 mg/mL) was added to each well. The plates were kept at 37 °C for 4 hours. Then, the plates were centrifuged at 1700 rpm for 10 min. To solubilize the formazam crystals, 50 µL of DMSO was added to each well and kept at 37 °C for 10 minutes. The plate readings were performed on a multi-reading spectrophotometer using a wavelength of 550 nm. The results were represented as a percentage of live parasites in relation to the untreated negative control.

2.6 Statistical analysis

All experiments were performed in triplicate and the results are shown as the average. For statistical analysis, one-way analysis of variance was used, followed by the Tukey test ($p < 0.05$).

3 Results and Discussion

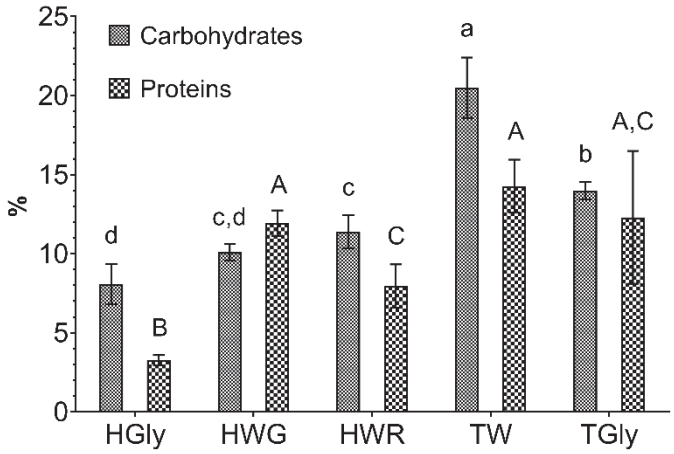
3.1 Carbohydrate and proteins contents in microalgal extracts

The content of carbohydrates and proteins were measured (Fig. 2.3) in each extract, since it has been reported that proteins and carbohydrates have a bioestimulant effect on vegetables and shows antimicrobial activity (MICHALAK; CHOJNACKA, 2015).

The aqueous extract obtained from the biomass of *T. obliquus* grown without the addition of glycerol (TW) showed the highest carbohydrate content, with 20%. While the protein content obtained in TW was 14%. On the other hand, the aqueous extract obtained from the *H. pluvilais* biomass cultivated with glycerol (HGly) yielded only 3% of proteins, a significantly lower content compared to the others extracts. HGly extract also had the lowest carbohydrate content, with 8%. A possible explanation for this low yield is the increase in the thickness of *H. pluvilais* cells when flagella loss occurs (ZHANG et al., 2017). This increase in thickness would, therefore, make it difficult to extract the compounds, requiring a longer extraction time or a strategy that allows cell disruption. However, the levels of carbohydrates and proteins in the extract obtained from the *H. pluvialis* red biomass grown without the addition of glycerol (HWR) were not as low as the values obtained in HGly extracts, which would explain a low content of these compounds in these biomasses. The biomass of *T. obliquus* cultivated with the addition of glycerol (TGly) yielded the extract with the second highest carbohydrate content, with 14%. While the protein content was 12%.

The protein content found in the extracts of *H. pluvialis* and *T. obliquus* did not reach values already reported in the literature. Navarro-López and colleagues, 2020) obtained 35% of proteins by cultivating *Scenedesmus obliquus*, however, the authors obtained the extracts through enzymatic hydrolysis and cell disruption, while in our study we chose to perform an aqueous extraction, as it is a low-cost method that produces an extract with wide applicability. Furthermore, the objective was to extract mainly polysaccharides.

FIGURE 2.3 – CONTENT OF CARBOHYDRATES AND PROTEINS FOUND IN THE MICRO-ALGAE EXTRACTS



LEGEND: Content of carbohydrates and proteins found in the extracts: HGly (aqueous extracts obtained green biomass of *H. pluvialis* cultivated with glycerol), HWG (aqueous extracts obtained green biomass of *H. pluvialis* cultivated without glycerol), HWR (aqueous extracts obtained red biomass of *H. pluvialis* cultivated without glycerol), TW (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol), and TGly (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol). The results shown are the averages ± SD. Same lowercase letters indicate that there is no significant difference in carbohydrate content ($p < 0.05$). Equal capital letters indicate that there is no significant difference between protein levels ($p < 0.05$).

3.2 Plant growth bioestimulant activity

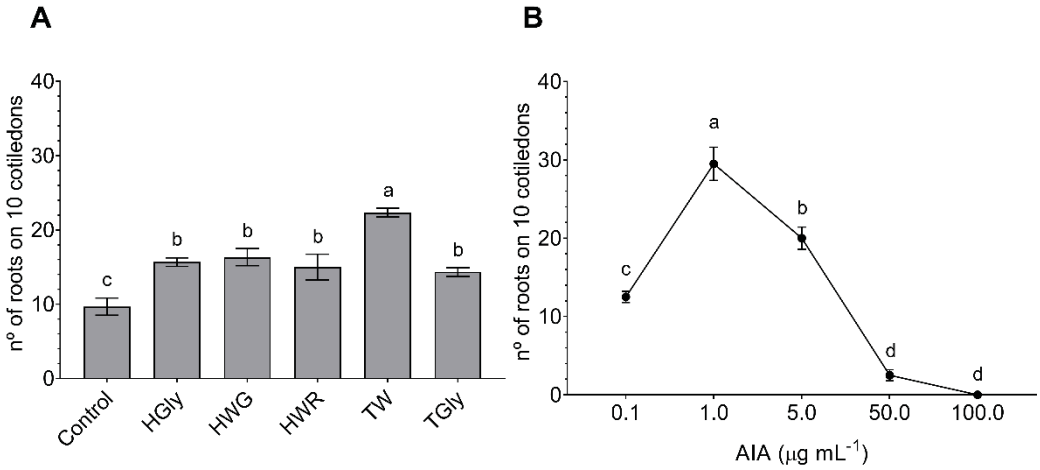
3.2.1 Auxin-like effect

The assays for auxin-like activity were performed on cucumber seeds for five days. Our results showed that all extracts induced root formation, with significantly higher results compared to the control (only water) (Fig 3.3). The largest number of roots was found in cotyledons treated with TW extract formed an average of 22 ± 0.6 roots. This value corresponds to 5 mg L^{-1} of auxin according to the reference curve with indole acetic acid (AIA) (Fig. 3.3A). Interestingly, TW is the extract with the highest carbohydrate content (Fig 2.3). Some studies show that polysaccharides and oligosaccharides can induce plant growth. Sairanen and collaborators (SAIRANEN et al., 2012) showed that the addition of exogenous glucose to seedlings of *Arabidopsis* increased the concentration of AIA in the plant. In addition, the accumulation of

endogenous AIA increased as the exogenous glucose content increased. The authors also show that the addition of exogenous glucose increased the level of gene transcripts related to the synthesis of AIA. Auxin is a plant growth regulator phytohormone with an important role in root induction (Navarro-López et al., 2020). Therefore, we believe a synergistic effect between molecules with auxin activity and total high carbohydrate content present in TW extract, induced the root formation and, possibly, the synthesis of endogenous auxin in cucumber cotyledons.

Navarro-López and collaborators (2020) found similar results with microalgae extracts obtained using different strategies. The authors used extract of *Scenedesmus obliquus* in cucumber seeds to evaluate the auxin-like effect and observed that cell disruption, followed by enzymatic hydrolysis, produced extracts capable of inducing root formation in 141,9 ±4.6% in relation to control.

FIGURE 3.3 – AUXIN-LIKE ACTIVITY FROM MICRO-ALGAE EXTRACTS

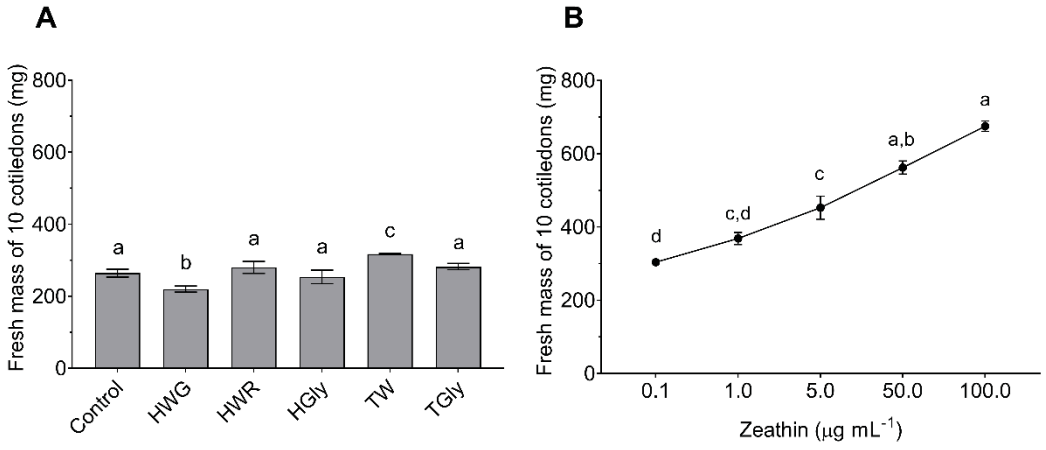


LEGEND: (A) Auxin-like activity of extracts: HGly (aqueous extracts obtained green biomass of *H. pluvialis* cultivated with glycerol), HWG (aqueous extracts obtained green biomass of *H. pluvialis* cultivated without glycerol), HWR (aqueous extracts obtained red biomass of *H. pluvialis* cultivated without glycerol), TW (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol), and TGly (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol).
(B) Reference curve made using indole acetic acid (AIA).
NOTE: The results shown are the averages ± SD. Same lowercase letters indicate that there is no significant difference ($p < 0.05$).

3.2.2 Cytokinin-like effect

The extracts obtained from the *H. pluvialis* and *T. obliquus* biomass were also evaluated for cytokinin-like effect activity, using cotyledons of cucumber seeds. Cotyledons had their masses measured on the first and last day of the test. Figure 5 shows the results obtained in this test and the reference curve using zeatin. Observed that the only extract with cytokinetic activity was TW, obtained by aqueous extraction of the biomass of *T. obliquus* grown without glycerol. At the end of three days, the cotyledons treated with the TW extract reached a mass of 318 mg, result is equivalent to 0.1 μg mL^{-1} zeatin (Fig. 5). In view of these results, three possibilities can be considered, the aqueous extracts of *H. pluvialis* and *T. obliquus* did not extract any compounds with cytokinin effect, or the extracted compounds are in a very low concentration, or, the extracts has some compound with inhibitory activity on plant growth. This inhibitory effect was also observed by Navarro-Lopes and colleagues Navarro-López et al. (2020), although some extracts have shown cytokine-type activity. The authors observed that extracts from *S. obliquus* at concentrations of 2 g L^{-1} there was inhibition of the increase in the mass of cucumber cotyledons. In addition, some authors have reported that seaweed extracts may contain compounds with an inhibitory effect on plant growth, especially abscisic acid Niemann; Dörffling (1980).

FIGURE 4.3 – CYTOKININ-LIKE ACTIVITY FROM MICRO-ALGAE EXTRACTS



LEGEND: (A) Cytokinin-like activity of extracts: HGly (aqueous extracts obtained green biomass of *H. pluvialis* cultivated with glycerol), HWG (aqueous extracts obtained green biomass of *H. pluvialis* cultivated without glycerol), HWR (aqueous extracts obtained red biomass of *H. pluvialis* cultivated without glycerol), TW (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol), and TGly (aqueous extracts obtained biomass of *T. obliquus* grown without glycerol).
(B) Reference curve made using zeatin.
NOTE: The results shown are the averages ± SD. Same lowercase letters indicate that there is no significant difference ($p < 0.05$).

Our results indicate that *H. pluvialis* and *T. obliquus* extracts at a concentration of 1 mg mL^{-1} have auxin activity, however, it is important to highlight that different extraction strategies must be evaluated, especially in the *H. pluvialis* biomass.

3.3 Antimicrobial and antileishmania activity

The extracts of *H. pluvialis* and *T. obliquus* were evaluated for antimicrobial activity against one Gram-positive bacteria, Vancomycin-Resistant Enterococcus (VRE) and one Gram-negative, *Acinetobacter baumannii*, and antilheismania activity. For the assay, the extracts HWG, HWR, HSG, HSR, HGly, TW, and TGly were used. The concentrations of the extracts were adjusted to 10 mg mL^{-1} and the final concentrations in the plates ranged from 6.7 mg mL^{-1} to 0.1 µg mL^{-1}

The concentrations of extracts tested in this study, did not show antileishmania or antimicrobial activity. A possible explanation for this observation is the low concentration of the extracts used in the tests, since antimicrobial activity of *H. pluvialis* extracts has already been reported in the literature. In addition, obtaining the extracts using water at room temperature may have limited the content of extracted compounds. The extraction performed with acetone, on the other hand, may have extracted compounds of interest, but these were solubilized in low concentration (10 mg mL^{-1}). Literature data that reported antimicrobial activity of *H. pluvialis* extracts, used hexane and ethanol as solvents, in addition the authors varying the extraction temperature from 50 °C to 200 °C Santoyo et al. (2009). In addition, the concentration of the extracts varied from 10 mg mL^{-1} to 250 mg L^{-1}

Santoyo et al. (2009). In a study with *Scenedesmus subspicatus*, the authors used ethanol, methanol, butanol, acetone, DMSO, and water to obtain the extracts Dantas et al. (2019). However, only the extracts obtained with the last three solvents showed some antimicrobial activity. It is worth noting that the methodology used to obtain the extracts was similar to that used in the present study.

Furthermore, the microorganisms used in this study*, A. baumannii* and ERV, are strains nosocomial and have resistance to several antibiotics. Strains of bacteria with multiple drug resistance have what is called intrinsic resistance (IR) (MUNITA; ARIAS, 2016). This IR is based on the production of enzymes that can eliminate the antimicrobial compound or prevent its binding to the target site, even without previous contact with the compound (D'COSTA et al., 2011). The strains used in this study show phenotypes with resistance to multiple drugs promoted by enzymatic and non-enzymatic mechanisms. Resistant strains of *A. baumannii* use both mechanisms. The enzyme mechanism is characterized by the ability to hydrolyze molecules with β-lactam rings, for example (MUNITA; ARIAS, 2016). On the other hand, the non-enzymatic mechanisms found in *A. baumannii* are characterized by the activation of efflux pumps (CHEN et al., 2017), decreased membrane permeability (LEE et al., 2017), and change in the target site, such as, for example, the fluoroquinolone resistance mechanism developed by a spontaneous mutation in three genes encoding gyrases and topoisomerase IV) (VRANCIANU et al., 2020). The strain of *Enterococcus* used in this study has resistance to vancomycin. However, resistance to vancomycin can evolve with other resistance mechanisms, including decreased cell permeability to various antibiotics (LEVITUS; REWANE; PERERA, 2020). The different mechanisms of resistance of *A. baumannii* and VRE, as well as the transfer and acquisition of determinants of resistance to antibiotics, hinder the work of selecting new compounds with antimicrobial activity.

Therefore, new strategies must be employed to obtain the extracts of *H. pluvialis* and *T. obliquus*. In addition, higher concentrations of the extracts should be used to perform the antimicrobial tests.

4. Conclusions

This was the first study to evaluate the effect of *H. pluvialis* extract on root formation and on the increase of cucumber cotyledon mass. In addition, extracts of *T. obliquus* and *H. pluvialis* were also evaluated for antimicrobial activity. Our results showed that the *T. obliquus* extract containing 20% of carbohydrates induced the largest number of roots and provided the greatest increase in the cotyledon mass, values equivalent to 5 μg mL^{-1} auxin and 0.1 μg mL^{-1} zeatin. *H. pluvialis* extracts, on the other hand, showed auxin activity, but no cytokinin activity was observed. The authors did not observe any antimicrobial or antileishmania activity in the extracts obtained with water or acetone from *H. pluvialis* and *T. obliquus* biomass. These results reinforce the authors' hypothesis that the extracts were used in a low concentration. Thus, new extraction strategies and new extract concentrations must be evaluated.

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CONCLUSÕES GERAIS

Nossos resultados mostram que a utilização de 20 $\mu mol_{fótons}\ m^{-2}\ s^{-1}$ de luz azul em conjunto com a privação de nitrogênio é uma estratégia que promove altos ganhos de astaxantina. A microalga *H. pluvialis* produziu 8 vezes mais astaxantina nos cultivos iluminados com luz azul quando comparada com os cultivos em luz branca. Da mesma maneira, a análise dos transcritos de genes carotenogênicos indicou que a abundância de cada gene foi maior nos cultivos com luz azul. Enquanto o número de transcritos de PII foi significativamente maior nos cultivos mantidos em luz branca, o nível de transcritos de *phot* foi similar nos cultivos mantidos em luz azul e vermelha. No entanto, não foi encontrado domínios de receptor de luz vermelha no gene phot. Esses resultados nos permitem inferir que a luz vermelha pode induzir a expressão do gene *phot* de maneira indireta. Em relação à composição bioquímica, não foi encontrada diferença no acúmulo de lipídeos. Por outro lado, os cultivos em luz branca produziram o maior teor de proteínas, alcançando 32%, enquanto os cultivos em luz vermelha acumularam o meio teor de carboidratos, chagando a 29%.

Por outro lado, a adição de 1 g L^{-1} de glicerol induziu o acúmulo de lipídeos, carboidratos e astaxantina em cultivos mixotróficos *H. pluvialis*. Porém, concentrações de glicerol de 5 e 10 g L^{-1} inibiram o crescimento. Já nos cultivos de T. obliquus, a melhor concentração de glicerol foi de 5 g L^{-1} e proporcionou uma produção de 1 g L^{-1} de biomassa com 23% de lipídeos.

Os resultados mostraram que os extratos obtidos de biomassas de *H. pluvialis* e *T. obliquus* cultivadas sob diferentes condições possuem diferentes teores de carboidratos e proteínas com diferentes respostas frente à atividade bioestimulante. O extrato que induziu o maior número de raízes e maior expansão celular foi obtido com água da biomassa de *T. obliquus* cultivada sem glicerol (TW). Infelizmente, os extratos de *H. pluvialis* e *T. obliquus,* nas concentrações variando de 6,7 mg mL^{-1} a 0,1 µ mL^{-1}, não inibiram o crescimento de bactérias. Embora o extrato TW tenha respondido com sucesso como bioestimulante de crescimento vegetal, esses resultados indicam que novas estratégias de extração e concentrações maiores de extratos devem ser avaliadas.

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