LISA MAYLIN SCHÜLER

IMPROVEMENT OF MICROALGAL BIOMASS USING PHYSIOLOGICAL AND GENETIC APPROACHES

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Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo Ciências Biológicas

Especialidade em Biotecnologia

Trabalho efetuado sob a orientação de:

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Lisa Maylin Schüler)

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Knowledge dissemination

Publications in indexed scientific journals

1. Schüler, L.M., Schulze, P.S.C., Pereira, H., Barreira, L., León, R. Varela, J. 2017. Trends and strategies to enhance triacylglycerols and high-value compounds in microalgae. Algal Research 25: 263–273. DOI: 10.1016/j.algal.2017.05.025

2. Schüler, L.M., Santos, T., Pereira, H., Duarte, P., Katkam, G.N., Florindo, C., Schulze, P.S.C., Barreira, L., Varela, J. 2020. Improved production of lutein and *β*-carotene by thermal and light intensity upshifts in the marine microalga *Tetraselmis* sp. CTP4. Algal Research 45 DOI: 10.1016/j.algal.2019.101732

3. Schüler, L.M., Katkam, G.N., Duarte, P., Pacines, C., Márquez, A.M.M., Léon-Bañares, R., Varela, J., Barreira, L. 2020. Improvement of carotenoid extraction from a recently isolated, robust microalga, *Tetraselmis* sp. CTP4 (Chlorophyta). Bioprocess and Biosystems Engineering DOI: 10.1007/s00449-019-02273-9

4. Schüler, L., Greque de Morais, E., Trovão, M., Machado, A., Carvalho, B., Carneiro, M., Maia, I., Soares, M., Duarte, P., Barros, A., Pereira, H., Silva, J., Varela, J. 2020. Isolation and characterization of novel *Chlorella vulgaris* mutants with low chlorophyll and improved protein contents for food applications. Frontiers in Bioengineering and Biotechnology 8:469 DOI: 10.3389/fbioe.2020.00469

5. Schüler, L.M., Bombo, G., Duarte, P., Santos, T.F., Maia, I.B., Pinheiro, F., Jacinto, R., Schulze, P.S.C., Pereira, H., Barreira, L., Varela, J. 2021. Carotenoid biosynthetic gene expression, pigment and *n*-3 fatty acid contents in carotenoid-rich Tetraselmis striata CTP4 strains under heat stress combined with high light. Manuscript submitted to Bioresource Technology

Congress participations with presentations

1. Schüler, L.M., Schulze, P.S.C., Katkam, G., Pereira, H., Barreira, L., Varela, J. 2017. A novel *Tetraselmis* sp. CTP4 as promising candidate for wastewater treatment. EUALGAE Training School, Almería, Spain, Oral presentation

2. Schüler, L.M., Duarte, P., Márquez, A.M.M., Léon-Bañares, R., Katkam, G.N., Varela, J., Barreira, L. 2018. Optimization of carotenoid extraction from a stress-resistant, lipid-rich chlorophyte, *Tetraselmis* sp. CTP4. AlgaEurope 2018, Amsterdam, Netherlands, Poster presentation

3. Schüler, L.M., Duarte, P., Barreira, L., Varela, J. 2019. Improvement of lutein and *β*-carotene production by abiotic growth factors in a robust marine microalga *Tetraselmis* sp. CTP4. EUROCAROTEN international conference, Lemesos, Cyprus, Oral presentation

4. Schüler, L.M., Duarte, P., Barreira, L., Varela, J. 2019. Isolation of carotenoid-hyperproducing mutants of *Tetraselmis* sp. CTP4 by fluorescence-activated cell sorting. AlgaEurope 2019, Paris, France, Poster presentation

5. Schüler, L.M., Duarte, P., Barreira, L., Varela, J. 2020. Carotenoid induction in *Tetraselmis* sp. CTP4 by abiotic stress. AlgaEurope 2020, E-Poster presentation

Thesis overview

General layout

This thesis is divided into seven chapters. It starts by providing background information on microalgae and lipophilic compounds in particular pigments, which is then followed by an extensive review about the induction of triacylglycerols, polyunsaturated fatty acids and carotenoids in microalgae with a discussion of the possible strategies for strain improvement. Afterwards, the development of the best extraction method of *Tetraselmis striata* CTP4 carotenoids is described. Optimal cultivation strategies to produce carotenoids by *Tetraselmis striata* CTP4 photoautotrophically were subsequently studied. Carotenoid-rich mutants were generated and the response of the carotenoid pathway in *Tetraselmis striata* CTP4 wild type and its mutants under different growth conditions was elucidated. Furthermore, strain improvement of the heterotrophically grown *Chlorella vulgaris* was performed. Finally, the overall outcome and future perspectives of this thesis are discussed.

Chapter-by-chapter outline

Besides providing information on commercial applications and production of microalgal pigments, a profound knowledge about the chemical features of pigments, their biosynthesis and functions is needed to improve pigment production in microalgae (Chapter 1). Moreover, the production of these lipophilic compounds is highly dependent on the microalgal species and environmental factors, which is reviewed in depth in Chapter 2. Both chapters highlight the importance of further improving microalgal strains, describing the methodologies available.

In Chapter 3, the best extraction of carotenoids of *Tetraselmis striata* CTP4 representing a microalga with a mechanically resistant cell covering (i.e., a theca) is described. Different cell disruption methods, extraction solvents and the suitability of the best extraction method for large scale applications are discussed. This methodology paved the way for the following experiments within the present thesis.

Accumulation of carotenoids in *Tetraselmis striata* CTP4 under different growth conditions was studied in Chapter 4. The effect of environmental factors such as light intensity, temperature, salinity and nutrient availability were addressed, pointing out the most important cues. A two-stage cultivation was proposed for best carotenoid production in this species. Moreover, as this microalga is known to produce high amounts of lipids, it was found that carotenoids did not accumulate under lipid-inducing conditions, unlike other microalgae such as *Dunaliella salina*.

The Chapters 5 and 6 comprise two different case studies of strain improvement using random mutagenesis and a strain selection on the herbicide norflurazon, namely the obligate

photoautotroph *Tetraselmis striata* CTP4 and the freshwater species *Chlorella vulgaris* grown under heterotrophic conditions, respectively. Carotenoid and EPA-rich mutants of *Tetraselmis striata* CTP4 were generated with a following enrichment by fluorescence activated cell sorting. Carotenoid biosynthesis under different growth conditions of wildtype and mutants and the expression of corresponding genes was studied in Chapter 5. However, in Chapter 6 chlorophyll-deficient mutants of *Chlorella vulgaris* with different colours were obtained and investigated in terms of growth, pigment and biochemical composition grown heterotrophically in the light and dark.

Finally, in Chapter 7, the overall work is discussed presenting the main conclusions in the strain improvement of *T. striata* (obligate photoautotroph) and *C. vulgaris* (non-obligate photoautotroph), proposing future research lines therewith.

Abstract

Microalgae are sustainable biological feedstocks of proteins, carbohydrates, and lipids rich in high-valuable carotenoids and *n*-3 fatty acids. To facilitate the application of microalgal biomass to food, feed and pharmaceutical markets, microalgal compounds extraction and production needs to be improved. In this thesis, carotenoid extraction was optimised using the euryhaline microalga *Tetraselmis striata* CTP4, which is known for its mechanical resistant cell covering. Glass bead-assisted disruption using wet biomass and acetone led to the best carotenoid recovery from this species. The same disruption method was afterwards successfully applied to the freshwater species *Chlorella vulgaris*. Thereafter, the carotenoid and EPA production of the obligate photoautotroph species *T. striata* was optimised by physiological and genetic approaches. The highest carotenoid content of 8.48 mg g^{-1} DW was achieved upon a thermal upshift from 20 to 35 °C under high light for only two days. The carotenoid profile contained high amounts of violaxanthin, lutein and *β*-carotene. However, upon random mutagenesis two norflurazon-resistant *T. striata* strains were isolated, displaying carotenoid contents of up to 10.2 mg g^{-1} DW. Comparative gene expression analysis of these mutants and the wildtype (CTP4) revealed the upregulation of several carotenogenesis-related transcripts in the novel strains. Interestingly, these mutants displayed also fatty acid profiles enriched in EPA. A similar genetic approach applied to *C. vulgaris* under heterotrophic growth resulted into chlorophyll-deficient mutants with high protein contents of up to 48.7% of DW. Additionally, the norflurazon-resistant strain of *C. vulgaris* showed a deficiency in carotenoid accumulation. Overall, this study revealed that *T. striata* is a promising candidate for carotenoids and EPA production. Moreover, the mutants of *C. vulgaris* broaden the potential of algal biomass for based food products. Furthermore, the study of randomly mutagenized strains provided further insights into the regulation of metabolic pathways, being a powerful tool for strain improvement.

Keywords: microalgae, pigments, polyunsaturated fatty acids, abiotic stress factors, random mutagenesis, FACS

Resumo

As microalgas são uma fonte rica e sustentável de proteínas, glícidos e lípidos, contendo ainda compostos de valor acrescentado, como carotenoides e ácidos gordos essenciais. Comparativamente às fontes tradicionais destes compostos (por exemplo, plantas superiores, frutas e animais), as microalgas apresentam diversas vantagens, uma vez que apresentam maiores produtividades sem necessidade de terreno arável. Além disso, muitas espécies poderem ser cultivadas durante todo o ano com recurso a água salgada ou mesmo residual diminuindo a pressão sobre os recursos de água doce. Apesar destas vantagens e da crescente procura por produtos naturais, apenas algumas espécies de microalgas são exploradas industrialmente para obtenção de compostos de elevado valor, como carotenóides e ácido eicosapentenóico (EPA). Contudo, para facilitar a aplicação da biomassa microalgal no mercado alimentar, rações e produtos farmacêuticos, tanto o processo de extração como de produção destes compostos necessitam ser melhorados.

Na presente dissertação, foi otimizada a extração de carotenóides na microalga eurialina *Tetraselmis striata* CTP4, reconhecida por apresentar uma cobertura celular resistente (teca). Um factor determinante para uma extração de rendimento elevado foi a aplicação de um método de disrupção celular. O método de rutura com a melhor recuperação de carotenóides para esta espécie foi com a utilização de biomassa húmida com recurso a esferas de vidro e acetona como solvente. Embora este método tenha sido desenvolvido para a análise e quantificação de carotenóides à escala laboratorial, o possível aumento de escala do processo para instalações industriais teve relevância na tomada de decisão em relação aos parâmetros da extração, nomeadamente na seleção de solventes, método de disrupção celular e pré-tratamento de biomassa. O mesmo método de disrupção foi posteriormente aplicado à espécie de água doce *Chlorella vulgaris*, também conhecida por ter uma parede celular de dupla camada.

Posteriormente, procedeu-se à otimização da produção de carotenoides para a espécie obrigatória fotoautotrófica *T. striata* CTP4 usando uma abordagem fisiológica. O objetivo final foi avaliar o uso desta espécie como *produtor-triplo,* ou seja*,* capaz de acumular simultaneamente carotenoides, triacilgliceróis (TAGs) e ácidos gordos polinsaturados de cadeia longa (LC-PUFA). Embora a depleção de azoto tenha demonstrado aumentar o conteúdo lipídico, o fator decisivo para a acumulação de carotenoides nessa espécie foi a reposição de azoto. Nessas condições, o teor mais elevado de carotenoides (8,48 ± 0,47 mg g $^{-1}$ Peso Seco) foi obtido com imposição de luz elevada, 170 µmol m^{-2} s^{-1}, e uma mudança térmica de 20°C para 35°C, durante dois dias. No perfil de pigmentos, detetaram-se violaxantina, luteína e *β*-caroteno com valores máximos de 1,64 ± 0,08; 3,17 ± 0,18 e 3,21 ± 0,18 mg g^{-1} PS, respetivamente. A luteína foi identificada nas células de *T. striata* CTP4 em

crescimento ativo como um pigmento fulcral na obtenção de luz e na fotoproteção. Além disso, verificou-se que o composto EPA se encontrava aumentado em células cultivadas em condições mesofílicas comparativamente às células sujeitas a *stress*, sendo responsável por 3,83 ± 0,21% do total de ácidos gordos. Resumindo, na espécie em estudo, o EPA e os pigmentos usados na captação de luz, como a violaxantina e o *β*-caroteno, acumulam-se quando sujeitas a cultivos de intensidades de luz e temperaturas mais baixas. Contrariamente, os corpos lipídicos, provavelmente compostos por TAGs, e os pigmentos fotoprotetores tal como a luteína, zeaxantina e *β*-caroteno, aumentam em condições consideradas de *stress*.

Paralelamente, foi melhorado o conteúdo em carotenoides para a estirpe *T. striata* CTP4, recorrendo ao método de mutagénese aleatória e combinado com a seleção de células ativadas por fluorescência (FACS) e crescimento em norflurazon. Foram isolados dois mutantes resistentes ao norflurazon, ED5 e B11, com valores mais elevados de carotenoides com resultados 1,5 vezes superiores ao do controlo, com conteúdos máximos de 10,2 ± 0,4 mg g^{-1} PS. Devido à resistência desses mutantes ao herbicida norflurazon, esperava-se um efeito visível na expressão do gene que codifica a fitoeno-dessaturase. De facto, verificou-se que os níveis de transcrição de outros genes envolvidos na via biossintética dos carotenóides encontram-se significativamente afetados em ambos os mutantes, independentemente das condições de crescimento. Mais especificamente, em condições de *stress*, os níveis de transcrição dos genes *PSY* e *PDS*, que codificam a fitoeno sintase e a fitoeno dessaturase encontram-se aumentados em 1,9 e 2,0 vezes, respetivamente, em células ED5 quando comparadas com o tipo selvagem (WT). Nas mesmas condições, os níveis de transcrição de *PSY* e *PDS* também sofreram uma regulação positiva no mutante B11. O conteúdo de clorofila em ambos os mutantes foi até 60% inferior ao do WT, sugerindo um aumento da sensibilidade destas células à luz. É de destacar, também, que os perfis de ácidos gordos desses mutantes mostraram um aumento em EPA em comparação com o WT, atingindo níveis até 8,7% do total de ácidos gordos. As mudanças observadas em diferentes classes de metabólitos sugerem, assim, que as mutações geradas têm um efeito pleiotrópico no metabolismo geral das células.

Uma abordagem genética semelhante à aplicada à *T. striata* CTP4 foi usada para melhorar a biomassa da espécie de *C. vulgaris* em crescimento heterotrófico. Após mutagénese química, uma estirpe amarela (MT01) e uma branca (MT02) foram isoladas representando uma diminuição de 80% e 99% no conteúdo total de clorofila e em comparação com a espécie selvagem. A cor amarela do MT01, no escuro, deveu-se principalmente à presença de luteína e na estirpe MT02 de *C. vulgaris* foi detetada uma deficiência na acumulação de carotenoides, com identificação de apenas o carotenoide incolor fitoeno.

Notavelmente, ambos os mutantes MT01 e MT02 registaram um teor de proteína 30% e 60% superior ao WT, atingindo 39,5% e 48,7% PS, respetivamente. Os resultados sugerem que a anulação parcial da biossíntese de pigmentos é um fator que pode promover o incremento dos teores de proteína nesta espécie. Tal está provavelmente relacionado com a alteração da regulação de compostos usados para o armazenamento de azoto através, por exemplo, de um desvio para proteína de compostos azotados usados na biossíntese de clorofila.

Em geral, este estudo revelou que estirpes melhoradas de *T. striata* CTP4 são promissoras para a produção de carotenoides e outros compostos considerados de alto valor, com aplicações nas indústrias nutra- e farmacêutica. No entanto, a redução da intensidade de cor verde e aumento do teor de proteína dos mutantes de *C. vulgaris* amplificam o potencial dos produtos alimentares à base de algas, como ingrediente análogos da carne. Curiosamente, o sucesso de qualquer mutagénese aleatória é altamente dependente da genética da espécie e do procedimento de seleção, pelo que esta tese fornece evidências adicionais que o desenvolvimento por meios genéticos de estirpes de microalgas biotecnologicamente relevantes é uma ferramenta muito poderosa tanto para reunir conhecimento para o melhoramento de estirpes, como para tornar produtos à base de microalgas economicamente sustentáveis.

Palavra chave: microalgas, pigmentos, ácidos gordos polinsaturados, fatores de stresse abiótico, mutagénese aleatória, FACS.

Table of Contents

List of abbreviations

AA	arachidonic acid
Ac	acetone
ACCase	acetyl-CoA carboxylase
ALA	α-linolenic acid
AMD	age-related macular degeneration
ANOVA	analysis of variance
ASE	accelerated solvent extraction
BHT	butylated hydroxytoluene
BKT	*β*-carotene ketolase
CAGR	compound annual growth rate
Car	carotenoids
CDB	conjugated double bonds
Chl	chlorophyll
CHYB	*β*-carotene hydroxylase
CYP97A5	cytochrome P450 *β*-ring hydroxylase
CYP97C3	cytochrome P450 *ε*-ring hydroxylase
DAG	diacylglycerol
Des	desaturase
DGAT	diacylglycerol acyltransferase
DGLA	dihomo-*γ*-linolenic
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DW	dry weight
Elo	elongase
EMS	ethyl methane sulfonate
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ETA	eicosatetraenoic
EtOH	ethanol
FACS	fluorescence activated cell sorting
FAME	fatty acid methyl esters
FAS	fatty acid synthase
G3P	glycerol-3-phosphate
GGPP	geranylgeranyl pyrophosphates

GLA	*γ*-linolenic acid
GMO	genetically modified organism
GPAT	glycerol-3-phosphate acyltransferase
GRAS	generally recognized as safe
HL	high light
HPH	high pressure homogenisation
HPLC	high pressure liquid chromatography
HS	high salinity
HT	high temperature
IBD	inflammatory bowel disease
IPP	isopentenyl pyrophosphate
LA	linoleic acid
LC-PUFA	long-chain polyunsaturated fatty acids
LCYE	*ε*-lycopene cyclase
LD	lipid droplets
LHC	light harvesting complex
LL	low light
LPA	lysophosphatidate
LS	low salinity
LT	low temperature
LYCB	lycopene *β*-cyclase
MAE	microwave-assisted extraction
MeOH	methanol
MEP	2-C-methyl-D-erythriol 4-phosphate
MNNG	*N*-methyl-*N*-nitro-*N*-nitrosoguanidine
MUFA	monosaturated fatty acids
ND	nitrogen depletion
NR	nitrogen repletion
NSY	neoxanthin synthase
PA	phosphatidic acid
PBR	photobioreactor
PC	phosphatidylcholine
PD	phosphorus depletion
PDS	phytoene desaturase
PETC	photosynthetic electron transport chain
PG	phosphatidylglycerol

PSY	phytoene synthase
PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP	reverse phase
SDA	stearidonic acid
SFA	saturated fatty acids
SFE	supercritical fluid extraction
TAG	triacylglycerol
TAGL	triacylglycerol lipase
TFA	total fatty acids
THF	tetrahydrofuran
UAE	ultrasound-assisted extraction
USD	United States dollar
UV	ultraviolet
VDE	violaxanthin de-epoxidase
Vis	visible
WT	wildtype
WW	wet weight
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase
Z-ISO	ζ-carotene isomerase

CHAPTER 1

General Introduction: Microalgae and pigments

1.1 Microalgae as natural source of lipophilic compounds

1.1.1 Microalgae

Microalgae are unicellular or colonial microscopic eukaryotic organisms, belonging to highly different phylogenetic clades. As primary producers, microalgae are ubiquitous across most environments, including extreme habitats such as salt ponds, acidic mine waters, deserts or the Antarctic ice (Brock, 1975; Kim et al., 2018; Seckbach, 1995). Because of their great adaptability to abiotic stress, some species are eurythermal and/or euryhaline, thus growing at different temperatures and/or in both seawater and freshwater, respectively. Microalgae can grow photosynthetically by converting the energy of sunlight to produce biomass and oxygen, while consuming CO_2, water, and nutrients, including nitrates, phosphates and iron. Most microalgae are obligate photoautotrophs and are unable to grow in the dark. Possible hypothesis for this is the lack of efficient uptake of substrates (e.g., lack of glucose transporters) and incomplete pathways or the absence of an enzymatic reaction in the central carbon metabolism (Chen and Chen, 2006). However, some microalgal species can grow hetero- or mixotrophically, displaying properties similar to those of non-photosynthetic microorganisms. Heterotrophic microalgae most probably evolved in light-limiting environments and are able to metabolize different carbon sources (Neilson and Lewin, 1974). Species capable of growing both hetero- and photoautotrophically have been reported in the genus *Chlorella*, though some studies describe other microalgae such as *Tetraselmis suecica* and *Neochloris oleoabundans* as being able to have both modes of nutrition as well (Azma et al., 2011; Hu et al., 2018; Morales-Sánchez et al., 2013). Because of their high biodiversity, microalgae produce a great variety of compounds, which enable them to survive and even thrive in a wide range of habitats. These compounds include proteins, lipids, polysaccharides, pigments and other bioactive compounds which can be used for several applications in the food, feed and fuel sectors (Walker et al., 2005). Therefore, microalgae are considered to be a promising natural and sustainable source of these compounds displaying several advantages over higher plants, which are traditionally exploited for these compounds. When euryhaline or marine microalgae are used, they do not compete with food production for freshwater and agricultural land (Gouveia, 2014). Furthermore, the areal and photosynthetic productivities of microalgae exceed those of terrestrial plants by up to 20 and 10-15 times, respectively, contributing to the overall carbon dioxide capture (Williams and Laurens, 2010; Yen et al., 2013). Moreover, most species can be produced all year long, especially those more robust and able to sustain different climatic variations. Nevertheless, large scale production of microalgal biomass still remains challenging due to low biomass and target

molecule productivities, wide environmental variations under outdoor conditions, light availability in dense cultures and contamination of cultures by competitors and predators (Pienkos and Darzins, 2009). Moreover, the utilization of large amounts of water and nutrients has led to high production costs.

1.1.2 Lipophilic compounds

Over the past decades, one important class of biochemical compounds that has gained increased attention in microalgal biotechnology are lipids and lipophilic compounds. Microalgal lipids can be classified in non-polar (e.g., triacylglycerols, sterols, free fatty acids, waxes and steryl esters) and polar lipids (phosphoglycerols and glycosylglycerols; Guschina and Harwood, 2013). Moreover, microalgae produce many lipophilic compounds, such as terpenes, tocopherols, carotenoids, quinines and chlorophyll. The main functions of lipids are either as structural or storage compounds by dividing compartments in the form of membranes and providing energy, respectively. Nevertheless, lipids can have functional roles in the photosynthetic complex or as signalling molecules in changing environments.

Non-polar lipids such as triacyclglycerols (TAGs) are storage lipids and can be found in lipid bodies in the cytosol (Chapter 2). TAGs are synthesized in the light and accumulated under stress conditions; however, under dark conditions the degradation of TAG provides energy to the cells. The fatty acids of TAGs are predominantly saturated with chain lengths of 16 or 18 carbons. Because of the need for renewable, sustainable fuels, microalgal TAGs have been considered as one of the most promising replacement of fossil fuels. However, microalgae are not only exploited for TAGs but also for hydrocarbons and terpenes to produce biofuel (Chisti, 2013).

Membrane lipids are composed of phospho- and glycolipids, which are important structural components of the thylakoids but also function in the biogenesis of the thylakoid membrane, folding and assembly of protein units in the photosynthetic complex and chloroplast development (Kobayashi, 2016). Membrane lipids usually contain high amount of polyunsaturated fatty acids (PUFA), which play an important role in several defence and signalling mechanisms (Martins et al., 2013). For example, under stress conditions, PUFA remove reactive oxygen species (ROS) and thus, prevent oxidative stress-induced cell damage. Even though oxidative molecules such as ROS and reactive nitrogen species (RNS) are important cell-signalling molecules, they lead to oxidative stress when their amount overcomes the antioxidant capabilities of a given cell (Lamers et al., 2008). The subsequent

oxidative stress leads to damage of cellular components including photosystems, proteins, lipids and DNA, which might result into cell death. However, some microalgal species are able to produce TAGs containing PUFA (Chapter 2). It has been proposed that under stress conditions these TAGs are reservoirs for membrane remodelling. Moreover, lipids of particularly marine microalgae are rich in the long-chain PUFA eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which are nutritionally important fatty acids, as they exert several beneficial effects on human health such as reduction of inflammation and the prevention of cardiovascular diseases (Adarme-Vega et al., 2012).

1.2 Microalgal pigments

The two groups of lipophilic pigments are chlorophylls and carotenoids which represent major classes of photosynthetic pigments in eukaryotic microalgae. Both groups of pigments contain a large system of conjugated double bonds (CDB), which is responsible for their characteristic absorption spectra of light in the UV/Vis range. The light that is not absorbed is captured by the human eye and gives the pigments their distinctive colour.

1.2.1 Carotenoids

Carotenoids represent a diverse group of over 1100 natural compounds that are composed of a hydrophobic 40-carbon isoprenoid backbone and can be classified by the absence or presence of oxygenated groups as carotenes (e.g., lycopene and β-carotene) and xanthophylls (e.g., lutein and violaxanthin), respectively (Figure 1.1). The number of CDB is decisive for the colour of carotenoids. For example, carotenoids with less than 7 CDBs are colourless (e.g., phytoene), while a larger number of CBD imparts colours from light yellow to deep red to carotenoids (Meléndez-Martínez et al., 2007). Furthermore, the CDB structure is responsible for the antioxidant activity of pigments by quenching singlet oxygen or scavenging of free radicals (Mascio et al., 1991).

Microalgae are capable of producing many different types of carotenoids; however, some of them are only synthesized in some algal taxa and, therefore, can be used as chemotaxonomic markers (Takaichi, 2011). The most distributed pigment within microalgal groups is β-carotene contrary to astaxanthin, canthaxanthin and lutein, which are exclusively found in chlorophytes. Fucoxanthin is only present in haptophytes, chrysophytes, bacillariophytes and fucoxanthin-containing dinophytes (Takaichi, 2011). Therefore, the

production of carotenoids is not a simple task and the knowledge of the biosynthetic pathways and the roles of the pigments are important to understand the factors influencing the production of these pigments and how microalgal pigment production can be improved.
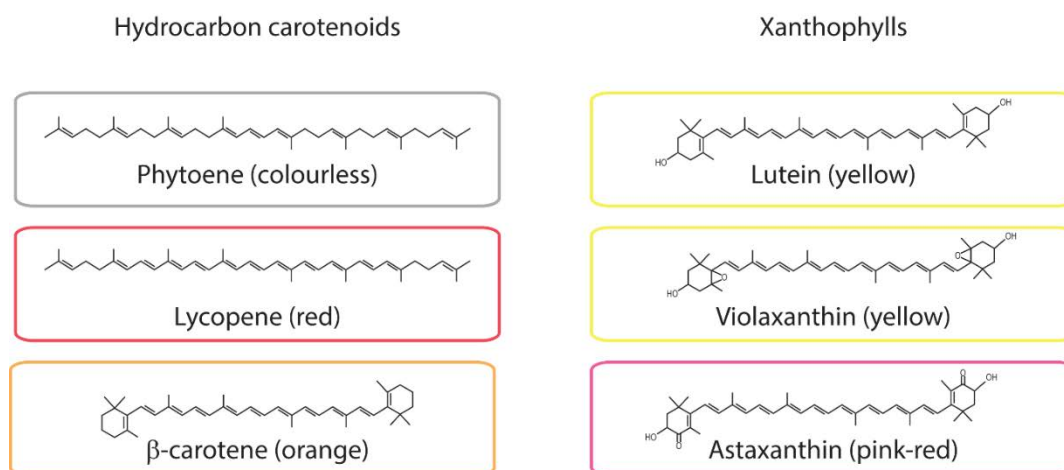
Figure 1.1. Structures and corresponding colours of common carotenoids in microalgae. In the left panel representative hydrocarbon carotenoids and in the right panel oxygenated derivates of hydrocarbon carotenoids, xanthophylls are displayed. Adapted from de de Carvalho and Caramujo (2017).

1.2.1.1   Biosynthesis

The biosynthesis pathway of carotenoids is complex, involving many enzymes and genes, which are not yet fully described in microalgae, but they have been deduced from the current knowledge of the biochemistry of higher plants. Carotenoids are synthesised in the chloroplast and are products of the plastidial 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway, which produces the precursor isopentenyl pyrophosphate (IPP) (Varela et al., 2015). Four molecules of IPP lead to the formation of geranylgeranyl pyrophosphates (GGPP) by the action of GGPP synthase. The first C_{40} carotenoid phytoene results from the condensation reaction of two GGPP molecules catalysed by phytoene synthase (PSY) (Figure 1.2). PSY is one of the most important regulators of the carotenoid biosynthesis and this is considered to be the rate-limiting step of initiation of the pathway (Chen et al., 2007; Yan et al., 2005).

Phytoene is converted to all-*trans* lycopene by four stepwise desaturations catalysed by phytoene (PDS) and *ζ*-carotene (ZDS) desaturases, followed by isomerization by *ζ*-carotene isomerase (Z-ISO). Lycopene is the first coloured carotenoid and the branching point in the biosynthetic pathway, leading either to *β*- or *α*-carotene. The cyclisation of both ends of lycopene leads to the biosynthesis of *β*-carotene containing two *β*-rings, a metabolic step catalysed by lycopene *β*-cyclase (LCYB). The hydroxylation of both *β*-rings by *β*-carotene

5

hydroxylase (CHYB) leads to zeaxanthin. This nonheme diiron monooxygenase preferentially hydroxylates the *β*-rings of *β,β*-carotenoids (Kim and DellaPenna, 2006). The following conversion of zeaxanthin via antheraxanthin to violaxanthin is catalysed by zeaxanthin epoxidase (ZEP) and can be reversed back to zeaxanthin by violaxanthin de-epoxidase (VDE), also known as the violaxanthin cycle. Neoxanthin synthase (NSY) is responsible for the formation of an allene group from violaxanthin resulting into neoxanthin. In some chlorophytes, *β*-carotene and zeaxanthin can be further converted into astaxanthin and canthaxanthin, respectively. These conversions are stepwise catalysed by *β*-carotene ketolases (BKT), which are known rate-limiting enzymes in the synthesis of those carotenoids.
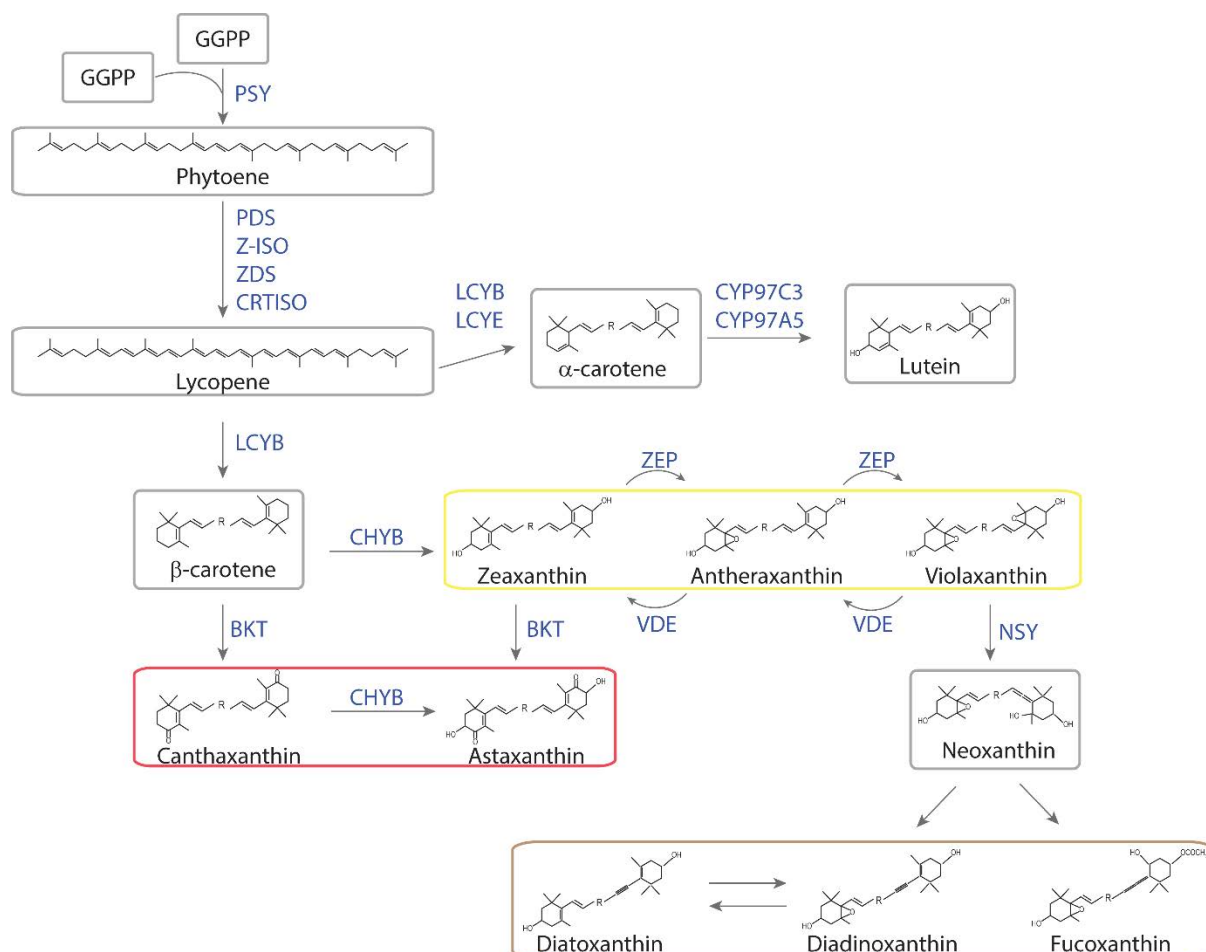
Figure 1.2. Carotenoid biosynthetic pathway in microalgae. Enzymes involved in the biosynthesis are displayed in blue: phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (Z-ISO), lycopene-*β*-cyclase (LCYB), lycopene-ε-cyclase (LCYE), *β*-carotene hydroxylase (CHYB), *β*-ring hydroxylase CYP97A5, ε-ring hydroxylase CYP97C3, *β*-carotene ketolase (BKT), zeaxanthin epoxidase (ZEP), violaxanthin de-epoxidase (VDE), neoxanthin synthase (NSY). The violaxanthin cycle is displayed in the yellow box. The carotenoids canthaxanthin and astaxanthin are only found in some chlorophytes (red box). Fucoxanthin, diadinoxanthin and diatoxanthin are only found in haptophytes and diatoms (brown box). Diagram derived from Takaichi (2011) and Varela et al. (2015).

In the other branch, lycopene is cyclised by LCYB and ε-lycopene cyclase (LCYE), resulting in the biosynthesis of α-carotene containing one β- and one ε-ring. The relative activities of LCYB and LCYE define the quantities of the carotenoids produced by each branch. The following hydroxylation step is catalysed by a different class of carotenoid hydroxylases than CHYB, namely the heme-containing cytochrome P450 monooxygenases (Kim and DellaPenna, 2006). The formation of lutein is catalysed in two steps by β-ring hydroxylase CYP97A5 followed by ε-ring hydroxylase CYP97C3 or vice versa. In some species, including *Tetraselmis* sp., loroxanthin or other carotenoids can be synthesised from lutein (Jin et al., 2003b).

Little is known about the enzymes involved in the biosynthesis of fucoxanthin and diadinoxanthin. However, a study in *Phaeodactylum tricornutum* revealed neoxanthin as the branching point for the formation of both carotenoids (Dambek et al., 2012). Diadinoxanthin is synthesised by a single isomerization of one of the allenic double bounds in neoxanthin, whereby two modification reactions, namely ketolation and acetylation, result in the formation of fucoxanthin. Diadinoxanthin can be converted to diatoxanthin by a reversible de-epoxidation reaction, the so called diadinoxanthin cycle. Both the diadinoxanthin and violaxanthin cycle serve as a photoprotective mechanisms under changing light intensities (Jahns et al., 2009; Lohr and Wilhelm, 1999).

1.2.1.2   Functions in microalgal cells

Carotenoids play essential roles in light harvesting and photoprotection of the photosynthetic machinery of microalgal cells (Figure 1.3). Light is the primary source of energy for photosynthetic organisms; however, both light intensity and quality affect photosynthesis and can even lead to cell death. Therefore, microalgae have developed mechanisms to maximise light-harvesting and minimise the damaging effects thereof, thus ensuring the survival under varying circumstances. The accessory light-harvesting pigments are located in the thylakoid membranes and are bound to peptides building a pigment-protein complex associated within the light harvesting complex (LHC) (Takaichi, 2011). As carotenoids absorb light differently when compared to chlorophyll, they expand the light absorbance spectrum of the microalgal cell, which is especially important under low light conditions. The close location of accessory pigments to the primary light-harvesting chlorophyll molecules ensures the efficient transfer of excitation energy, initiating the photochemical actions of photosynthesis (Figure 1.3A). Light-harvesting pigments are neoxanthin, violaxanthin, β-carotene and fucoxanthin, while lutein is a semi-photosynthetic pigment due to a lower efficiency of the

energy transfer (Mulders et al., 2014b). However, during exposure to excess light, photoprotective pigments accept excess energy from chlorophyll and dissipate it into heat, hence, reducing photoinhibition (Figure 1.3B).

A. Light harvesting pigments

B. Photoprotective pigments

C. Structural pigments
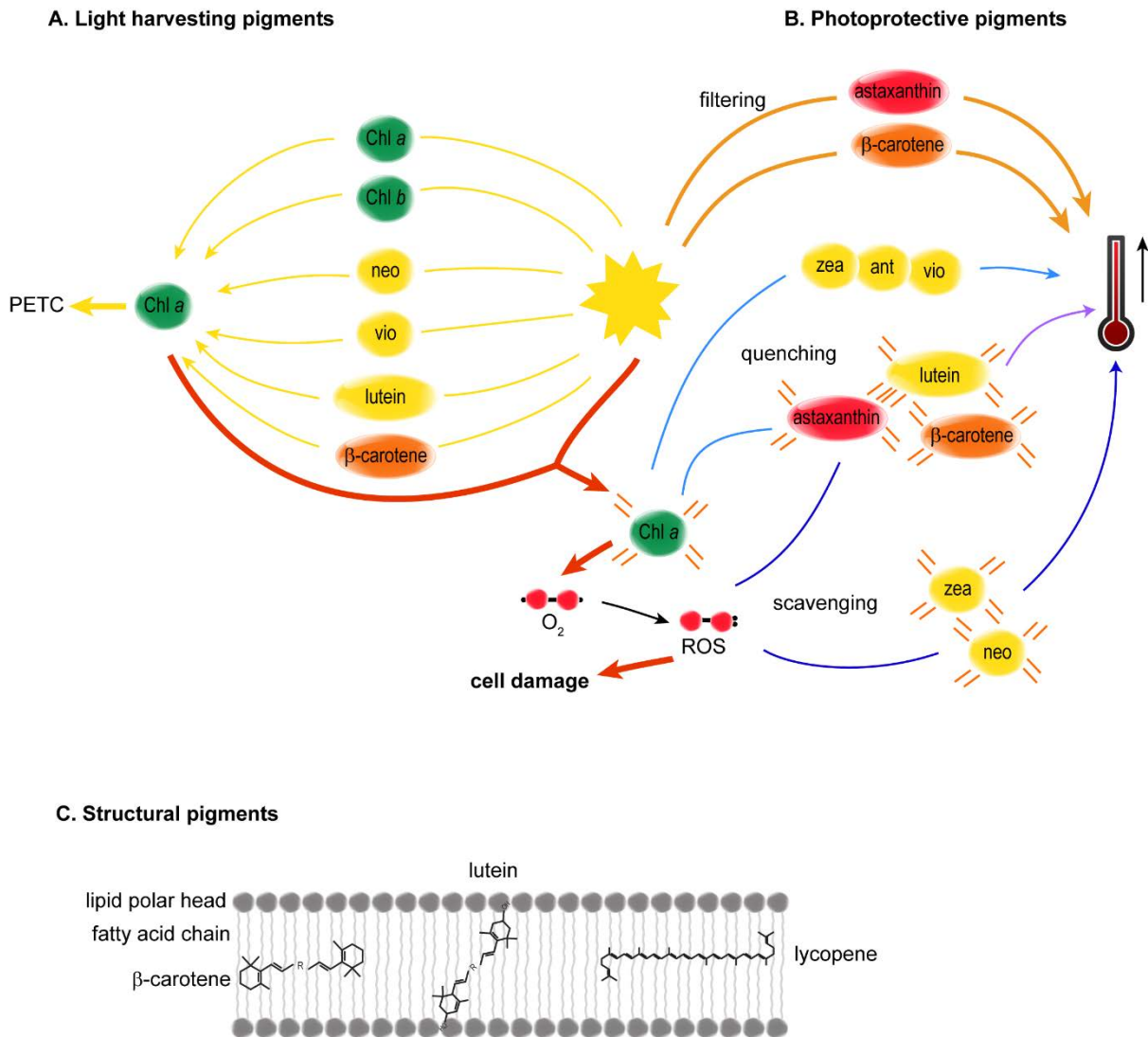
Figure 1.3. Functions of carotenoids in cells of Chlorophytes. Pigments have essential functions in light harvesting and are usually located in close proximity to the primary light harvesting chlorophyll *a* molecule (A). The light energy is transferred to the photosynthetic electron transport chain (PETC) where it is converted into chemical energy (yellow arrows). However, under excess light, chlorophyll *a* can enter a triplet excited state (orange lines) and convert molecular oxygen into reactive oxygen species (ROS), leading to cell damage (red arrows). Photoprotective pigments prevent this process by converting excess energy into heat (B). Carotenoids with a filtering role directly absorb excess light (orange arrows); carotenoids with a quenching role accept the energy from chlorophyll *a* and acquire a triplet excited state (light blue arrows); carotenoids with a scavenging role react with ROS (dark blue arrows). Moreover, carotenoids have also a structural role by spanning membranes, stabilizing them mechanically and being able to regulate lipid fluidity (C). Partially adapted from Mulders et al. (2014b) and Tan et al. (2014).

Photoprotective pigments can be divided by their action into three different groups, namely quenching, scavenging and filtering (Mulders et al., 2014b). Pigments with a filtering role are not bound to the photosynthetic apparatus, as they protect chlorophyll by the absorbance of harmful radiation. Astaxanthin and *β*-carotene belong to this functional group of carotenoids, when stored in lipid bodies in the cytosol or stroma, respectively. However, pigments with quenching and scavenging roles are, like accessory light harvesting pigments, located closer to the photosynthetic machinery. Astaxanthin, lutein and *β*-carotene quench the energy of triplet excited chlorophyll *a* leading to the prevention of the formation of ROS (Mulders et al., 2014b).

Furthermore, the pigments of the violaxanthin cycle, zeaxanthin and violaxanthin, and the diatoxanthin cycle are quenchers. In the process of quenching, the carotenoids acquire a triplet excited state that can be converted back safely to the ground state, releasing heat (Varela et al., 2015). The carotenoids with a scavenging role are astaxanthin, *β*-carotene, lutein and neoxanthin, thus reacting with ROS and thereby preventing cell damage. Besides high light intensities, other stress factors such as high salinities or heat can lead to ROS production resulting into the damage of LHCs. For example, under higher salinities, *Dunaliella salina* accumulates high amounts of *β*-carotene, an important mechanism to cope with oxidative stress (Borowitzka et al., 1990). Additionally, carotenoids, particularly xanthophylls, can span membranes, thereby stabilizing the structure and most probably affecting membrane properties (Figure 1.3C). After freeze-thaw, zeaxanthin has been found to be increased in Arctic bacteria, suggesting its role in the modulation of membrane stability (Singh et al., 2017). In a study of *Chlamydomonas* sp. ICE-L, lutein may improve the integrity of membranes, a crucial role for adaptation to freezing temperatures (Yi-Bin et al., 2017). A possible mechanism for this structural role is that there is recent evidence that carotenoids can influence membrane fluidity and abrogation of carotenoid biosynthesis prevents this adaptation from taking place (Seel et al., 2020). Moreover, xanthophylls may play a similar function in stabilizing membranes in organism under other membrane-influencing conditions such as thermal and salinity stress (Havaux et al., 1996). Taken together, the functions of carotenoids are versatile, and one pigment can have several functions depending on the location within the cell or environmental conditions to which the cells are exposed.

1.2.1.3 Induction of carotenoids

The accumulation of carotenoids is highly dependent on environmental cues as these compounds improve the survival under changing conditions. For example, microalgae found

in extreme environments such as high saline lakes or in the Antarctic ice display orange to red colours due to their high carotenoid contents. These are important findings which contribute to the investigation to manipulate microalgal carotenoid production and other lipophilic compounds by abiotic stress factors (Chapter 2). Nutrient depletion, high light intensities and salinity have been found to be the most important factors for increased β-carotene, astaxanthin and canthaxanthin contents in chlorophytes. These observations were confirmed by expression profiles of important carotenoid pathway enzymes such as PSY, PDS, LCYB and BKT which have been found to be up-regulated in *Haematococcus pluvialis*, *Chlorella zofingiensis*, *Chlamydomonas reinhardtii* and *Dunaliella salina* (Bohne and Linden, 2002; Coesel et al., 2008; Cordero et al., 2012; Couso et al., 2012; Gao et al., 2015; Mao et al., 2018). Conversely, increased lutein and fucoxanthin contents have been found to be correlated with optimal growth conditions (Chapter 2).

A different approach to improving the accumulation of certain carotenoids is the utilization of carotenoid pathway inhibitors. For example, an increase in phytoene was achieved in *Dunaliella bardawil* and *Chlorococcum* sp. by blocking phytoene desaturase with inhibitors such as norflurazon and fluoridone, respectively (Laje et al., 2019; León et al., 2005). The inhibition of lycopene cyclase with triethylamine resulted into an accumulation of lycopene in *D. bardawil* (Liang et al., 2016). The accumulation of higher carotenoid contents by stress conditions or inhibitors, however, are often accompanied with reduced growth. A solution to this hurdle is usually the use of a two-stage production system where high concentrations of biomass are produced first, then followed by a carotenoids accumulation phase.

1.2.2   Chlorophyll

Chlorophyll is the well-known green pigment ubiquitous in all photosynthetic organisms. The basic structure of chlorophyll is a tetrapyrrole with a magnesium bound to the centre of the ring and usually with a phytol side chain attached. Different substituent groups on the pyrrole rings give rise to chlorophylls *a*, *b*, *c*, *d* and *f*, which display different green hues: blue-green, brilliant green, yellow-green, brilliant forest green and emerald green, respectively (Mulders et al., 2014b). Chlorophyll *a* is the most abundant pigment in all microalgae, while chlorophyll *b* is mainly found in chlorophytes and chlorophyll *c* in heterokontophytes, haptophytes and dinophytes (Takaichi, 2011). Chlorophylls *d* can be found in rhodophytes and most recently chlorophyll *f* has been identified in cyanobacteria (Chen et al., 2012; Manning and Strain, 1943). However, the isolation of chlorophyll from its natural environment leads to

loss of the magnesium atom and the formation of the olive-grey phaeophytin (Humphrey, 2004).

Chlorophyll is bound to proteins in LHCs located in the thylakoid membranes of the chloroplast. Chlorophyll *a* is essential in the photochemistry of photosynthesis, as it is able to transfer directly the excitation energy to the photosynthetic electron transport chain (PETC), where it is converted to chemical energy. Microalgae have evolved under light-limiting conditions resulting in an increase of chlorophyll molecules and a larger chlorophyll antenna size to maximise light utilization (Koh et al., 2020). Besides the function as light harvesting pigment, chlorophyll also have a minor role as nitrogen storage compounds representing 0.1-1.8% of total N (Lourenço et al., 2004).

Chlorophyll contents of microalgae change with culture conditions, which is dependent on the species and the stage of cultivation (da Silva Ferreira and Sant'Anna, 2017). In general, nutrient depletion (N and P), darkness, and extreme high light conditions decrease chlorophyll contents, while optimal growth conditions, low light intensities and high temperature increase chlorophyll contents.

1.3   Applications and commercial production of pigments

The global carotenoids market is expected to grow at a compound annual growth rate (CAGR) of 4.2% from USD 1.5 billion in 2019 to USD 2 billion by 2026 ("Carotenoids market by type, application, region - Global forecast 2026," 2020). Conversely, the global chlorophyll market represents a smaller market but also growing at an estimated CAGR of 7.5% from 279.5 million in 2018 to USD 463.7 million by 2025 ("Chlorophyll extract market size, share | Industry report, 2026," 2020). This increase is mainly due to the usage of natural colourants in food/feed rather than the use of chemically synthesised pigments. Health issues such as hyperactivity disorder in children, hypersensitivities and possible toxicities have been related with the intake of synthetic dyes (Amchova et al., 2015). Because of the growing importance of higher food quality, safety and sustainability, organic and natural sources are prioritized by food producers. Furthermore, the market growth is attributed to innovative technologies for improved extraction of pigments from biological matrices. Moreover, the use of pigments in preventive healthcare and on the improvement of the immune system is contributing to the growth of "natural" pigment market (Figure 1.4).

Astaxanthin and *β*-carotene are two of the most utilized carotenoids, followed by lutein, lycopene and zeaxanthin. The market for astaxanthin as animal feed and nutraceutical was

expected to have increased to USD 800 million and USD 300 million by 2020, respectively. The market for lutein is expected to grow at the highest CAGR due to its application to pharmaceutical and human and animal nutrition products. In addition, innovative pigments such as fucoxanthin, canthaxanthin and phytoene are entering the market as well.

1.3.1 Food and feed

The largest application of carotenoids is in the feed sector, accounting for 46% of the total carotenoid market in 2018, mainly due to their colour and nutritional properties ("Carotenoids market by type, application, region - Global forecast 2026," 2020). The utilization of astaxanthin and β-carotene in the feed for salmon, trout and crustaceans enhances the desired red/pink colour of the fish muscle (Lorenz and Cysewski, 2000), while lutein supplementation in poultry feed increases the yellow colour of egg yolk (Englmaierová et al., 2013). In addition to their colour, the antioxidant properties of carotenoids improve immunity, fertility and the general health status of animals, as well as reduce ROS generation. The inhibition of the former is essential to prevent cell damage, and the accompanied food degradation and development of off-flavours.

Moreover, pigments are included in a broad range of food and beverages to improve their colour. Although chlorophylls are used as food colourants, there are disadvantages related with their application due to its low stability. Chlorophylls are sensitive to pH, light and temperature leading to the formation of degradation products accompanied by colour loss (Martins and Silva, 2002). A more stable compound can be produced by chemical modification in replacing the magnesium atom by copper resulting into chlorophyllin. Nevertheless, chlorophyll might have undesirable sensory characteristics such as a "grassy" taste, colour and odour with low consumer acceptance in meat and dairy products (Karan et al., 2019; van Lelyveld and Smith, 1989; Wold et al., 2005).

1.3.2 Health care

The health and nutraceutical markets are currently the fastest growing sectors with increased demand for dietary supplements, growing at a CAGR of 4.5% from 2018 to 2025 ("Carotenoids market size, share and trends | Industry analysis - 2025," 2018). Changes in lifestyle and the rising health consciousness of an aging population are responsible for the increased consumer demand for natural products with health-promoting properties. Besides

other lifestyle factors, the prevention of many medical conditions, such as cancer, cardiovascular and degenerative diseases as well as deceleration of aging have been linked to a healthy diet with a sufficient intake of antioxidant pigments (Figure 1.4).

As humans cannot synthesize pigments *de novo*, they must be obtained from the diet usually in the form of fruits, vegetables, or microalgae. One essential function in human health is the conversion of carotenoids into retinoids, precursors of vitamin A. The most important provitamin A carotenoid is *β*-carotene contributing to the prevention of vitamin A deficiency. Vitamin A is well-known for its essential role in growth, embryonic development, reproduction and vision (Zile, 1998). Besides, the benefits of carotenoids for human health are related with their antioxidant activity; however, other biological activities, such as pro-oxidant, enhancement of gap junction communication, alteration of immune function, modulation of signalling pathways, regulation of gene transcription or modulation of membrane properties may contribute to their health promoting effects (Ambati et al., 2019). Chlorophyll also displays antioxidant, anti-inflammatory, anticancerogenic and antimutagenic properties and its consumption may be beneficial to human health (García et al., 2017). The increased demand for "natural" pigments and their application in the nutraceutical market provides a great opportunity for pigments derived from microalgal biomass.

Anti-cancer properties have been attributed to several carotenoids and chlorophylls, indicating the prevention of different types of cancers in humans, including bladder, breast, hepatic, intestinal, leukemic, lung, oral, and prostate cancer (Figure 1.4). Lycopene is the most studied carotenoid, most probably due to its greater effect on cancer prevention than other carotenoids (Nishino et al., 2002). Lycopene intake correlated inversely with prostate cancer (Schwarz et al., 2008). Other studies reported a protective effect of lycopene and chlorophyll against colon cancer (Balder et al., 2006; Erhardt et al., 2003). Furthermore, canthaxanthin and astaxanthin have been shown effective against bladder and prostate cancer by inducing apoptosis (Palozza, 1998; Tanaka et al., 1994). A different example is the use of *Dunaliella salina* extracts enriched with pigments displaying anticancerogenic effects towards oral cancer (Chiu et al., 2017). More specifically, violaxanthin isolated from *Dunaliella tertiolecta* or *Chlorella ellipsoidea* showed antiproliferative effects against human mammary and colon carcinoma cell lines, respectively (Cha et al., 2008; Pasquet et al., 2011). Furthermore, several studies have demonstrated that fucoxanthin, which can be extracted in large amounts from diatoms or the haptophyte *Tisochrysis lutea* presents anti-cancer effects (Kumar et al., 2013).

Furthermore, carotenoids are highly researched due to their anti-inflammatory properties and may help in reducing the risk for diseases including arthritis, asthma, multiple

sclerosis, inflammatory bowel disease (IBD), and atherosclerosis. Several studies claimed anti-inflammatory properties of astaxanthin and fucoxanthin and revealed the responsible mechanisms of action (Kim et al., 2010; Ohgami et al., 2003; Shiratori et al., 2005). Astaxanthin mainly derived from *Haematococcus pluvialis* reduced oxidative stress and inflammation and may prevent atherosclerotic cardiovascular disease (Fassett and Coombes, 2012). Furthermore, *β*-carotene-rich *Dunaliella tertiolecta* slowed down Crohn´s disease in rats, a type of IBD which causes inflammation of the digestive tract (Lavy et al., 2003)

Eye health
lutein
zeaxanthin
β-carotene

Anti-diabetic
astaxanthin
β-carotene
lutein
zeaxanthin
lycopene

Liver health
β-carotene
astaxanthin

Cancer prevention
β-carotene
lutein
zeaxanthin
violaxanthin
astaxanthin
lycopene
phytoene

Fertility
β-carotene
lutein
zeaxanthin

Brain - cognitive functions
astaxanthin
lutein
lycopene

Cardiovascular health
astaxanthin
lycopene
lutein
zeaxanthin

Maternal and infant nutrition
zeaxanthin
lutein

Skin protection
β-carotene
lycopene
lutein
astaxanthin

Immunity
zeaxanthin
lutein

Figure 1.4. Carotenoids and their relationship with human health. Adapted from Newtrition ("Power Shakes: How to Make Healthy Foods Even More Healthier | AgroDay,")

More recently, the importance of carotenoids in cognitive functions, metabolic health, during pregnancy and infancy has been reported (Figure 1.4) (Meléndez-Martínez, 2019). Long lasting oxidative stress in the brain is related to cognitive decline accompanying Alzheimer´s disease (Mattson, 2004). Extracts from *Chlorella* sp. containing *β*-carotene and lutein have been shown to prevent cognitive impairment in an age-dependent dementia mice model (Nakashima et al., 2009). Lutein is the dominant carotenoid in the human brain of infants and adults and may be beneficial in the maintenance of cognitive health also in healthy

human (Craft et al., 2004; Vishwanathan et al., 2011). Moreover, lutein and zeaxanthin are present in the human milk and very important for visual and cognitive development in infants (Giordano and Quadro, 2018). Lutein and zeaxanthin, which can be isolated from *Chlorella* spp., are major pigments in the eye and may prevent the development of age-related macular degeneration (AMD), cataract and retinitis pigmentosa (Johnson, 2014). AMD is one of the major causes for visual impairment and blindness in elder people. The protective functions of these important macular pigments are related with the absorption of blue light and their antioxidant activity, thus preventing light damage (Krinsky et al., 2003). Moreover, an increased dietary intake of lutein and zeaxanthin may improve visual performance in healthy adults (Kvansakul et al., 2006).

A novel function of carotenoids is related with the control of obesity which is major contributing factor to the development of diabetes or cardiovascular diseases. Fucoxanthin extracted from *Phaeodactylum tricornutum* has shown anti-obesity effects in mice (Koo et al., 2019). The anti-obesity and anti-diabetic activities of astaxanthin have been widely study. For example, astaxanthin has been reported to inhibit inflammation and liver fibroses in obese mice (B. Kim et al., 2017). Moreover, astaxanthin seems to improve cognitive function in diabetes mice (X. Li et al., 2016). The dietary intake of carotenoids, such as *β*-carotene and lutein, has been proven to be effective against development and progression of type 2 diabetes mellitus (Katyal, 2013; Sluijs et al., 2015). Moreover, a chlorophyll-rich spinach diet prevented obesity and promoted changes in the gut microbiota in mice (Li et al., 2019).

Finally, the antioxidant activity of carotenoids is also responsible for their protective function from UV damage, thus, improving health and appearance of the human skin (Sathasivam and Ki, 2018). A supplementation study using *β*-carotene, lutein and lycopene was able to show a reduction in UV-induced erythema (Heinrich et al., 2003). Finally, fibroblasts treated with an astaxanthin extract derived from *Haematococcus pluvialis* have been shown to lead to enhanced skin proliferation and repair in the human dermis (Chou et al., 2016).

1.3.3 Cosmetics and personal care products

In cosmetic industries, carotenoids and chlorophylls are included in anti-aging creams, anti-irritant peelers and skin care products due to their antioxidant properties and nutritional value to skin and hair. The absorbance of UV radiation by pigments protects the skin against the sunlight and gives skin a glowing appearance, additionally, pigments aid in the colouring

effect of tan lotions (Stahl and Sies, 2012). Furthermore, carotenoids in the skin may be reversely correlated with skin aging. A higher concentration of lycopene in the skin of women aged between 40 to 50 years has been correlated with smoother skin, presenting fewer furrows and wrinkles (Darvin et al., 2008). Chlorophyll is able to mask odours and is therefore, applied in hygiene products, such as deodorants and toothpaste (Mourelle et al., 2017)

1.3.4   Commercial production of pigments

Microalgae mass production is performed in either closed photobioreactors (PBR) or open systems. PBRs can be classified as airlift or bubble columns or, in alternative, tubular or flat panel PBRs, whereas open systems can be implemented as rectangular, raceway or thin layer cascade ponds. The selection of the cultivation system depends on several factors such as the environmental conditions, risk of contamination, capital and operational costs, level of controllability of system parameters, species to cultivate and final product application (Aitken and Antizar-Ladislao, 2012). Microalgae successfully developed for mass production of carotenoids include *Dunaliella salina*, *Haematococcus pluvialis, Chlorella* spp., *Scenedesmus* spp. and *Phaeodactylum tricornutum* (Ambati et al., 2019; Del Campo et al., 2007).

Dunaliella salina is a marine microalga accumulating large amounts of β-carotene under high salinity, temperature and irradiation stress and under nutrient limitations (Ben-Amotz, 1999; Borowitzka et al., 1990). Therefore, this alga is produced in open rectangular growth ponds in hot countries with high solar irradiance and where hypersaline water is available. Nevertheless, *D. salina* can also be grown under controlled conditions in tubular 55-L PBRs reaching 10% DW of carotenoids, containing mainly β-carotene and lutein (García-González et al., 2005).

H. pluvialis is a freshwater microalga which produces large amounts of astaxanthin commonly in a two-stage cultivation consisting of a first step in which fast growing green microalgae are produced in tubular PBRs and in a second stage, when astaxanthin accumulation is induced by exposure of the alga to environmental stressors, in open ponds. In this way, biomass containing 2.5% DW of astaxanthin was produced at industrial scale (25,000-L PBRs; Olaizola, 2000). A new approach is the one-step cultivation of *H. pluvialis*, which comes with the advantage of a continuous production and the use of only one PBR. Biomass of *H. pluvialis* containing 1.26% DW of astaxanthin was produced continuously in an outdoor 50-L tubular PBR and astaxanthin production was induced by constant nitrogen limitation and dilution of the culture to achieve high irradiation (García-Malea et al., 2009).

Despite the large accumulation of astaxanthin, *H. pluvialis* is a slow-growing microalga, easily susceptible to contamination and high light intensities are needed for astaxanthin induction, thus impeding several commercial applications (Fábregas et al., 2001; Imamoglu et al., 2009). A potential alternative producer of astaxanthin is the freshwater alga *Chlorella* (*Chromochloris*) *zofingiensis* due to its faster growth and robustness. *C. zofingiensis* can be cultivated under heterotrophic growth conditions to achieve high biomass and, in a second step, astaxanthin production can be induced by high light and nitrogen starvation, giving a content of 0.112% DW of astaxanthin (Zhang et al., 2017).

Another important microalgal genus with well-established mass production is *Chlorella*, which is mainly composed of freshwater microalgae with a well-balanced biochemical profile. The major pigments of *Chlorella* are chlorophyll *a* and *b* accounting for up to 5-7% DW (Koutra et al., 2021; Sarkar et al., 2020). Lutein, astaxanthin and *β*-carotene, however, can be found in lower quantities.

Although several lutein producing microalgae have been identified, no commercial production of this xanthophyll is available yet. In a pilot-scale, 50-L bubble column PBR operated semi-continuously, *C. vulgaris* containing 7.6 mg g^{-1} DW of lutein was produced (McClure et al., 2019). *C. sorokiniana* was produced in 50-L column PBR in a two-stage strategy including a mixotrophic phase to produce biomass followed by a phototrophic phase leading to the production of 9.51 mg g^{-1} DW of lutein (Xie et al., 2020). Furthermore, microalgae of the genus of *Muriellopsis* are known for their high lutein contents (4-6 mg g^{-1} DW) which have been grown outdoors in 100-L open tank (Blanco et al., 2007). However, a novel microalgal species *Coccomyxa onubensis* has been grown in 800-L tubular PBR obtaining biomass with 10 mg g^{-1} DW of lutein (Fuentes et al., 2020). Moreover, freshwater species of the genus *Scenedesmus* are promising producers of both astaxanthin and lutein. *S. almeriensis* cultivated in a 28.5-L bubble column photobioreactor showed lutein contents of 8.54 mg g^{-1} DW (Molino et al., 2019), whereas the combination of nitrogen deficiency with light and salinity stress has led to the production of canthaxanthin and astaxanthin, 1.73 and 1.11 mg g^{-1} DW, respectively, in *S. rubescens* (Jo et al., 2020).

Innovative species for the production of fucoxanthin are *Isochrysis galbana* and *Phaeodactylum tricornutum* with contents as high as 18 and 10 mg g^{-1} DW of fucoxanthin, respectively (Gao et al., 2017; Kim et al., 2012).

1.4   Strain improvement

Pigment production of microalgae at large-scale is still challenging and only few strains have been established (see 1.3.4). To overcome these limitations and to reduce production costs, novel strains with improved productivities of the desired compounds are required. A promising strain for industrial scale production of carotenoids needs to possess certain key characteristics, such as fast growth rate, robustness, high carotenoid content, capability of growing in changing environmental conditions, and be easy to harvest. In addition, it should have the ability to grow in low-cost media, such as wastewater, brackish or saline water. Although that is a plus, it is not always compatible with the application of a product derived from biomass grown under such conditions.

Apart from strain improvement by genetic or metabolic engineering, random mutagenesis is preferred for industrial applications. Random mutagenesis is a non-GMO strategy and comes with the advantage that previous knowledge of the biosynthetic pathways is not required (also see Chapter 2). Improved microalgal strains are generated upon exposure to mutagenic agents followed by selection of the mutant with the desired traits. Different chemical or physical mutagenic agents can be applied, such as ethyl methane sulfonate (EMS), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) or UV radiation. Exposure to UV-C radiation (254 nm) induces the formation of pyrimidine dimers at dipyrimidine sites leading to DNA damage or lesions (Ikehata and Ono, 2011). On the other hand, chemical agents alkylate the DNA leading to substitutions of nucleotides, insertions or deletions. For example, EMS alkylates specifically guanine resulting in G/C to A/T transitions while MNNG induces a wide spectrum of mutations (Singer and Kusmierek, 1982). The result of random mutagenesis is often the generation of hundreds of mutant colonies, making a good screening method for the targeted mutant necessary. Mutants can be selected by different properties such as growth performance measured by absorbance, fluorescence or visual appearance. For example, the UV-induced mutant of *Phaeodactylum tricornutum* with higher fucoxanthin contents has been selected by best growth performance in 96-well plates (Yi et al., 2015). Different studies of *Dunaliella salina* and *D. tertiolecta* generated high zeaxanthin accumulating mutants by EMS treatment followed by visual screening and selection of less green colonies (Jin et al., 2003a; M. Kim et al., 2017). On the contrary, a lutein-deficient, violaxanthin producing mutant of *Chlorella vulgaris* was generated by EMS using a selection by fluorescence imaging (Kim et al., 2020). In a different study, a high chlorophyll-containing (6.7% of DW), halotolerant mutant of *Chlorella vulgaris* was obtained after UV exposure and selection of dark green coloured colonies (Nakanishi and Deuchi, 2014). However, a more selective process of mutant

screening is the utilization of carotenoid biosynthesis pathway inhibitors, such as nicotine, norflurazon or fluoridone. Several herbicide-resistant mutants of *H. pluvialis* were obtained by random mutagenesis containing high amounts of astaxanthin (Chen et al., 2003; Sandesh Kamath et al., 2008). High carotenoid producing mutants of *Chlorella zofingiensis* were isolated using MNNG and the selection by the inhibitor diphenylamine (Huang et al., 2018). Other studies generated high lutein producing mutants of *Chlorella sorokiniana* by random mutagenesis using MNNG followed by the selection using lycopene-cyclase inhibitor nicotine (J.-H. Chen et al., 2017; Cordero et al., 2011b). Fluorescence activated cell sorting (FACS) is a powerful tool to isolate individual cells with desired cell characteristics (Pereira et al., 2018b). For example, a correlation between the lipidic dye Nile red and *β*-carotene contents in *D. salina* was used to isolate carotenoid-hyperproducing mutants of this strain (Mendoza et al., 2008).

A different approach for improved utilization of microalgae as carotenoid production platform is the reduction of the antenna size. A truncated antenna size not only improves photosynthetic efficiencies and biomass production but also reduces the amount of chlorophyll. More specifically, *Chlorella vulgaris*, *Chlorella saccharophila*, *Chlorella sorokiniana*, *Nannochloropsis gaditana*, *Cyclotella* sp. and *Chlamydomonas reinhardtii* mutants with pale green colours obtained by EMS or UV treatment have been isolated (Cazzaniga et al., 2014; Dall'Osto et al., 2019; de Mooij et al., 2015; Huesemann et al., 2009; Patil et al., 2020; Perin et al., 2015; Shin et al., 2016).

1.5 Extraction of microalgal pigments

The extraction of carotenoids from microalgal biomass can be a very challenging task and involves several considerations. As microalgae are a diverse group of organisms, they possess diverse biochemical properties and thus extraction is highly species-dependent (McMillan et al., 2013). Moreover, extraction is dependent on the chemical structure of the carotenoids and the perspective application as food, pharmaceutical or cosmetics. Therefore, the establishment of common protocol for carotenoid extraction of microalgae is not possible and has to be developed for each strain (Chapter 3; Cerón-García et al., 2018). Furthermore, during extraction several precautions have to be taken to avoid extreme light, heat and oxygen as carotenoids are sensitive once outside of their natural environment. The harvested microalgal biomass is a wet paste with different amounts of water. Common industrial drying processes such as freeze-, spray- or drum drying can be applied to the biomass to facilitate further extraction or to improve biomass storage as microbial and chemical decomposition is

reduced by the lower water activity. However, the drying process can lead to isomerization or degradation of carotenoids. Freeze-drying has been considered the mildest method, as it is performed under low temperatures and vacuum. Nevertheless, the drying process has a high energy consumption and accounts for 70-75% of the processing costs. Therefore, the direct use of wet biomass in the extraction is preferred.

1.5.1 Pre-treatment

Several microalgae are covered by rigid cell walls with varying chemical composition and structure which are dependent on the microalgal species and the cell stage. For example, *Haematococcus pluvialis* in the cyst stage is known for its 1.8-2.2 µm thick, multi-layered cell wall (Hagen et al., 2002). The cell walls of *Chlorella vulgaris* and *Neochloris oleoabundans* are composed of two layers protecting the cells from environmental stressors (Burczyk and Hesse, 1981; Rashidi and Trindade, 2018). These cell walls prevent the penetration of the extracting solvent into the cell and thus an efficient mass transfer of the carotenoids to the solvent. Therefore, depending on the cell wall composition an appropriate pre-treatment of the biomass needs to be selected. Cell disruption methods include chemical, mechanical/physical and biological means. Organic solvents, acids, bases, detergents, hydrogen peroxide, ozone or ionic liquids haven been used as chemical pre-treatments in the disruption of microalgae (D.-Y. Kim et al., 2016). Biological pre-treatments, for example, enzymatic hydrolysis of the cell wall, have been considered a very mild treatment suitable for thermolabile compounds such as carotenoids (Gerken et al., 2013). These methods, however, display several disadvantages such as contamination of the extract, prolonged reaction times when enzymes are used, and expensive reagents leading to increased processing costs. Therefore, the preferred pre-treatment usually involves mechanical or physical means. Disruption of the cells can be achieved by bead beating, grinding, cryogenic grinding, ultra-sonication, dispersion, osmotic shock, cooking, freeze-thawing, among others (Saini and Keum, 2018). The most common disruption methods for both laboratory and pilot-scale processes are bead-beating, grinding and high-pressure homogenisation (HPH). However, bead milling has been shown to be the most efficient method for the recovery of carotenoids (Chapter 3) and is also available at large scale (Taucher et al., 2016). Nevertheless, mechanical methods have higher energy consumption than enzymatic or chemical pre-treatments. Moreover, some methods produce excess heat that needs to be controlled leading to additional cooling costs.

1.5.2 Solvent extraction

Traditionally, solvent extraction is used for the recovery of carotenoids from microalgal biomass. The choice of the solvent is the most critical factor for efficient carotenoid recovery, being dependent on the microalgal species and on the polarity of the carotenoids to extract. More polar solvents such as ethanol, methanol and acetone are suitable to extract xanthophylls, while "non-polar" solvents such as ethyl acetate, tetrahydrofuran, hexane or chloroform are preferable for the extraction of carotenes and carotenoid-esters (Saini and Keum, 2018). Apart from the use of a single solvent, solvent mixtures have been also applied successfully, such as dichloromethane/methanol, chloroform/methanol, acetone/petroleum ether, and hexane/ethanol (Soares et al., 2016). However, the use of solvents has been associated with health and safety issues, having also known environmental hazards. For food processing, ethanol, acetone and hexane have been given the status of "generally recognized as safe" (GRAS) solvents and the combination thereof is a good choice to extract both xanthophylls and carotenes. Conventional solvent extraction is cheap and easy to scale up; however, large amounts of solvent need to be used, being a time-consuming process.

Besides other methods, e.g., microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE, sometimes also called PLE), supercritical fluid extraction (SFE) has been proposed as an alternative to conventional solvent extraction (Singh et al., 2015). SFE is considered an environmentally friendly process due to the recycling of CO_2 and the elimination of toxic solvents when supercritical CO_2 is used. Furthermore, SFE is less time consuming than solvent extraction and can lead to the extraction of high purity compounds. However, the recovery of xanthophylls such as lutein from *Scenedesmus almeriensis* is very poor by SFE (Macías-Sánchez et al., 2010). Moreover, sophisticated equipment and the use of dry biomass make this method expensive, hampering its widespread application (Rammuni et al., 2019).

1.5.3 Saponification

As carotenoids are lipophilic compounds, they are co-extracted with other lipids and chlorophyll. Moreover, xanthophylls such as astaxanthin are often esterified with fatty acids. The presence of chlorophyll and esterified carotenoids interferes with the chromatographic analysis of carotenoid extracts due to co-elution and absorbance in the same wavelength (Chapter 3; Bijttebier et al., 2014). Moreover, degradation products of chlorophyll contribute to oxidised odour and off-flavours hampering the application of the extract (Wold et al., 2005).

The removal of chlorophyll as well as the ester bonds of carotenoids in microalgal oils can be performed by alkaline saponification (T. Li et al., 2016). However, this method also led to a significant decrease in protein, lipid and carbohydrate content. Moreover, saponification leads to degradation, isomerization and other structural changes in carotenoids (Kimura et al., 1990). Recently, in a study done on *Isochrysis galbana*, chlorophyll was removed selectively by the basic resin Ambersep 900 OH, preserving the natural composition of esterified compounds such as carotenoids and glycerolipids (Bijttebier et al., 2014). However, this study did not evaluate the overall biochemical compositions of the biomass.

1.6   *Tetraselmis striata* CTP4

The genus *Tetraselmis* (Stein, 1878) belongs to the class Chlorodendrophyceae and the phylum Chlorophyta (Massjuk, 2006). The motile, unicellular cells are generally cordiform, elliptical or almost spherical shaped with sizes ranging from 6-25 µm and possess four equal flagella, which arise from an anterior pit. The cells contain a single, U-shaped chloroplast containing a centred pyrenoid and an eyespot. A distinguishing mark of this genus is the thin cell covering, a so called "theca", which is derived from the extracellular fusion of primitive scales (Manton and Parke, 1965). Asexual division occurs in the non-motile stage with daughter cells positioned in reversed orientation. Daughter cells develop new thecae and flagella within the parental theca, which eventually disrupts and releases the progeny cells. *Tetraselmis* species can be found in marine and freshwater ecosystems (John et al., 2002) and are often euryhaline and eurythermal. The best-known species are *T. chui* and *T. suecica*, being widely used in aquaculture as feed for juvenile molluscs, rotifers and shrimp larvae due to their biochemical composition and nutritional value (Meseck et al., 2005; Sharawy et al., 2020).

Tetraselmis sp. CTP4 was isolated from the natural park of Ria Formosa in the Algarve, Portugal by a FACS procedure sorting for lipid-rich strains (Pereira et al., 2016). Phylogenetic analysis by 18S rDNA gene classified this strain to be a member of the *Tetraselmis striata/convolutae* clade. More recently, due to the full sequencing of its genome, it has been confirmed that the 18S, 28S, 5.8S, ITS1 and ITS2 sequences of CTP4 are virtually indistinguishable from those of two strains classified as *T. striata,* namely LANL and SAG 41.85 (J. Varela, personal communication). Cells of this strain represent a large cell size ranging from 15-30 µm depending on culture conditions (Figure 1.5). Because of its ability to grow at 5-35 °C and culture survival of temperatures up to 50 °C, this strain is considered eurythermal. Furthermore, *T. striata* CTP4 is euryhaline and can be grown in urban

wastewater or fertilized seawater with salinities ranging from 5 to 100 ppt (Santos, 2014; Schulze et al., 2017), outcompeting most contaminants. All these advantages made it possible to scale up cultures of *T. striata* CTP4 to industrial 100-m^3 photobioreactors (Pereira et al., 2018a). Additionally, a low-cost harvesting procedure can be achieved by sedimentation, as CTP4 cells are large and usually lack flagella at late exponential or stationary phase, settling down to 18% of the original culture volume in 6 h (Pereira et al., 2016; Trovão et al., 2019). This robust strain possesses a well-balanced biochemical profile containing 20-32% of protein, 7-50% of lipids and about 18% of carbohydrates depending on culture conditions, thus making it suitable for nutritional applications or as feed stock for biodiesel production (Pereira et al., 2019, 2016). Moreover, cells of *T. striata* CTP4 synthesize significant levels of chlorophyll, carotenoids and *n*-3 polyunsaturated fatty acids, which are known to display biological activities and are associated with promoting human health (Pereira et al., 2019). The presence of these compounds along with the robustness of this species, makes it an interesting candidate to investigate carotenoid production and further strain improvement.
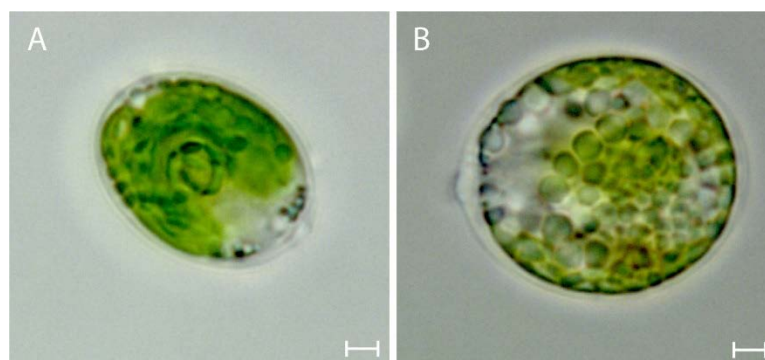
Figure 1.5. Microscopic observation of *Tetraselmis striata* CTP4 grown under nitrate replete (A) and deplete (B) conditions. Scale bar = 5 μm

1.7 *Chlorella vulgaris*

Chlorella vulgaris belongs to the class Trebouxiophyceae and is one of the core chlorophyte species (Figure 1.6). The cells are spherical, subspherical or ellipsoid shaped without flagella and with sizes ranging from 2-10 μm. The single chloroplast of *C. vulgaris* is cup-shaped and with or without the presence of pyrenoids. Asexual reproduction occurs by autosporulation, whereby the mother cell ruptures and releases 4 daughter cells (Safi et al., 2014). *C. vulgaris* is widely distributed in freshwater as well as in marine and terrestrial ecosystems. By the early 1900, German scientists discovered that *Chlorella* is an

unconventional food source because of its high protein content (Milledge, 2011). Nowadays, this species is one of the few approved for human consumption and is produced in large-scale for feed and food, and for nutraceutical and cosmetic applications. *Chlorella vulgaris* displays high growth rates, resistance to wide environmental conditions and contaminants. Furthermore, it can be grown photoauto-, mixo- or heterotrophically in a broad range of bioreactors. The biomass is composed of 42-58% of proteins, 5-40% of lipids and 12-55% of carbohydrates depending on the growth conditions (Safi et al., 2014). *C. vulgaris* is one of the most researched species because of its biochemical composition containing also pigments such as chlorophyll, lutein and *β*-carotene as well as other bioactive compounds.

Chlorella vulgaris is grown industrially under photoautotrophic or heterotrophic conditions in the facilities of Allmicroalgae Natural Products S.A., Portugal. A two-stage cultivation using heterotrophically grown *C. vulgaris* to inoculate photoautotrophic 100 m^3 PBRs yielded a faster scale-up procedure and biomass containing protein and chlorophyll contents of $52.2 \pm 1.3\%$ of DW and 24.0 ± 1.6 mg g^{-1} DW, respectively (Barros et al., 2019). Because this species is already being used in industrial applications, its robustness and adaptability to changing conditions make this microalga an interesting candidate for further strain improvement.
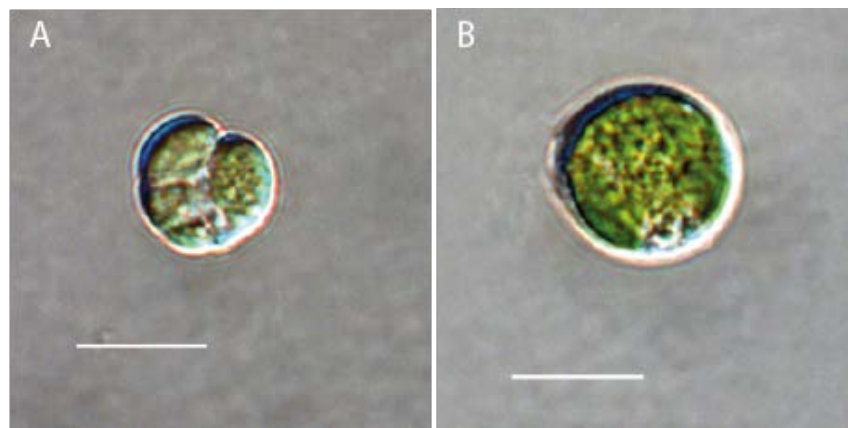
Figure 1.6. Microscopic observation of *Chlorella vulgaris* dividing (A) and resting (B) cells. Scale bar = 5 μm

1.8   Thesis aims and objectives

Although the research on pigment production by microalgae is an emerging field, little is known on how these compounds are produced under a broad range of growth conditions by microalgae of the genus *Tetraselmis*. In addition, only a few reports on improved strains for carotenoid production and the analysis of expression profiles of carotenogenic genes in microalgae are available.

Therefore, the main aim of this thesis is the upgrade of the biomass value of the marine microalgae *Tetraselmis* sp. CTP4. Particular focus is given on the improvement of carotenoid contents by physiological as well as genetic approaches. However, during the execution of this thesis, this aim was extended to *Chlorella vulgaris* in order to compare the generation of mutants between an obligate (*T. striata*) and non-obligate (*C. vulgaris*) photoautotroph.

Taking these aims in consideration, the specific objectives are as follows:

-   Improve the extraction and analysis of carotenoids from a robust microalga *Tetraselmis striata* CTP4
-   Investigate the influence of abiotic stress on carotenoid production and profile of *Tetraselmis striata* CTP4
-   Isolate carotenoid-rich mutants of *Tetraselmis striata* CTP4 and compare carotenoid and corresponding gene expression profiles under changing growth conditions
-   Isolate mutants of *Chlorella vulgaris* with differential pigment contents under heterotrophic growth conditions

Overall, this thesis is expected to provide new insights into pigment metabolism and carotenogenic gene expression under various environmental conditions as well as in the generation of mutants of obligate and non-obligate photoautotrophic microalgae.

CHAPTER 2

Trends and strategies to enhance triacylglycerols and high-value compounds in microalgae

Trends and strategies to enhance triacylglycerols and high-value compounds in microalgae

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Highlights

- TAG and carotenoids can be co-induced under stress, e.g., nitrogen depletion

- LC-PUFA usually accumulate during optimal growth, at low light or low temperature

- LC-PUFA-rich algae are able to partition LC-PUFA into TAG

- *Parietochloris incisa* has been identified as a potential triple-producer

- High-throughput methods are powerful tools to isolate algal strains with improved traits

Abstract

Microalgae are important sources of triacylglycerols (TAGs) and high-value compounds such as carotenoids and long-chain polyunsaturated fatty acids (LC-PUFAs). TAGs are feedstocks for biofuels or edible oils; carotenoids are used as pigments in the food and feed industries; and LC-PUFAs are beneficial for human health, being also key to the correct development of fish in aquaculture. Current trends in microalgal biotechnology propose the combined production of biofuels with high-value compounds to turn large-scale production of microalgal biomass into an economically feasible venture. As TAGs, carotenoids and LC-PUFAs are lipophilic biomolecules, they not only share biosynthetic precursors and storage sinks, but also their regulation often depends on common environmental stimuli. In general, stressful conditions favour carotenoid and TAG biosynthesis, whereas the highest accumulation of LC-PUFAs is usually obtained under conditions promoting growth. However, there are known exceptions to these general rules, as a few species are able to accumulate LC-PUFAs under low light, low temperature or long-term stress conditions. Thus, future research on how microalgae sense, transduce and respond to environmental stress will be crucial to understand how the biosynthesis and storage of these lipophilic molecules are regulated. The use of high-throughput methods (e.g., fluorescent activated cell sorting) will provide an excellent opportunity to isolate *triple-producers*, i.e., microalgae able to accumulate high levels of LC-PUFAs, carotenoids and TAGs simultaneously. Comparative transcriptomics between wild type and triple-producers could then be used to identify key gene products involved in the regulation of these biomolecules even in microalgal species not amenable to reverse genetics. This combined approach could be a major step towards a better understanding of the microalgal metabolism under different stress conditions. Moreover, the generation of triple-producers would be essential to raise the biomass value in a biorefinery setting and contribute to meet the world´s rising demand for food, feed and energy.

Keywords: TAG; Carotenoids; Long-chain polyunsaturated fatty acids; Microalgae; Biotechnology; Stressors

2.1 Commercially available species of microalgae and their evolutionary relationships

Phototrophic microalgae are of great interest as microscopic factories for the production of biomolecules for the energy and added-value compound markets. Compared with terrestrial plants, microalgae do not compete with crop plants for arable land. In addition, they have higher areal productivities, in some cases 20 times higher than those of terrestrial plants (Williams and Laurens, 2010), thus being a good alternative feedstock for feed, food and fuel. Only about 20 species are currently commercialised, mainly belonging to the unranked Stramenopiles / Haptista lineages or to Archaeplastida (Burki et al., 2016). Most prominent candidates of the Stramenopiles / Haptista lineages (Figure 2.1) include the genera *Nannochloropsis* and *Phaeodactylum* due to their high contents of the long-chain polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. These important microalgal-based ω-3 fatty acids find their applications in aquaculture, animal feed and nutraceutical industry and can replace those obtained from fish meal or oils. Furthermore, microalgae of the Archaeplastida lineage produced industrially are usually chlorophytes, such as *Dunaliella*, *Haematococcus* and *Chlorella*.
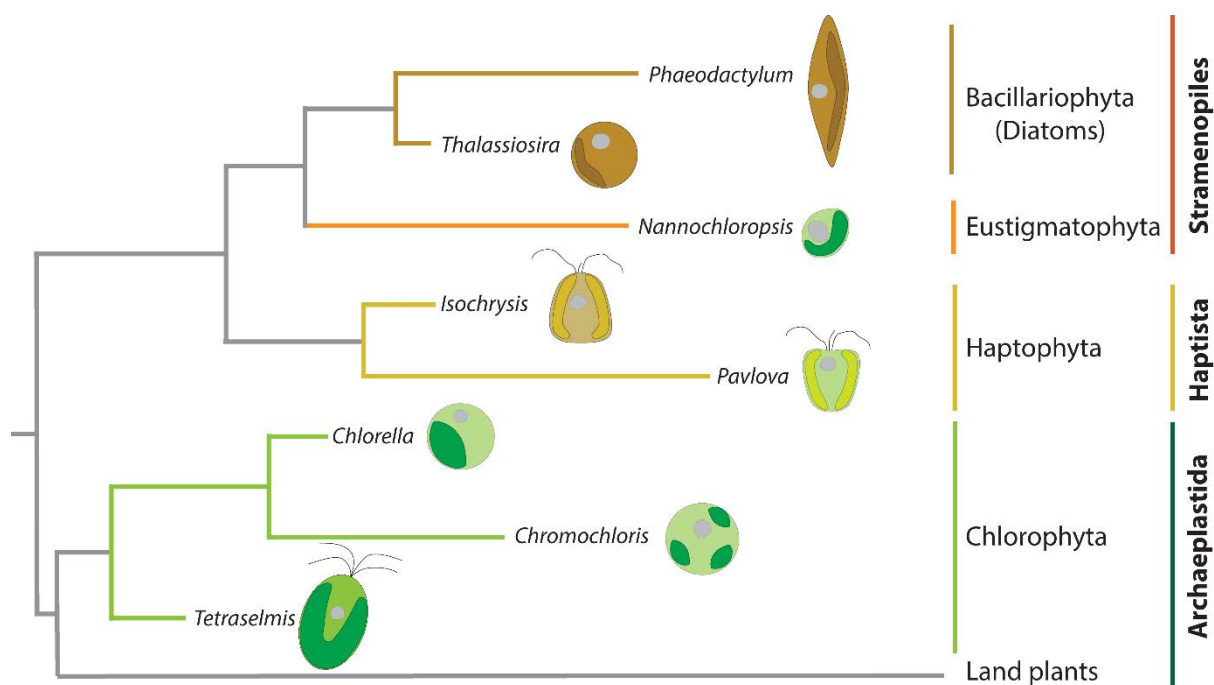
Figure 2.1. Evolutionary relationships of target microalgal lineages for combined production of TAGs, LC-PUFAs and carotenoids. Branch length does not represent distance values (adapted from Burki et al., 2016).

These algae are rich in the carotenoids *β*-carotene, astaxanthin and canthaxanthin used as pigments in food and feed (Borowitzka, 2013). Microalgae are also source of other carotenoids such as lutein and fucoxanthin which are utilized as antioxidants. Although, there have been efforts to use microalgal triacylglycerols (TAGs) as feedstock for biofuels, there is a current trend in microalgal research to combine the biosynthesis of LC-PUFA-rich TAGs with the accumulation of other high-valuable lipophilic compounds, such as carotenoids. In this sense, microalgal TAGs would be used as edible oil in food and feed applications as well as a vehicle for carotenoids.

The production of large amounts of TAGs, LC-PUFAs and carotenoids by microalgae depends on the species and growth conditions. Environmental stimuli such as nutrient availability, light intensity and temperature, among other factors, further increase the accumulation of TAGs and high-value compounds as a response to unfavourable growth conditions, thus leading to improved survival of the microalgal cell. Nevertheless, the biosynthesis, signalling pathways and mechanisms responsible for their accumulation are not yet fully understood (Li-Beisson et al., 2015).

This review focuses on the microalgal production of TAGs, LC-PUFAs and carotenoids as well as methodologies promoting their accumulation and pathways involved in the biosynthesis of these high-value biomolecules and lipids. Even though several reviews on each class of biochemicals have been published (Khozin-Goldberg et al., 2011; Klok et al., 2014; Martins et al., 2013; Minhas et al., 2016; Mulders et al., 2014b; Pienkos and Darzins, 2009; Varela et al., 2015), to the best of our knowledge a discussion on how the combined production of all three could be achieved in the same microalga is lacking. As these three classes of compounds can be considered to be lipids or lipophilic molecules, they share biosynthetic precursors, common environmental stimuli and accumulate in lipophilic "sinks", such as lipid droplets and bilayers. Results from various studies using environmental factors for the induction of the three compounds are summarized. For each biomolecule class, induction methodologies are compared and the most promising species to become *triple-producers* are indicated. We further provide an overview of current and future strategies to improve TAGs, LC-PUFAs and carotenoid production via the selection of promising target species and high-throughput isolation of naturally occurring mutants thereof. Knowledge gaps are discussed and future lines of research for the improvement of the biotechnological applications of microalgae are suggested.

2.2   General views of lipid metabolism of microalgae

Microalgal lipids can be classified as polar (e.g., phospho- and glycolipids) and neutral or non-polar (e.g., TAGs, sterols and waxes; Khozin-Goldberg and Cohen, 2011). The assembly of polar lipids and TAGs can occur in the chloroplast envelope or at the ER membrane, depending on the microalgal species (Figure 2.2). Polar lipids usually accumulate in membranes, such as the plasma, ER and thylakoid membranes, as well as the inner and outer membranes of the chloroplast and mitochondria, whereas TAGs are often deposited in plastidial or cytosolic lipid droplets. Both polar lipids and TAGs can be composed of LC-PUFAs that are synthesized at the ER by specific desaturases and elongases (Figure 2.2). Nevertheless, TAGs usually have high levels of saturated and monounsaturated fatty acids to maximize molecule packing, whereas membrane lipids tend to contain higher contents of LC-PUFAs (Guihéneuf and Stengel, 2013; Henderson et al., 1990).

Even though the exact topology of the enzymes involved in carotenoid biosynthesis has not been fully elucidated, it is often surmised that carotenoids are synthesized in the lipid compartment (Figure 2.2). Based on studies in land plants and microalgae, the biosynthesis of carotenoids can occur in several places in the chloroplast, namely the plastidial envelope, plastoglobuli and thylakoid membranes, which might also depend on the function of the synthesized carotenoids (Shumskaya and Wurtzel, 2013). For example, in *Dunaliella salina* var. *bardawil*, it has been proposed that *β*-carotene can be synthesized in the plastidial envelope as well as in plastoglobuli (Davidi et al., 2015). Furthermore, carotenoids are a very diverse group of 40-carbon isoprenoid biomolecules with more than 750 structures and different functions, whose biosynthetic pathways may occur in a given microalgal lineage and be partially absent in another (Varela et al., 2015). The xanthophylls (i.e., oxygen-containing carotenoids) astaxanthin and lutein occur mainly in Archaeplastida, whereas fucoxanthin and vaucheriaxanthin are mainly present in Stramenopiles / Haptista microalgae; *β*-carotene can be found in all three major microalgal lineages (Takaichi, 2011).
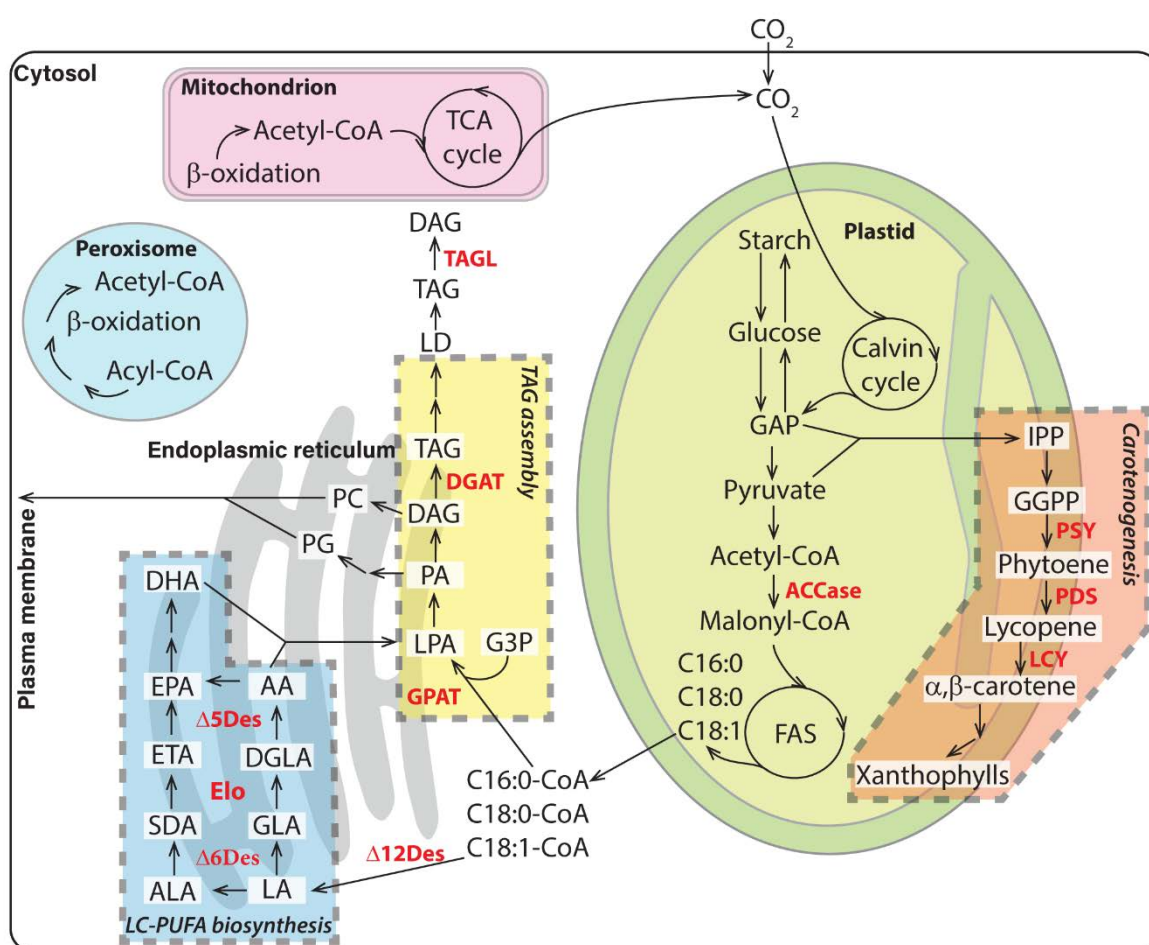
Figure 2.2. Simplified scheme of the compartments and the metabolic pathways of carotenogenesis, fatty acid synthesis and triacylglycerols (TAGs) assembly in microalgae. For simplicity, TAG assembly is only shown at the endoplasmic reticulum (ER) membrane, although it can also occur in the chloroplast envelope. Long-chain polyunsaturated fatty acids (LC-PUFAs) biosynthesis occurs at the ER membrane and the fatty acids are incorporated into phospholipids, glycolipids or TAGs in the cytoplasmic or plastidial membranes. Examples of LC-PUFAs are: linoleic (LA), *α*-linolenic (ALA), γ-linolenic (GLA), stearidonic (SDA), arachidonic (AA), dihomo-γ-linolenic (DGLA), eicosatetraenoic (ETA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids. When synthesized in large amounts, TAGs are usually packed into lipid droplets (LD) in the stroma or cytosol. The backbone glycerol-3-phosphate (G3P) is esterified with three fatty acids to form TAGs, including the intermediates lysophosphatidate (LPA), phosphatidic acid (PA) and diacylglycerol (DAG). During TAG assembly, phospholipids such as phosphatidylglycerol (PG) and phosphatidylcholine (PC) are also synthesized and are later incorporated into the plasma, ER and / or thylakoid membranes. Although the topology of carotenoid biosynthesis is not fully known, current evidence suggests that it occurs mainly at the plastidial envelope, though it might also occur in the stroma, thylakoid membrane and plastidial lipid droplets (also known as plastoglobuli). Carotenogenesis starts with isopentenyl pyrophosphate (IPP), which is synthesized from the precursor molecules pyruvate and glyceraldehyde-3-phosphate (GAP). After several condensation steps, geranylgeranyl pyrophosphate (GGPP) is converted into phytoene, the first carotenoid to be synthesized. Stepwise desaturation reactions lead to lycopene, which can follow two different routes, yielding either *α*- or *β*-carotene. Their hydroxylation can give rise to high-value xanthophylls: e.g., lutein and astaxanthin. Enzymes found upregulated under inductive conditions or used in attempts at metabolic engineering in strains under- or overexpressing them are shown in red: acetyl-CoA carboxylase (ACCase), fatty acid synthase (FAS), desaturase (Des), elongase (Elo), glycerol-3-phosphate acyltransferase (GPAT), diacylglycerol acyltransferase (DGAT), triacylglycerol lipase (TAGL), phytoene desaturase (PDS), phytoene synthase (PSY), and lycopene cyclase (LCY).

2.3 Induction of biosynthesis and accumulation of TAGs, LC-PUFAs or carotenoids in microalgae

Under optimal growth conditions microalgae are able to display fast growth rates with doubling times as low as 10 h (e.g., *Chlamydomonas reinhardtii*; Griffiths and Harrison, 2009), producing biomass with low amounts of lipids. However, under sup-optimal growth conditions algae tend to accumulate neutral lipids, carbohydrates and / or carotenoids and are thus able to adapt to environmental extremes. The biosynthesis and accumulation of specific compounds depend, however, on the genetics of the microalgal species, growth phase, nutrient availability, salinity, temperature and irradiation. The research focusing on how TAGs, carotenoids and LC-PUFAs can be induced rests heavily on the evolutionary lineage of the microalga under study. Recent trends indicate that Stramenopiles microalgae (e.g., *Nannochloropsis*) are often researched for their TAGs and LC-PUFAs content, whereas chlorophytes (e.g., *Chlorella*) are the target of most reports on TAGs and carotenoids.

2.3.1 TAG induction

Microalgae such as *Chlorella vulgaris*, *Chromochloris* (syn. *Chlorella*) *zofingiensis*, *Chlorococcum littorale*, *Nannochloropsis oceanica*, *Neochloris oleoabundans* and *Scenedesmus obliquus* are able to accumulate large amounts of TAGs under stress conditions. A common trigger is nutrient depletion, which also occurs upon entry into stationary phase. These non-optimal conditions lead to decreased cell growth and proliferation. In turn, this may entail a lower need for membrane lipids and a larger amount of fatty acids being diverted to TAG assembly (Fan et al., 2011; Goodson et al., 2011; Li-Beisson et al., 2015).

Apart from being a mechanism of carbon and energy storage, TAG biosynthesis and assembly might have additional functions (Hu et al., 2008). In photosynthetic organisms, the energy of photons captured by the photosystems or associated antennae provide electrons able to reduce $NADP^+$ to NADPH. The reduced form of this electron carrier is then oxidized by biosynthetic pathways, becoming available to photosynthesis once again. As the biosynthesis of lipids requires roughly the double amount of NADPH than that of protein or carbohydrates, pathways leading to TAG accumulation might therefore protect microalgal cells from excess electrons produced under stress conditions. This can take place when the capacity of the photosynthetic electron transport chain is exceeded and electrons accumulate, resulting in the generation of reactive oxygen species (ROS).
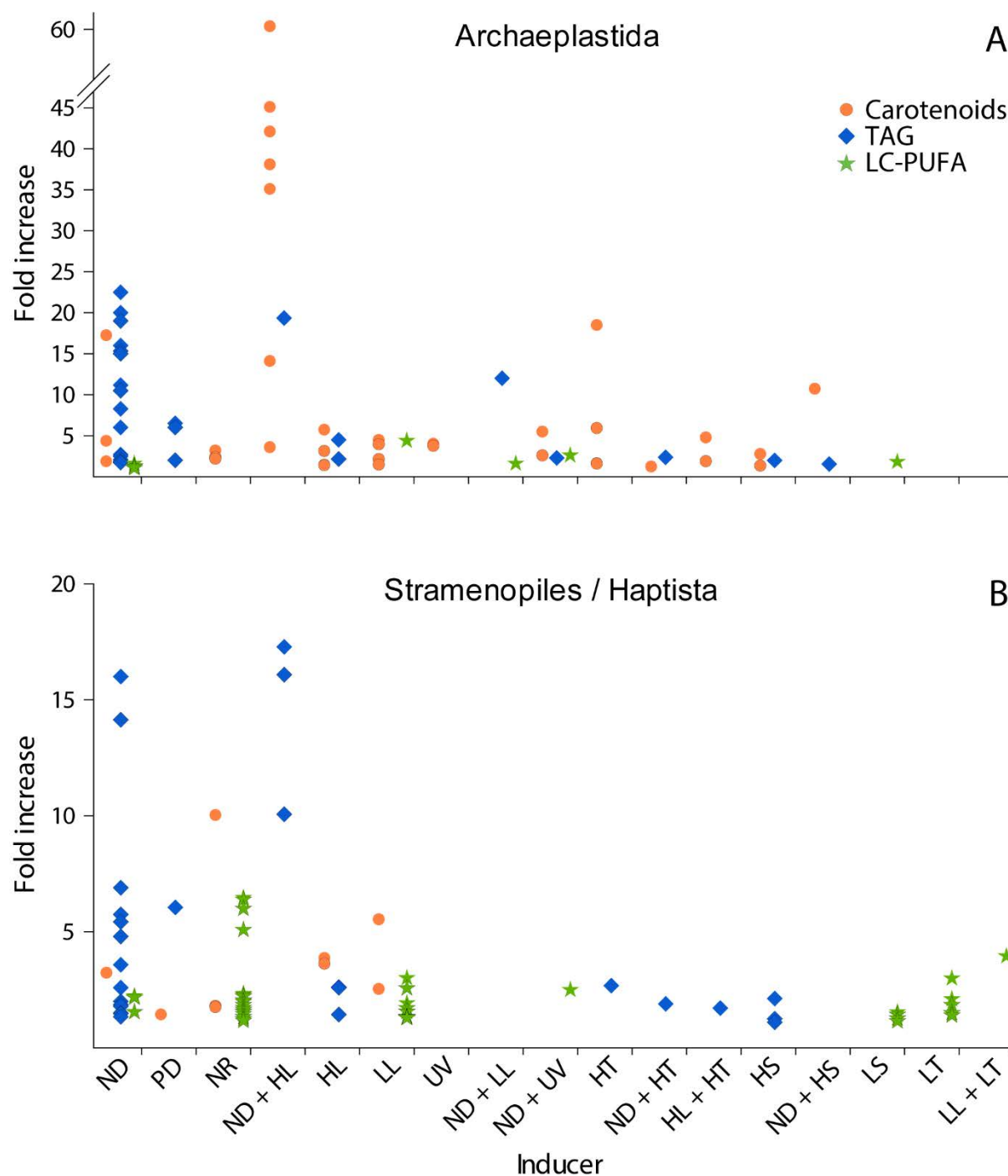
Figure 2.3. Fold increase of TAGs, carotenoids and LC-PUFAs in microalgal species belonging to the evolutional lineage of Archaeplastida (chlorophytes, A) and Stramenopiles / Haptista (diatoms, eustigmatophytes and haptophytes, B) under different inducers. The fold increase of each compound was calculated as ratios of the amount under control and inducing conditions to normalize the data. This analysis includes 86 studies on TAGs (blue diamonds), carotenoids (orange circles) and / or LC-PUFAs (green stars) induction methods, respectively (Table S2.1 in supplementary data). The inducers were as follows: nitrogen depletion (ND), phosphorus depletion (PD), high light (HL), low light (LL), UV-C radiation (UV), high temperature (HT), low temperature (LT), high salinity (HS), low salinity (LS) or nitrogen repletion (NR) and combinations thereof.

Although ROS are constantly produced in the cell and may have an important function as signalling molecules (Lamers et al., 2008), an imbalance of ROS can lead to oxidative stress resulting in damage of photosystems and oxidation of DNA, protein and lipids.

Regardless of the evolutionary lineages, nitrogen depletion was the most tested induction technique with an up to 20-fold increase (Figure 2.3), leading to contents as high as 45% TAGs of dry weight (DW) after starving *C. vulgaris* for 14 days (Breuer et al., 2012). Other species accumulating high amounts of TAGs (35-42% of DW) under nitrogen depletion are *N. oleoabundans*, *C. zofingiensis, C. littorale, S. obliquus, Nannochloropsis gaditana* and *N. oceanica* (Breuer et al., 2012; Cabanelas et al., 2016; Meng et al., 2015; Mulders et al., 2014a; Simionato et al., 2013). For some microalgae (e.g., *Nannochloropsis oculata* and *C. zofingiensis*), TAG content was improved from 32 to 40% TAGs of DW by exposing them to higher light intensities (250-350 µmol m^{-2} s^{-1}; Liu et al., 2016; Ma et al., 2016).

The existence of common inducers for TAGs in microalgae of different lineages could be the result of the conservation of the TAG assembly pathway. As a matter of fact, genes coding for key enzymes of the TAG assembly (e.g., acyltransferases) resembling those present in plant cells are also found in microalgae (Li-Beisson et al., 2015). This assertion can be supported by studies showing the upregulation of diacylglycerol acyltransferase (DGAT), an enzyme catalysing the last step of the TAG assembly, in *C. reinhardtii* and *N. oceanica* under nitrogen starvation (Figure 2.2). Under nitrogen starvation and high light, the NADPH levels as well as the enzymatic activity of DGAT and acyl-CoA carboxylase (ACCase) increase (Ma et al., 2016). Hence, besides TAG assembly, fatty acid biosynthesis appears to be induced by these two stressors as well. Nevertheless, the effectiveness of nitrogen depletion as inducer of TAGs and fatty acid biosynthesis is species- and also time-dependent. Furthermore, phosphorus depletion has also been shown to be an efficient inducer of TAG biosynthesis in *Scenedesmus* sp., *Monodus subterraneus* and *C. vulgaris*, though yielding a more modest increase (≈ 6-fold) as compared to that obtained under nitrate depletion (Figure 2.3; Chia et al., 2013; Khozin-Goldberg and Cohen, 2006; Liang et al., 2013; Wu et al., 2015). In fact, depletion of nutrients is one of the preferred triggers of TAG accumulation, as a decrease in nutrient levels added to cultures can significantly reduce costs in large-scale processes.

High salinity leads to oxidative stress, a condition that has been described to induce the accumulation of lipids in both freshwater and marine microalgae, probably as a response to an unfavourable environment for growth and the need for storing energy-rich compounds to be able to survive to a harsher environment (Pancha et al., 2015). However, further research

on the mechanisms of lipid upregulation under high salinity is still needed. The marine species *Nannochloropsis salina* and *Dunaliella tertiolecta* accumulated double the amount of TAGs under growth in high salinities of 58 ppt compared to 29 ppt (Bartley et al., 2013; Takagi et al., 2006). In *Scenedesmus* sp., a salinity shift from freshwater to 23 ppt resulted in a TAG content of 25% of DW, whereas under nitrogen depletion TAGs were accumulated up to 38% of DW (Breuer et al., 2012; Pancha et al., 2015). Thus, salinity as inducer of TAG accumulation is apparently not as efficient as nutrient depletion. Nevertheless, addition of salt to the growth media can reduce the risk of contamination and therefore halotolerant species might be suitable candidates for production in outdoor ponds.

High light showed an effect on TAG accumulation in the chlorophyte *D. salina* var. *bardawil*, as well as in the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* accumulating up to 39.5% of DW (Brown et al., 1996; Nogueira et al., 2015; Rabbani et al., 1998).

Another strategy for high TAG accumulation is the combination of stress factors, such as nitrogen depletion and high temperature (33 °C), which caused a 2-fold increase in *Scenedesmus obtusus* and *N. salina* (Fakhry and Maghraby, 2015; Xia et al., 2016). A small dose of UV-C radiation under nitrogen depletion was able to double the TAG content in *Tetraselmis* sp. in only 2 days (Sharma et al., 2014). Conversely, the combination of high light (300 µmol m^{-2} s^{-1}) and thermal stress (30 °C) was effective for the inducing TAG accumulation (up to 32% of DW) in the haptophyte *Isochrysis galbana* (Nogueira et al., 2015).

2.3.2 LC-PUFA induction

In most microalgal species, LC-PUFAs are mainly found in membrane lipids such as phospholipids and glycolipids, as these are important structural and functional components of the cell membranes, acting also in cellular signalling and playing an important role in the physiology of the microalgal cell (Martins et al., 2013). Furthermore, EPA and DHA are considered to be important to maintain optimal membrane fluidity upon temperature, salinity or light intensity shifts. Additionally, LC-PUFAs, especially those of the ω-3 series, can act as antioxidants, protecting microalgal cells from oxidative damage caused by ROS (Okuyama et al., 2008; Richard et al., 2008).

Large amounts of LC-PUFAs can be found in Stramenopiles and Haptista microalgae, probably due to the presence of an ω-3 desaturase able to convert arachidonic acid (AA) to EPA or specific Δ5 elongase and Δ4 desaturase activities for the synthesis of DHA from EPA

(Figure 2.2). The synthesis of EPA and DHA is elevated under conditions promoting growth such as nitrogen and phosphorus repletion, most probably due to elevated synthesis of membranes in actively growing cells (Figure 2.3). Under nitrogen repletion, the highest EPA contents of total fatty acids (TFA) were observed in *N. oceanica* (32% of TFA) and *Pavlova lutheri* (29% of TFA), whereas *P. tricornutum* (30% of TFA) and *I. galbana* (14% of TFA) displayed the highest DHA contents (Liu et al., 2013; Meng et al., 2015; Tonon et al., 2002). Furthermore, low light conditions are often reported as a factor for increasing LC-PUFA contents, which might be due to an increase in thylakoid membranes to counterbalance the lower light availability (Berner et al., 1989; Fisher et al., 1998). EPA, a major chloroplast fatty acid in the eustigmatophyte *Nannochloropsis* sp., increased up to 38% of TFA when exposed to low light intensity (50-60 µmol m^{-2} s^{-1}; Ma et al., 2016; Mitra et al., 2015b; Van Wagenen et al., 2012). Conversely, the DHA content increased from 12.6% to 19.2% and 8% to 14% of TFA in *P. lutheri* and *I. galbana*, respectively, under high light (460 µmol m^{-2} s^{-1}; Guihéneuf et al., 2009; Tzovenis et al., 1997). Higher levels of this LC-PUFA might be advantageous to microalgal cells due to their antioxidant properties, thus preventing the photooxidation of key components of the photosynthetic apparatus (Thompson et al., 1990; Tzovenis et al., 1997).

Another important inducer of LC-PUFAs is temperature. Thermal shifts cause alterations in the fluidity of the cytoplasmic and thylakoid membranes, which in turn can be regulated through their fatty acid composition. Low temperatures increase lipid order in membranes, whereas high temperatures cause the opposite effect, enhancing the fluidization of membranes. Membrane fluidity is important for the function of light harvesting complexes, photosystems and membrane proteins such as translocators, sensor proteins and ion channels (Los et al., 2013). Therefore, under low temperature, usually more PUFAs are synthesized and incorporated into the membrane to maintain its correct fluidity. Decreasing temperatures (13-17 °C) increase 2-3 times the EPA and DHA contents in several mesophilic species (Jiang and Gao, 2004; Tatsuzawa and Takizawa, 1995; Van Wagenen et al., 2012). The application of both low light (30 µmol m^{-2} s^{-1}) and low temperature (10 °C) even led to a 4-fold increase of EPA content (28% of TFA) in phospholipids in *Nannochloropsis* sp. (Mitra et al., 2015b).

Furthermore, salinity might also alter the fatty acid composition of microalgae. Under high salinity, there is usually a trend towards a higher saturation of the fatty acids. The adjustment of membrane permeability needed to avoid an extensive influx of Na$^+$ and Cl$^-$ ions might explain this response. The opposite trend is seen at low salinities, which seem to promote higher unsaturation levels in fatty acids. In *Nannochloropsis* sp. and *I. galbana*, low

salinity of 10 ppt led to increased contents of EPA and DHA, respectively (Gu et al., 2012; Pal et al., 2013; Renaud and Parry, 1994).

Within the Archaeplastida lineage, the chlorophytes *Parietochloris incisa* and *Tetraselmis* sp. have been reported to accumulate significant amounts of LC-PUFAs. Under nitrogen depletion, EPA increased marginally in *Tetraselmis* sp. (10% of TFA), which might be further triggered by exposure to UV-C radiation (Sharma et al., 2014; Tsai et al., 2016). *P. incisa* is known for its high amounts of AA, which can reach 60% of TFA under low light (30 µmol m^{-2} s^{-1}) and nitrogen depletion (Solovchenko et al., 2008a).

2.3.3 Carotenoid induction

In photoautotrophic organisms, carotenoids are essential accessory pigments in the photosystems and light harvesting complexes, playing an important role in energy transfer during photosynthesis (Varela et al., 2015). Furthermore, under stress conditions, such as excess light, heat stress and nutrient depletion, the accumulation of ROS like singlet oxygen and free radicals can be quenched and scavenged by carotenoids, respectively. Because of this protective role, a few carotenoids (e.g., lutein and *β*-carotene) are essential components of the photosynthetic machinery, being present in the thylakoid, plastidial, ER, and mitochondrial membranes. However, there are other carotenoids (e.g., astaxanthin and canthaxanthin) that are only synthesized when specific environmental cues stimulate their accumulation.

The best induction technique for carotenoids in chlorophytes is high light under nutrient depletion resulting in increases of up to 60-fold due to very low initial carotenoid contents (Figure 2.3). *H. pluvialis* displayed the highest astaxanthin content (30 mg g^{-1} DW) upon exposure to a light intensity of 540 µmol m^{-2} s^{-1} under nitrogen depletion (Imamoglu et al., 2009). Under similar conditions, in *C. zofingiensis*, astaxanthin accumulated as high as 4.9 mg g^{-1} DW, which is more than double the concentration (2.4 mg g^{-1} DW) obtained upon nitrogen depletion (Liu et al., 2016; Mulders et al., 2014a). Another producer of astaxanthin is *N. oleoabundans* with a content of 6.9 mg g^{-1} DW under nitrogen starvation (Urreta et al., 2014). The concomitant exposure to high light might improve, however, these values in the future. Concerning *β*-carotene, the highest production was observed in *D. salina* (70 mg g^{-1} DW) under high light (1000 µmol m^{-2} s^{-1}) and nitrogen depletion (Lamers et al., 2010). Nevertheless, in this species, high light and salt stress can only induce carotenoid accumulation when nutrients are limiting. Under these conditions, the levels of *PSY* and *PDS*

transcripts encoding the enzymes responsible for the initial steps in carotenogenesis (Figure 2.2) have been found to be upregulated (Coesel et al., 2008). However, in *C. vulgaris* and *P. incisa*, high light has been applied to enhance *β*-carotene contents 3- and 2-fold, respectively (Seyfabadi et al., 2011; Solovchenko et al., 2008b).

Another important carotenoid in Archaeplastida is the xanthophyll lutein, a structural component of the light harvesting complex, occurring thus in the thylakoid membranes rather than in lipid droplets. The multiple functions of this orange pigment range from light harvesting to protection of the microalgal cell from excess light (Mulders et al., 2014a). Thus, high lutein concentrations can be found under conditions promoting photosynthetic efficiency and cell growth. Lutein-rich species under nitrogen repletion are *Muriellopsis* sp*., N. oleoabundans* and *Chlorella sorokiniana* with contents of up to 20 mg g^{-1} DW (Del Campo et al., 2000; Urreta et al., 2014). However, heat stress (33 °C) led to enhanced accumulation of lutein in *Muriellopsis* sp*.* and *Scenedesmus almeriensis* (Del Campo et al., 2000; Sánchez et al., 2008). Light intensity and quality are also important factors for lutein accumulation. The highest induction was observed in *Tetraselmis suecica* under short exposure to UV-C-radiation resulting in a 4-fold increase (5 mg g^{-1} DW; Ahmed et al., 2015). In *C. zofingiensis*, the lutein content doubled under a relatively low light intensity of 90 µmol m^{-2} s^{-1} (Del Campo et al., 2004). This enhanced lutein content under low light conditions could be due to a larger chloroplast and a concomitant increase in the number of thylakoids and associated pigment molecules, resulting in a more efficient light absorption and utilization (Berner et al., 1989). Nevertheless, in *S. almeriensis*, high light (1700 µmol m^{-2} s^{-1}) increased the levels of lutein, which could be further enhanced (up to 0.54% of DW) by heat stress (44 °C; Sánchez et al., 2008).

In Stramenopiles and Haptista microalgae, the highest increase in the carotenoids vaucheriaxanthin and *β*-carotene (≈ 4-fold) was reported in *N. gaditana* upon exposure to high light (1600 µmol m^{-2} s^{-1}; Camacho-Rodríguez et al., 2015). Under nitrogen depletion, *N. gaditana* and *N. oceanica* showed a 3-fold increase of vaucheriaxanthin, canthaxanthin and zeaxanthin (Simionato et al., 2013; Solovchenko et al., 2014), whereas the levels of fucoxanthin in the haptophyte *I. galbana* and in the diatom *P. tricornutum* under nitrogen repletion and low light (13.5 µmol m^{-2} s^{-1}) increased from 0.06 to 0.15 and 0.08 to 0.2 mg g^{-1} DW, respectively (Gómez-Loredo et al., 2016). However, further research on microalgae of these evolutional lines is needed to find carotenoid producers and the best induction techniques.

2.4 Induction of TAGs and / or carotenoids and LC-PUFAs

2.4.1 Induction of both TAGs and LC-PUFAs

In most microalgae, under stress conditions such as nutrient depletion or in stationary phase, accumulated TAGs are mainly composed of saturated and monounsaturated fatty acids. Under these conditions, usually a decline of LC-PUFAs can be observed. Nevertheless, in *N. oculata*, *P. lutheri, P. tricornutum, T. pseudonana* and *P. incisa*, LC-PUFAs have been found to increase under TAG-inductive conditions (Guihéneuf and Stengel, 2013; Khozin-Goldberg et al., 2002; Tonon et al., 2002; Yu et al., 2009). In *P. incisa*, under nitrogen starvation, the biosynthesis of AA was elevated due to transcriptional upregulation of the genes encoding Δ12, Δ5 and Δ6 desaturases, followed by accumulation of this fatty acid in TAGs rather than in phospho- or glycolipids (Bigogno et al., 2002; Iskandarov et al., 2010). LC-PUFAs deposited in TAGs could be used for an expedite adjustment of the composition of the cell membranes in response to a fast changing environment, avoiding a slower cycle of re-synthesis, assembly and deployment (Khozin-Goldberg et al., 2005). Alternatively, the observed increase in phospholipid / diacylglycerol acyltransferase (PDAT) and / or phospholipases in *P. tricornutum* suggests that LC-PUFAs (e.g. EPA) can be remodelled from membrane lipids into TAGs (Mus et al., 2013). This inverse flow, however, needs to be further investigated. Interestingly, species reported for being able to partition LC-PUFAs into TAGs seem also to contain high amounts of EPA, DHA or AA, but this apparent trend needs to be further researched to ascertain whether this holds true for a larger spectrum of microalgal species and whether upon long-term stressful conditions (> 14 days) these LC-PUFAs are diverted to TAGs as described previously (Tonon et al., 2002).

2.4.2 Induction of both TAGs and carotenoids

In some species, TAGs and carotenoids accumulate under the same stress conditions, namely nitrogen depletion, alone or in combination with high light. A possible explanation for this could be that lipophilic carotenoids need TAG-rich lipid droplets in order to accumulate (Ben-Amotz et al., 1989). Moreover, it has been suggested that both TAG accumulation and carotenogenesis are interdependent. In *D. salina*, a correlated increase in oleic acid, one of the most abundant fatty acids in TAGs / lipid droplets, and *β*-carotene has been reported, a likely consequence of their co-accumulation in stromal lipid globules (Rabbani et al., 1998). Conversely, astaxanthin accumulation in freshwater *H. pluvialis* and *C. zofingiensis* was found to take place in cytosolic lipid droplets, probably due to the export of this xanthophyll from the

plastid to the cytoplasm. These extrathylakoidal carotenoids could protect the chloroplast from photooxidation under unfavourable conditions such as excess light (Liu et al., 2014; Wang et al., 2003). This suggests that in other species, such as *N. gaditana* or *N. oleoabundans*, the accumulated carotenoid esters under stress conditions might be deposited in lipid droplets as well (Simionato et al., 2013; Solovchenko et al., 2014).

2.4.3 Induction of both LC-PUFAs and carotenoids

In studies of Stramenopiles / Haptista microalgae carotenoids and LC-PUFAs have been found to be elevated under conditions promoting growth or low light. These conditions promote membrane adjustments and an increase in antenna size as described above. The LC-PUFAs are incorporated into polar lipids of non-photosynthetic and thylakoid membranes. Carotenoids under these conditions have an important function in the light harvesting process. Therefore, in species such as *N. oceanica, P. tricornutum* and *I. galbana*, high amounts of EPA and DHA accumulate together with *β*-carotene, violaxanthin and fucoxanthin (Gómez-Loredo et al., 2016; Liu et al., 2013; Ma et al., 2016; Meng et al., 2015; Wu et al., 2016).

2.5 Strategies to improve TAG, LC-PUFA and carotenoid production

2.5.1 Triple-producers

From what has been said above, one of the key goals of current efforts in the field of microalgal biotechnology is to find triple-producers, i.e., microalgal species or strains able to accumulate TAGs, LC-PUFAs and carotenoids simultaneously. A general trend seen in the literature is that, when compounds of two of the three classes of biochemicals increase, compounds of the third class often decrease. For example, LC-PUFAs usually do not accumulate when TAGs and carotenoids accumulate, although LC-PUFAs accumulate together with carotenoids under decreasing amounts of TAGs. A possible solution to this problem and an important feature of a triple-producer would be the incorporation of LC-PUFAs in TAGs as found in species discussed previously (see section 2.4.1). A second feature of such a microalga would be the accumulation of carotenoids in lipid bodies. In this manner, one stress factor like nitrogen depletion could induce the accumulation of high amounts of edible oils containing LC-PUFA-rich TAGs and carotenoids.

Table 2.1 Possible target species for improvement towards simultaneous production of LC-PUFAs in triacylglycerols (TAGs) and carotenoids and their productions found in literature.

| Species | Inducer | TAGs [% DW] | Carotenoids [mg g^{-1} DW] | | | | | LC-PUFAs [% TFA] | | | References |
			Ast	β-car	Lut	Vau	Fuc	AA (20:4)	EPA (20:5)	DHA (22:6)	
Archaeplastida											
C. zofingiensis	ND + HL	12-40	4.9-6.3								(Liu et al., 2016; Orosa et al., 2001; Pelah et al., 2004)
N. oleoabundans	ND	12-42	6.9								(Breuer et al., 2012; Klok et al., 2013; Pruvost et al., 2009; Urreta et al., 2014)
	NR				19.4						(Urreta et al., 2014)
P. incisa	ND	32						47-60			(Khozin-Goldberg et al., 2002; Solovchenko et al., 2008a, 2008b)
	HL			9.3[b]	11.6[b]						(Solovchenko et al., 2008b)
Haptista											
I. galbana	HL + HT	32									(Nogueira et al., 2015)
	LL						0.15			17	(Gómez-Loredo et al., 2016; Liu et al., 2013)
P. lutheri	ND	75[a]							11	9	(Guihéneuf and Stengel, 2013; Tonon et al., 2002)
Stramenopiles											
N. gaditana	ND	38									(Mitra et al., 2015a; Simionato et al., 2013)
	HL			1.8[c]		1.5[c]					(Camacho-Rodríguez et al., 2015)
	LL								38		(Mitra et al., 2015a)
N. oculata	ND + HL	40-43							170[d]		(Ma et al., 2016; Tonon et al., 2002; Van Vooren et al., 2012)
	NR / LL								20-35		(Huang et al., 2013; Ma et al., 2016)
N. oceanica	ND	35									(Meng et al., 2015)
	NR / LS			0.4					16-32		(Meng et al., 2015; Solovchenko et al., 2014)
P. tricornutum	ND / HL	23-40							150[d]		(Breuer et al., 2012; Nogueira et al., 2015; Tonon et al., 2002)
	NR + LL						5.5	20-30		3	(Gómez-Loredo et al., 2016; Wu et al., 2016)
T. pseudonana	ND	14							375[d]	40[d]	(Brown et al., 1996; Tonon et al., 2002)
	NR								16-32		(Brown et al., 1996; Tonon et al., 2002)

The inducers were as follows: nitrogen depletion (ND), high light (HL), low light (LL), low UV-C-radiation (LU), high salinity (HS), low salinity (LS) or nitrogen repletion (NR). Carotenoids are: astaxanthin (Ast), β-carotene (β-car), lutein (Lut), fucoxanthin (Fuc) and vaucheriaxanthin (Vau). LC-PUFAs are arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Data in units other than those given in the header for a particular column are indicated with numbers in superscript, namely: [a] – % of TFA, [b] – nmol ml^{-1}, [c] – mg L^{-1} day^{-1}, [d] – fg cell^{-1}. More detailed information can be found in Table S2.1 in the electronic version of this dissertation.

From the data gathered in the sections above, the most likely species to be a possible triple-producer without genetic modification is *P. incisa* (Table 2.1). In this species, the accumulation of TAGs containing AA could be accompanied by high *β*-carotene and / or lutein contents under stress conditions (Bigogno et al., 2002; Khozin-Goldberg et al., 2002; Solovchenko et al., 2008a). Nevertheless, so far, no study has reported on the best conditions for the co-accumulation of all three biotechnologically relevant classes of compounds and respective contents.
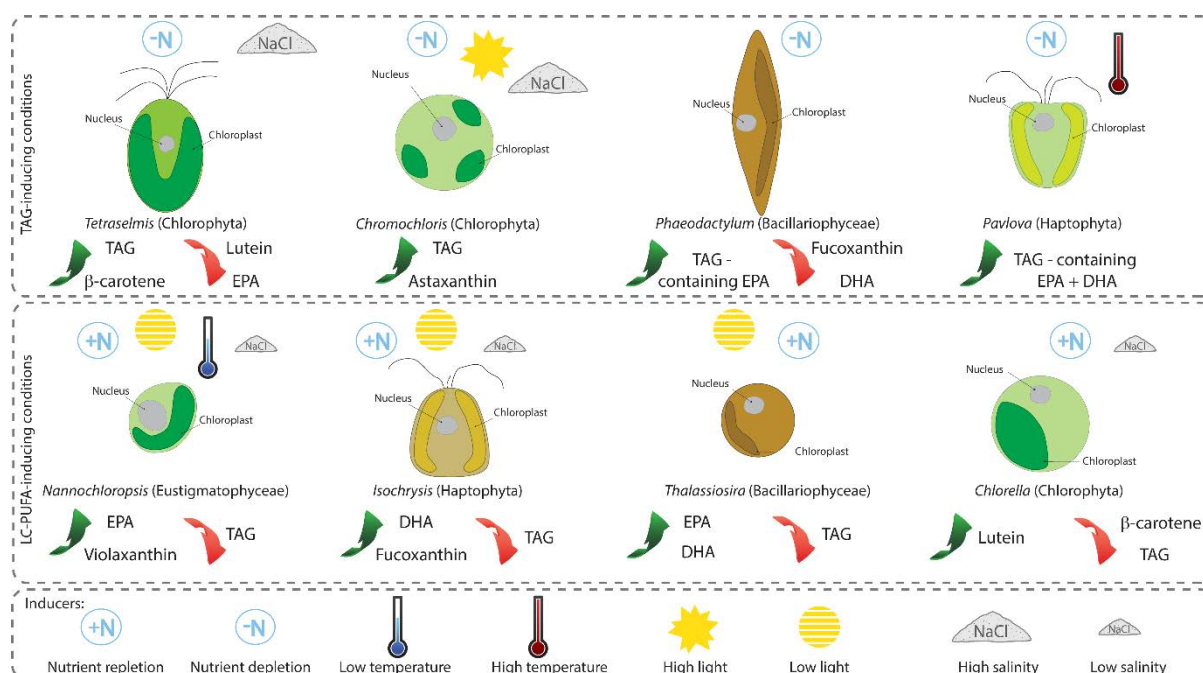
Figure 2.4. Simplified illustration of microalgal species with their relative content of TAGs, LC-PUFAs and carotenoids upon exposure to different inducers applied alone or in combination. In the upper panel TAG-inducing stressor such as nutrient depletion, high temperature, high light and high salinity are shown, whereas stressors such as low temperature, low light and nutrient replete conditions usually lead to elevated LC-PUFA contents as represented in the lower panel.

Alternative candidates as triple-producers are microalgae belonging to the genus *Nannochloropsis* (Figure 2.4). These microalgae are known for their high EPA contents up to 38% of TFA under nutrient sufficient growth conditions (Mitra et al., 2015b; Solovchenko et al., 2014) and have been shown to divert EPA into TAGs upon exposure to long-term starvation (Tonon et al., 2002). The main carotenoid of this genus is vaucheriaxanthin, whose contents increase also under TAG-inductive conditions. The low market value of this carotenoid might hinder further research, though. Alternatively, zeaxanthin produced by this species is more promising in terms of market value and applications as antioxidant and food

colorant (Borowitzka, 2013). Thus, the concurrent production of LC-PUFAs in TAGs and carotenoids in these species is a high possibility that should be further investigated in order to improve the content of both classes of high-value compounds using a common trigger.

Other species of interest are the haptophytes *I. galbana* and *P. lutheri* as well as the diatoms *P. tricornutum* and *T. pseudonana* due to their high contents of EPA and DHA that are partitioned into TAGs under nitrogen depletion (Figure 2.4). These microalgae belonging to the Stramenopiles and Haptista evolutionary lines contain significant amounts of fucoxanthin (Takaichi, 2011). However, this biomedically important xanthophyll decreases upon exposure of these microalgae to stress conditions. These microalgae can be improved, however, in the near future, by screening for mutants able to accumulate fucoxanthin together with LC-PUFA-rich TAGs under same growth conditions.

Eventually, the species *C. zofingiensis* and *N. oleoabundans* show potential as triple-producers due to the accumulation of high amounts of TAGs and astaxanthin. Nevertheless, the lack of significant amounts of LC-PUFAs hampers their use as a triple-producer. However, this drawback might be addressed by metabolic engineering or mutant selection via high-throughput methods.

2.5.2 Metabolic engineering

Metabolic engineering is one important tool for the improvement of specific biosynthesis pathways, which can also be employed to improve our knowledge of microalgal metabolic and cellular processes. Several authors have proposed that genetic engineering is the key to enhancing productivity of TAGs (reviewed by Bellou et al., 2016; Goncalves et al., 2016; Radakovits et al., 2010) or carotenoids and modifying the PUFA profile towards those fatty acids that better conform to the need of target industrial or nutritional applications (Hamilton et al., 2015). Among the metabolic engineering strategies to boost TAGs, efforts to overexpress enzymes involved in the biosynthesis of fatty acids (Dunahay et al., 1995) to manipulate TAG assembly (Hsieh et al., 2012; Liang and Jiang, 2013; Sanjaya et al., 2013) or to enhance the supply of carbon precursors or NADPH needed for the synthesis of TAGs (Talebi et al., 2014; Xue et al., 2015) have been reported. Other strategies involve the inhibition of competitive pathways such as starch biosynthesis or *β*-oxidation of lipids. For example, in *N. oceanica* under nitrogen starvation the LC-PUFA biosynthesis is inhibited by transcriptional downregulation of Δ12 desaturase catalysing the conversion of oleic acid into linoleic acid, a precursor of LC-PUFAs (Simionato et al., 2013). Interestingly, this limitation

can be overcome by overexpressing the endogenous enzyme, leading to increased accumulation of LC-PUFAs in TAGs under nitrogen starvation (Kaye et al., 2015). In *P. tricornutum* the overexpression of DGAT led to increased oil droplets with a simultaneous increase in EPA in TAGs (Niu et al., 2013). The same strategy also was used in *C. reinhardtii* with different results depending on the DGAT isoenzyme chosen (Iwai et al., 2014; La Russa et al., 2012). The partitioning of EPA, but not of DHA, into TAGs in *P. tricornutum* has already been shown by Tonon et al. (2002). Nevertheless, a different study in *P. tricornutum* used metabolic engineering of several desaturases and elongases leading to increased DHA synthesis with an ensuing accumulation in TAGs (Hamilton et al., 2014). A few attempts at engineering the biosynthesis of carotenoids have targeted the *PSY* and *PDS* genes encoding phytoene synthase and desaturase, respectively. These enzymes catalyse the first two steps of the carotenoid biosynthetic pathway, thus diverting isoprenoid compounds into this pathway (Figure 2.2). The successful expression of exogenous PSY in the model organism *C. reinhardtii* led to an increase in lutein (Cordero et al., 2011a). Furthermore, keto-carotenoids such as astaxanthin that are not present in the wild-type of *C. reinhardtii* or *D. salina* can be synthesized by the expression of the *BKT* gene encoding *β*-carotene ketolase (Anila et al., 2016; León et al., 2007). Nevertheless, the engineering of metabolic pathways is often challenging and overproduction of the desired compound is difficult to achieve. One reason for this is that the targeted gene may not be responsible for the bottleneck of the pathway, as one pathway can include several rate-limiting steps, and thus several enzymes need to be overexpressed. Very recently, a novel approach called "transcriptional engineering" has been put forward. Instead of targeting genes encoding metabolic enzymes, the overexpression of transcription factors or regulatory elements might be a possible methodology to enhance multiple components of one or several metabolic pathways (Bajhaiya et al., 2016). One starting point could be the transcriptional engineering of the LC-PUFA synthesis as genes of desaturases are expressed according to a similar pattern and could be co-transcribed as an operon (Iskandarov et al., 2011).

2.5.3 Fluorescent activated cell sorting as a powerful tool for strain improvement

Although metabolic engineering shows great promise, it is often restricted by the limited knowledge of the biosynthesis and storage of TAGs, carotenoid and LC-PUFAs and by current regulatory restrictions on genetically modified organisms (GMOs). To overcome this issue, fluorescent activated cell sorting (FACS) can be used to isolate hyper- or hypoproducing mutant strains (Montero et al., 2011). For this approach, the aforementioned

target species are interesting candidates due to the accumulation of high amounts of carotenoids and the partitioning of LC-PUFAs into TAGs. However, for this rapid selection, algal cells need to be stained with a fluorescent dye for the desired compound. The isolation of lipid-rich strains has been achieved for *T. suecica*, *Chlorella* sp. and *Nannochloropsis* sp. by either Nile-red or the non-toxic BODIPY 505/515 staining procedure coupled with repeated rounds of FACS (Doan and Obbard, 2012; Lim et al., 2015; Manandhar-Shrestha and Hildebrand, 2013). The lipid-rich microalgae can either derive from spontaneous mutations or from random mutagenesis using ethyl methane sulfonate or exposure to high doses of UV radiation. Furthermore, repeated rounds of mutant selection ensure the stability of genetic mutations (Figure 2.5). Carotenoid hyperproducing strains of *D. salina* have been isolated by the same method. As carotenoid and neutral lipid accumulation seem to be connected, Nile-red can be used as indirect marker of carotenoids (Mendoza et al., 2008). However, to the best of our knowledge, there have not been any successful attempts at isolating LC-PUFA-rich microalgae by FACS due to the inability of current fluorochromes to specifically stain polyunsaturated fatty acids. However, it has been reported an increase of LC-PUFAs in high lipid content strains mutagenized by UV radiation (Lim et al., 2015). As a matter of fact, the generation and the selection of stable mutants by FACS coupled with the elucidation of changes at the level of the genome and transcriptome could generate interesting data concerning what regulatory components (e.g., key enzymes, transcription factors or signal-transducing pathways) are responsible for enhanced carotenoid and lipid biosynthesis in hyperproducing mutants. Finding these molecular switches responsible for such traits could provide further knowledge of how microalgal cells sense the environment and how they respond to it in terms of accumulation and compartmentalization of TAGs, carotenoids and LC-PUFAs, especially under nitrogen starvation. A particular issue worth investigating, and related to this applied objective, is to learn how microalgae remodel their membranes as part of the response to growth-enhancing conditions and / or environmental stress.

2.6   Conclusion

Microalgae are rich sources of multiple bioactive compounds such as LC-PUFAs, TAGs and carotenoids with applications in food, feed and fuel markets. Recent trends in microalgal biotechnology point to the simultaneous accumulation of these high-valuable compounds and lipids by one microalgal species to turn the production into an economically feasible venture. Analysis of the current state of the art revealed that the chlorophyte *P. incisa* is a potential triple-producer, being able to accumulate the three lipophilic classes of

compounds that are amenable to be used in different streams in a biorefinery. Furthermore, oleaginous species of Stramenopiles lineage such as *N. oculata* or *P. tricornutum* show the ability of a similar production process. However, concomitant carotenoid accumulation is yet to be achieved. Oleaginous chlorophytes usually display large accumulation of carotenoids under stress conditions, but only marginal EPA and DHA contents. Most probably, the best strategy to obtain triple-producers will be to look for carotenoid-rich strains containing TAGs composed of LC-PUFAs. Metabolic engineering of the LC-PUFA biosynthesis in microalgae of Stramenopiles has already proven that under specific genetic backgrounds microalgae synthesize high amounts of LC-PUFAs and incorporated these into TAGs under nitrogen depletion.
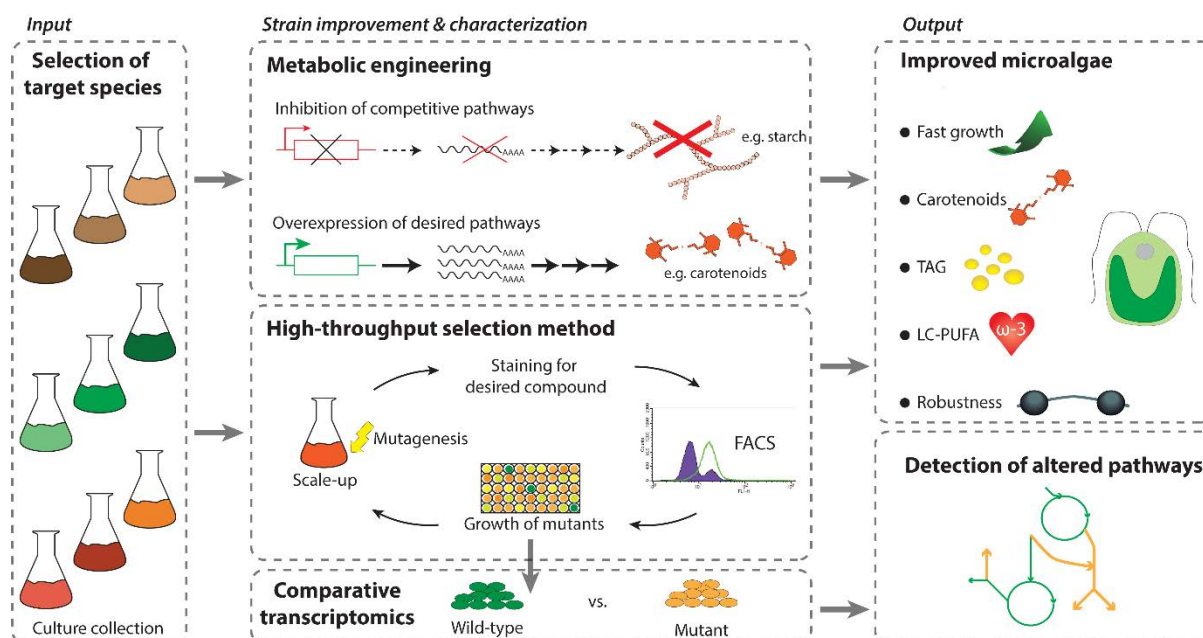
Figure 2.5. Strategies to improve TAG, LC-PUFA and carotenoid production in microalgae. Potential microalgal strains can be selected from a culture collection and improved via metabolic engineering (e.g., site-directed mutagenesis) and / or high-throughput methods (e.g., random mutagenesis) coupled with FACS. These processes may result in microalgae displaying fast growth rates, robustness against environmental changes and contaminants as well as the ability to produce carotenoids, TAGs and LC-PUFAs simultaneously. Furthermore, the study of the genetics of these improved strains can lead to the detection of altered biosynthetic pathways and novel key molecular players via comparative transcriptomics between wild-type and mutant.

Instead of the generation of genetically modified microalgae, naturally occurring lipid-, LC-PUFA- or carotenoid-hyperproducing mutants can be obtained by FACS-based selection (Figure 2.5). Even though both methods have been successfully applied to microalgae for one or the other compound, the genetic analysis of the derived mutants is lacking, which could

lead to the detection of genes or transcription factors responsible for the higher accumulation of valuable compounds. Thus, further knowledge about the metabolic pathways and especially the mechanism for the partitioning of LC-PUFAs into TAGs can be acquired, leading to a better understanding of the response of the microalgal metabolism to different stress factors. The species and techniques suggested in this review will hopefully guide the research into the direction of the production of multiple valuable compounds by microalgae, making them a promising sustainable resource of food, feed and energy.

Acknowledgements

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Improvement of carotenoid extraction from a recently isolated, robust microalga, *Tetraselmis* sp. CTP4 (Chlorophyta)

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Abstract

In recent years, there has been increasing consumer interest on carotenoids particularly of marine sustainable origin with applications in the food, cosmeceutical, nutritional supplement and pharmaceutical industries. For instance, microalgae belonging to the genus *Tetraselmis* are known for their biotechnologically relevant carotenoid profile. The recently isolated marine microalgal strain *Tetraselmis* sp. CTP4 is a fast-growing, robust industrial strain, which has successfully been produced in 100-m^3 photobioreactors. However, there are no reports on total carotenoid contents from this strain belonging to *T. striata / convolutae* clade. Although, there are several reports on extraction methods targeting chlorophytes, extraction depends on the strength of cell coverings, solvent polarity and the nature of the targeted carotenoids. Therefore, this article evaluates different extraction methods targeting *Tetraselmis* sp. CTP4, a strain known to contain a mechanically resistant theca. Here, we propose a factorial experimental design to compare extraction of total carotenoids from wet and freeze-dried microalgal biomass using four different solvents (acetone, ethanol, methanol or tetrahydrofuran) in combination with two types of mechanical cell disruption (glass beads or dispersion). The extraction efficiency of the methods was assessed by pigment contents and profiles present in the extracts. Extraction of wet biomass by means of glass bead-assisted cell disruption using tetrahydrofuran yielded the highest amounts of lutein and *β*-carotene (622 ± 40 and 618 ± 32 µg g^{-1} DW, respectively). Although acetone was slightly less efficient than tetrahydrofuran, it is preferable due to its lower costs and toxicity.

Keywords: Marine microalgae, Glass beads, Lutein, RP-HPLC, Wet biomass

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3.1 Introduction

Carotenoids are synthesized by all photosynthetic organisms, as they play important roles in light harvesting and photoprotection (Varela et al., 2015). They are composed of a 40-carbon isoprenoid backbone, responsible for colours ranging from yellow to red. Carotenoids are lipophilic compounds that can be divided into two groups: carotenes—non-oxygenated hydrocarbons (e.g., α-carotene and β-carotene)—and xanthophylls—molecules containing oxygenated groups as, for example, lutein, violaxanthin, zeaxanthin and astaxanthin.

Recently, intensive research has been carried out to produce carotenoids from biological sources, mainly due to the importance of these pigments in terms of human health (e.g., decreased risk of degenerative and cardiovascular diseases, possible cancer prevention or cataracts) and nutrition (Eggersdorfer and Wyss, 2018; Fiedor and Burda, 2014; Johnson, 2002). Furthermore, carotenoids find their applications in biotechnology as antioxidants, colorants for aquaculture feed and food, as well as ingredients for cosmeceuticals and pharmaceuticals (Guedes et al., 2011; Vílchez et al., 2011). Borowitzka et al. (2013) estimated the global market value of carotenoids to be about 1.2 billion USD, with β-carotene, lutein and astaxanthin representing approximately 60% of the total market. Microalgae are a promising biological resource of high-value biomolecules such as carotenoids, vitamins and polyunsaturated fatty acids (Guedes et al., 2011). These microscopic, mainly photosynthetic organisms, display high photosynthetic efficiencies, fast growth rates and are cultivated in large photobioreactors with high productivities of metabolites (Sánchez et al., 2008).

Carotenogenesis in microalgae occurs in the chloroplast from where the synthesized compounds are transported to different locations inside the cells. Carotenoids that are important for light-harvesting are located in thylakoid membranes, whereas other carotenoids, such as β-carotene and astaxanthin, are bound to lipids inside either cytosolic or plastidial lipid droplets, which may have a protective function by precluding the photooxidation of cellular components, in particular under unfavourable conditions (Ben-Amotz et al., 1989; Mulders et al., 2014b). However, the precise location of biosynthesis and storage of a given carotenoid is highly dependent not only on the genetics of the microalgal strain, but also on the growth conditions.

For an efficient extraction process, it is essential to ensure the complete disruption of the cell, including cell coverings, plasma and plastidial membranes, which are important barriers preventing the release of the pigments to the solvent. Furthermore, differences in the composition of these cell structures need to be considered. For example, *Tetraselmis* microalgae are known for their theca, a cell covering derived from the fusion of

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primitive scales and known for its mechanical strength (Becker et al., 1998; Siegel and Siegel, 1973). Cell disruption can be achieved by mechanical (e.g., pestle / mortar, bead milling, ultrasound, and homogenizers) or non-mechanical (e.g., freezing / thawing, heating, osmotic shock, and alkaline lysis) methods (Fernández-Sevilla et al., 2010). Aside from efficient cell lysis, another crucial factor in any extraction procedure is the choice of the extracting solvent system. The ideal solvent needs to be able to penetrate the microalgal cells and show high affinity to carotenoids. Polar solvents such as acetone, ethanol and methanol extract xanthophylls more efficiently, whereas chloroform, hexane and tetrahydrofuran (THF) are non-polar solvents with higher affinity to carotenes and esterified carotenoids (Saini and Keum, 2018). Besides the solubility of carotenoids in the solvents, its price and toxicity are important criteria for the selection of a given solvent for an industrial process. Moreover, it is important to minimize the degradation of carotenoids at all steps of the process. These pigments once outside the microalgal cell are very sensitive to UV radiation, oxygen and high temperature (Arvayo-Enríquez et al., 2013; Saini and Keum, 2018). Although pigment extraction is a common effort in microalgal biotechnology, a literature search (Table 3.1) on extraction methods of microalgal carotenoids revealed the inexistence of a common extraction protocol. Furthermore, for the same genus different protocols are applied, highlighting the importance of the development of carotenoid extraction method for each microalgal strain. In this study, the microalga *Tetraselmis* sp. CTP4, a robust and euryhaline species, recently isolated from the Ria Formosa in Faro, Portugal was selected (Pereira et al., 2016). This fast-growing species displays lipid contents of up to 33% of its dry weight with properties suitable for biodiesel production (Pereira et al., 2016). Furthermore, it has been successfully grown semi-continuously in industrial photobioreactors and harvested by natural settling, resulting in a biomass paste with only 20% of water within 6 hours (Pereira et al., 2018a). Thus, *Tetraselmis* sp. CTP4 is a good candidate for the co-production of biofuels and high-value products using a biorefinery approach. Therefore, the present work is focused on the optimization of carotenoid extraction, particularly the commercially important lutein and *β*-carotene from a mechanically robust microalga.

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Table 3.1. Carotenoid extraction protocols from microalgal biomass found in the literature.

Species	Type of biomass	Cell disruption	Solvent system	References
Chlorophyta				
Botryococcus braunii	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
Chlorella vulgaris	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
		sonication	methanol:water (9:1)	(Goiris et al., 2015)
		glass beads	100% acetone	(Batista et al., 2013)
Chlorella zofingineses	freeze-dried	bead beating	methanol:chloroform (2:2.5)	(Mulders et al., 2015)
Coelastrella sp. F50	freeze-dried	bead beating	methanol:dichlormethane (75:25)	(Hu et al., 2013)
Dunaliella salina	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
	wet	vortex (5-10 min)	acetone:water (8:2)	(León et al., 2005)
	wet	vortex (10 s) and sonication (10 min)	methanol:chloroform (2:2.5)	(Lamers et al., 2010)
Dunaliella tertiolecta	freeze-dried	mortar and sonication (10min)	acetone (8:2)	(Huang et al., 2016)
Haematococcus pluvialis	freeze-dried	pestle and mortar	hexane/ ethyl acetate /hot water	(Goiris et al., 2012)
		glass beads	100% acetone	(Batista et al., 2013)
Neochloris oleoabundans	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
		cryogenic grinding, shaking for 3 h at 452 rpm	100% acetone	(Castro-Puyana et al., 2013)

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Table 3.1 (continued)

Species	Type of biomass	Cell disruption	Solvent system	References
Parachlorella kessleri	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
Scenedesmus obliquus	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
Tetraselmis chui	frozen	grinding and sonication (5 min)	acetone:water (9:1)	(Garrido et al., 2009)
	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
Tetraselmis gracilis	frozen	20 h	acetone:water (9:1)	(Lourenço et al., 1997)
Tetraselmis marina	wet	grinding and sonication (5min)	acetone:water (9:1)	(Garrido et al., 2009)
	freeze dried	65 °C, 1 h	DMSO	(Dahmen-Ben Moussa et al., 2017)
Tetraselmis rubens	frozen	grinding and sonication (5 min)	acetone:water (9:1)	(Garrido et al., 2009)
Tetraselmis subcordiformis	frozen	grinding and sonication (5 min)	acetone:water (9:1)	(Garrido et al., 2009)
Tetraselmis suecica	frozen	grinding and sonication (5 min)	acetone:water (9:1)	(Garrido et al., 2009)
	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
	freeze-dried	sonication	methanol:water (9:1)	(Goiris et al., 2015)
	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
	freeze-dried	30 min incubation	ethanol:water (3:1)	(Sansone et al., 2017)
	frozen		acetone:water (9:1)	(Abiusi et al., 2014)

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Table 3.1 (continued)

Species	Type of biomass	Cell disruption	Solvent system	References
Tetraselmis suecica	frozen	sonication	methanol	(Wright et al., 1997)
	frozen	grinding and sonication (5 min)	95% methanol	(Zapata et al., 2000)
	wet	sonication	acetone:water (9:1)	(Borghini et al., 2009)
	freeze-dried	incubation of 30 min, vortex, sonication 10 min RT	acetone:methanol (7:3)	(Di Lena et al., 2019)
Tetraselmis tetrathele	frozen	grinding and sonication (5 min)	acetone:water (9:1)	(Garrido et al., 2009)
	wet	60 °C, 30 min	ethanol	(El-Kassas and El-Sheekh, 2016)
Tetraselmis wettsteinii	frozen		acetone: methanol (7:3)	(Egeland et al., 1995)
Tetraselmis sp.	wet	sonication, 65 °C, 30 min	ethanol	(Dammak et al., 2017)
Tetraselmis sp.	freeze-dried	3 h at RT	ethanol	(Maadane et al., 2015)
Tetraselmis sp. DS3	freeze-dried	bead beating	methanol:dichlormethane (75:25)	(Tsai et al., 2016)
Tetraselmis sp. M8	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
Eustigmatophyta				
Nannochloropsis gaditana	freeze-dried	sonication (10 min)	100% methanol	(Cerón-García et al., 2010)
Nannochloropsis oculata	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)

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Table 3.1 (continued)

Species	Type of biomass	Cell disruption	Solvent system	References
Haptophyta				
Diacronema vlkianum	freeze-dried	glass beads	100% acetone	(Batista et al., 2013)
Isochrysis galbana	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
		glass beads	100% acetone	(Batista et al., 2013)
Isochrysis sp.	freeze-dried	pestle and mortar	hexane/ ethyl acetate /hot water	(Goiris et al., 2012)
Pavlova lutheri	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
Bacillariophyta				
Chaetoceros calcitrans	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
		pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
Chaetoceros muelleri	freeze-dried	pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
Phaeodactylum tricornutum	freeze-dried	pestle and mortar	ethanol:water (3:1)	(Goiris et al., 2012)
		pestle and mortar, vortex	100% acetone, hexane, 10% NaCl (2:2:1)	(Ahmed et al., 2014)
		sonication	methanol:water (9:1)	(Goiris et al., 2015)

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3.2 Materials and Methods

3.2.1 Microalgal biomass

Tetraselmis sp. CTP4 was cultivated in the laboratory as described previously (Pereira et al., 2016; Schulze et al., 2017). Upon harvesting, the biomass was immediately frozen at -20 °C. Freeze-dried biomass was obtained upon lyophilisation for 24 h and stored in a desiccator at room temperature in the dark. Freeze-dried biomass can be made available upon request.

This article does not contain any studies with human participants or animals performed by any of the authors.

3.2.2 Optimization of carotenoid extraction

Different conditions, such as type of biomass, cell disruption method and solvent, were tested using a factorial experimental design to find the best method for extracting carotenoids from *Tetraselmis* sp. CTP4 (Figure 3.1). For that purpose, the extraction was conducted on both wet and freeze-dried biomass with the use of four different extraction solvents—ethanol (EtOH), acetone, methanol (MeOH) and tetrahydrofuran (THF)—and with the application of different cell disruption methods—mechanical dispersion or glass bead milling. Extraction solvents were of analytical grade except for THF, which was of HPLC grade to ensure the absence of peroxides. During all extraction steps, samples were kept on ice and in the dark to avoid pigment degradation. For the extraction, about 3 mg dry weight (DW) of each type of biomass were resuspended in 3 mL of ice-cold solvent. Afterwards, cells were lysed by means of mechanical dispersion using an IKA Ultra-Turrax T18D Basic apparatus (IKA-Werke GmbH, Staufen, Germany) at 25000 rpm during 2 cycles of 45 s or, alternatively, 0.7 g of glass beads (425-600 µm) were added and tubes were vortexed on an IKA Vortex Genius 3 shaker (IKA-Werke GmbH, Staufen, Germany) at maximum speed for 2 min. Ultra-Turrax mechanical dispersion produces a considerably amount of heat. So, even though the samples were kept on ice throughout the whole process, this method was applied for a shorter period than bead milling. To collect the supernatant, samples were centrifuged at 10 °C, 8000×g for 5 min. The extraction procedure was repeated until both the pellet and the supernatant became colourless.

Extracts were combined and dried using a gentle nitrogen flow and resuspended in 5 mL of 100% acetone for spectrophotometric analysis of total chlorophyll and carotenoid

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content. The extraction protocols that yielded the best results in the spectrophotometric analysis were analysed by HPLC to assess the pigment profile. To this end, the extracts were dried, resuspended in methanol (0.7 mL) followed by filtration using PTFE filters (0.2 µm). HPLC analysis was performed immediately after resuspension to avoid pigment degradation.

All experiments were carried out in triplicate and average values are reported. To determine significant differences, variance analysis (ANOVA) was performed at a confidence level of 95% using Tukey HSD post-hoc test. These statistical tests were performed with SPSS (release 25.0, SPSS Inc., Chicago, IL) software.

3.2.3 Determination of total chlorophyll and carotenoid contents

3.2.3.1 Spectrophotometric estimation of pigments

The absorbance (A_{nnn}) of the extracts was measured by spectrophotometry in a Spectronic Unicam 3000 UV-Vis (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) at three different wavelengths—662, 645 and 470 nm—to estimate their pigment composition. The concentrations of chlorophyll a (Chl a) and b (Chl b) and total carotenoids (TCar) in µg mL^{-1} were estimated using the following equations (Lichtenthaler and Wellburn, 1983):

$$\text{Chl } a = 11.75 \, A_{662} - 2.35 \, A_{645}$$

$$\text{Chl } b = 18.61 \, A_{645} - 3.96 \, A_{662}$$

$$\text{TCar} = \frac{1000 \, A_{470} - 2.27 \, \text{Chl } a - 81.4 \, \text{Chl } b}{227}$$

3.2.3.2 RP-HPLC analysis of carotenoids profile

The carotenoid profile of the extracts was analysed using a Merck Hitachi LaCrom Elite HPLC (Darmstadt, Germany) equipped with a diode-array detector (450 nm) using a LiChroCART RP-18 (5µm, 250x4 mm, LiChrospher) column, as described by Couso et al. (2012) with slight modifications. The mobile phase consisted of acetonitrile:water (9:1; v/v) as solvent A and ethyl acetate as solvent B and the gradient program applied was: 0–16 min, 0–60% B; 16–30 min, 60% B; 30–32 min 100% B and 32-35 min 100% A. Identification of the pigments was performed based on their retention times and confirmed by comparison of UV-Vis spectra with those of commercial standards. For quantification, external calibration curves were performed for neoxanthin, violaxanthin, lutein and β-carotene. All pigment standards

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were supplied by Sigma-Aldrich (Sintra, Portugal). All HPLC grade solvents were purchased from Fisher Scientific (New Hampshire, USA).
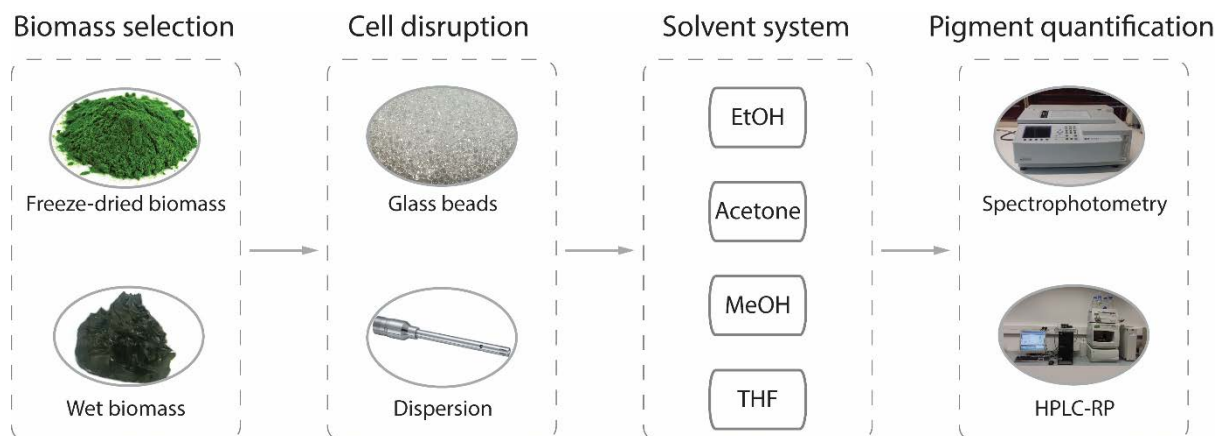
Figure 3.1. Workflow of methods used for pigment extraction from *Tetraselmis* sp. CTP4. All methods were tested on both freeze-dried and wet biomass. The cell disruption was achieved by either mechanical dispersion or grinding with glass beads. Four different solvents were used to extract the pigments, namely ethanol (EtOH), acetone, methanol (MeOH) and tetrahydrofuran (THF). Chlorophyll and carotenoid contents of each extract were analysed by spectrophotometry. Pigment profiles of the four best extracts were analysed by RP-HPLC. All methods were performed in triplicate.

3.3 Results and discussion

3.3.1 Optimization of pigment extraction of *Tetraselmis* sp. CTP4

The first parameter addressed in the optimization of pigment extraction was the type of biomass. Both freeze-dried and frozen biomass paste (wet biomass) were analysed, as these are two common types of industrial processing of microalgal biomass. The highest amount of both chlorophylls and carotenoids from *Tetraselmis* sp. CTP4 was recovered from wet biomass as compared to those from freeze-dried microalgae, up to 3- and 2.5-fold more, respectively (Figure 3.2). Freeze-drying has been considered a mild method to dehydrate the biomass without significant losses of pigments. However, long-term storage (> 35 days) at room temperature caused losses of carotenoids in *Phaeodactylum tricornutum*, and a decreased lutein content in *Scenedesmus almeriensis* of about 50% after 20 days (Cerón-García et al., 2010; Ryckebosch et al., 2011). Therefore, the lower recovery of pigments from freeze-dried biomass could be an effect of long-term storage conditions, as samples were processed 31 days after lyophilisation. In addition, extraction from wet biomass brings the advantage of being a simple method that omits the drying step, a costly and time-consuming process.

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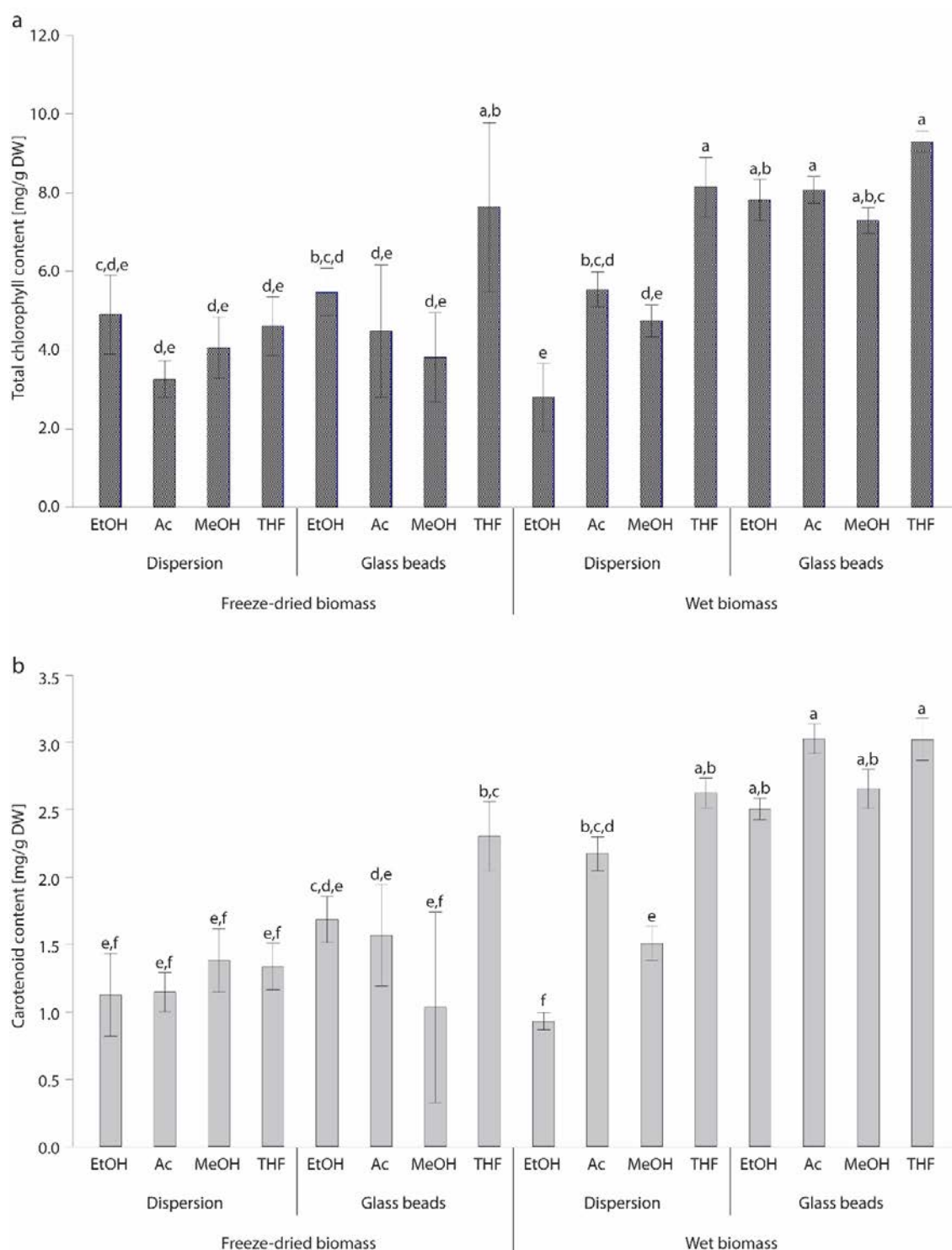
Figure 3.2. Total chlorophyll (a) and total carotenoid (b) contents of extracts prepared from lyophilized and wet *Tetraselmis* sp. CTP4 biomass. To optimize the extraction conditions, different solvents were tested, namely ethanol (EtOH), acetone (Ac), methanol (MeOH) and tetrahydrofuran (THF). Different cell disruption methods were also tested: dispersion or glass bead milling. Each extraction was performed in triplicate and results are reported as means ± standard deviation. Different letters indicate significant differences ($p < 0.05$) using one-way ANOVA with post hoc Tukey HSD test.

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Tetraselmis sp. CTP4 has medium-sized cells (9-12 μm; Pereira et al. 2016) and is known for its strong theca. Therefore, two different mechanical cell disruption methods were applied to ensure complete cell disruption and enhance carotenoid extraction. When freeze-dried biomass was used, differences between both disruption methods are not obvious mainly because the extracted amounts of both chlorophylls and carotenoids were low (Figure 3.2). A notable exception was THF that was significantly more efficient as an extracting solvent when using glass beads as a cell disruption method. However, when extraction was done on wet biomass, disruption by glass beads was significantly more efficient than by dispersion, for all pigments (Figure 3.2). The only exception was dispersion in combination with THF as solvent, which yielded high chlorophyll and carotenoid contents (Figure 3.2). Nonetheless, glass bead-assisted disruption significantly improved pigment recovery from wet biomass of CTP4 (1.5-fold) compared with dispersion (Figure 3.2). It is possible that the observed difference is partially related to the different time periods used for cell disruption by the two methods. Due to the higher amount of heat generated by the Ultra-Turrax dispersion, the application of this method was limited to 2 cycles of 45s while bead milling could be applied for 2 minutes without any noticeable heating of the sample. Since elevated temperatures can lead to metabolites degradation, heating production during cell disruption by Ultra-Turrax can be a strong limitation (Arvayo-Enríquez et al., 2013; Saini and Keum, 2018). Other authors have found similar results. For example, Taucher et al. (2016) achieved better disruption of *Chlorella zofingiensis* (cell size range: 2-15 μm) by ball milling rather than with mechanical dispersion. Another study in *Coelastrella* sp., an alga with a thick cell wall, reported also bead milling as a successful process for extracting carotenoids (Hu et al., 2013). Concerning *Tetraselmis*, glass bead milling was used to extract carotenoids in only one report (Tsai et al., 2016). Other studies on *Tetraselmis* species used cell disruption by sonication (Di Lena et al., 2019; Garrido et al., 2009; Goiris et al., 2015; Wright et al., 1997; Zapata et al., 2000). However, ultrasounds can lead to the cleavage of water to free •OH and •H radicals, which can damage the extracted carotenoids, and should therefore be avoided (Petrier et al., 1992). Another disadvantage of sonication is that it can only be applied to small-sized samples, whereas bead or ball milling have already been implemented at industrial scale. For example, a so called DYNO-mill, was successfully used for the disruption of *Tetraselmis* wet biomass to isolate protein (Geciova et al., 2002; Schwenzfeier et al., 2011). Therefore, a scale-up of this extraction method is conceivable, though involving high-energy consumption. Other industrially scalable methods include high-pressure homogenization, which has also been applied successfully to rupture microalgae including from the *Tetraselmis* genus (Spiden et al., 2013).

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Another important parameter is the selection of an appropriate solvent for an efficient extraction. However, in our study, the choice of solvent appears to be less important since no significant changes in the extraction yields of both chlorophylls and carotenoids were found, when glass beads were used for cell disruption. This might be related to the fact that all the chosen solvents are polar solvents and appropriate for the extraction of these compounds. The best chlorophyll yields were obtained using THF, which led to of 8.14 ± 0.82 mg g^{-1} DW. The best extraction yields for all carotenoids from CTP4 were obtained with THF and acetone: 3.02 ± 0.16 and 3.03 ± 0.11 mg g^{-1} DW, respectively (Figure 3.2b). Indeed, acetone is the most commonly used solvent alone or in combination with other solvents for carotenoid extraction from *Tetraselmis* spp. cells (Table 3.1). However, when dispersion was used as cell disruption method, the choice of solvent is rather important and THF or acetone were the most efficient for pigment extraction. These results indicate that the right combination of cell disruption and solvent needs to be selected for improved extraction of pigments from microalgal biomass. Nevertheless, THF has a tendency to form explosive peroxides for long storage as well as during distillation. Therefore, to minimize this problem, the purchased THF should be protected with an antioxidant molecule, such as butylated hydroxytoluene (BHT) (Khachik et al., 1986; Rivera and Canela, 2012).

3.3.2 Optimization of individual carotenoid extraction of *Tetraselmis* sp. CTP4 biomass

To optimize the selectivity of the extraction methods on individual carotenoids, RP-HPLC was used for analysis. Direct injection of extracts in acetone under the RP-HPLC conditions used in this paper showed broad peaks with shoulders, in particular for the polar xanthophylls, thus, leading to poor quantification (Figure 3.3a). Therefore, prior to injection, the solvent of the extracts was changed from acetone to methanol, which resulted in a better resolution of the peaks and good peak integration (Figure 3.3b). This observation is in agreement with other publications on RP-HPLC methods for the quantification of microalgal pigments (van Leeuwe et al., 2006; Wright et al., 1991; Zapata and Garrido, 1991). As an example, the absorbance spectrum of lutein is shown with a shoulder at 422 nm, and absorbance maxima at 448 and 476 nm (Figure 3.3b, small picture). However, once the extract was resuspended in methanol, analysis was performed immediately to avoid pigment degradation as methanol is known to promote formation of chlorophyll allomers, which can cause overlapping peaks with lutein (Figure 3.3c; Porra et al., 1997).

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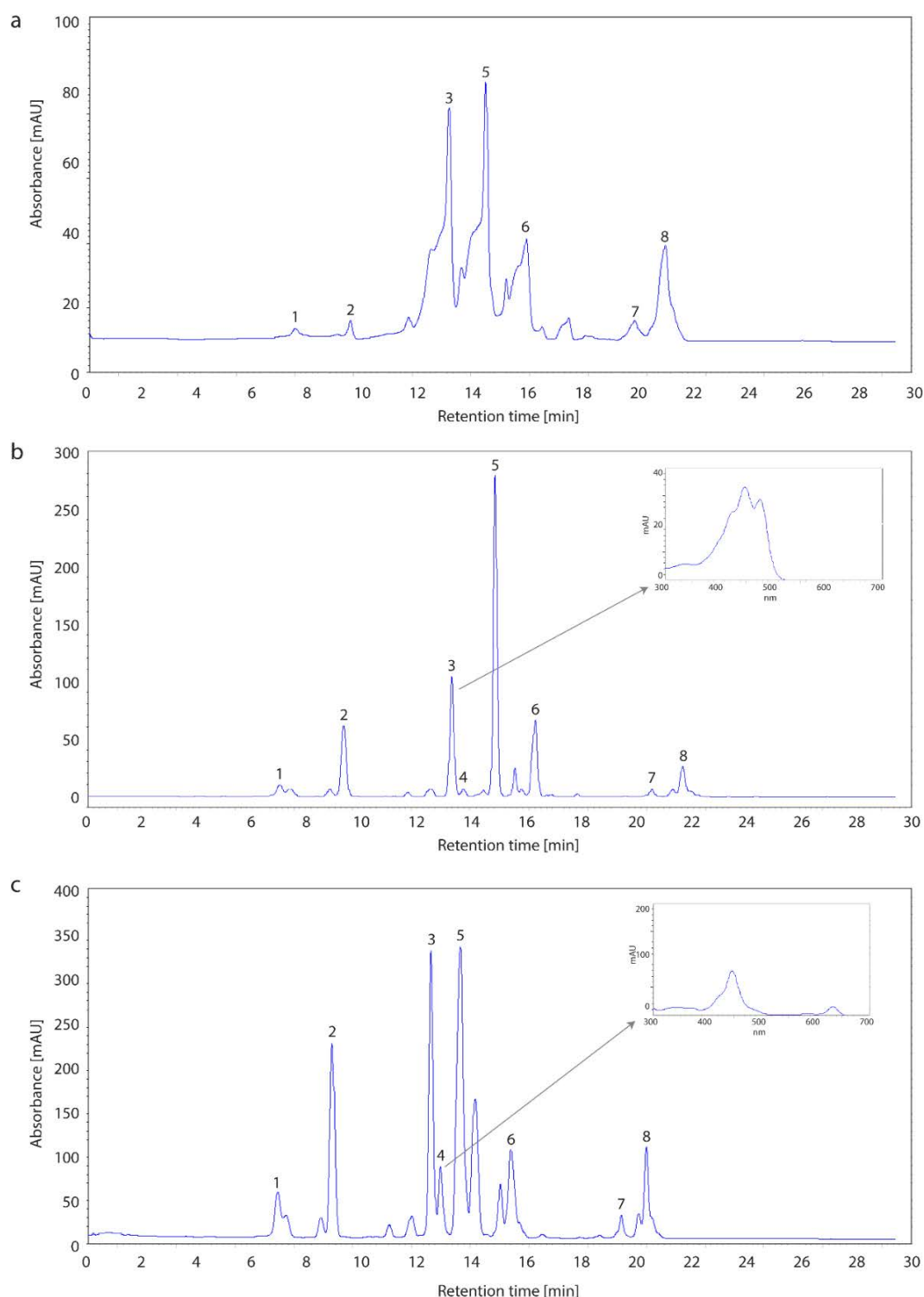
Figure 3.3. HPLC chromatograms of pigment extract of *Tetraselmis* sp. CTP4 at 450 nm. Extraction was carried out from wet biomass using acetone and cell disruption by glass beads. (a) Chromatogram of extracts injected in acetone, showing broad peaks and poorer resolution. (b) Chromatogram of the extract, in methanol, injected immediately after pigment extraction revealing well shaped and separated peaks. The spectrum of peak 3 showed the typical profile of lutein (small picture). (c) Injection after 24 h in methanol showing overlapping of peaks 3 and 4. The small picture shows the spectrum of peak 4 with an absorbance peak at 680 nm, typical of chlorophyll. The identified pigments were: 1) neoxanthin, 2) violaxanthin, 3) lutein, 4) zeaxanthin, 5) chlorophyll *b*, 6) chlorophyll *a*, 7) *α*-carotene, 8) *β*-carotene.

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Nevertheless, the focus of the RP-HPLC analysis was on carotenoids rather than chlorophyll. Therefore, only the most efficient extraction methods for carotenoids obtained by spectrophotometry were investigated, namely extractions from wet biomass using acetone or THF and cell disruption using glass beads or mechanical dispersion. RP-HPLC analysis of *Tetraselmis* sp. CTP4 carotenoid profile showed that the most dominant carotenoids were neoxanthin, violaxanthin, lutein and *β*-carotene (Figure 3.3a) which is in agreement with reports from other authors for different *Tetraselmis* species (Ahmed et al., 2014). The quantification of these four carotenoids in the extracts confirmed the results obtained by spectrophotometric analysis: glass bead-assisted extraction led to a 2.4-fold average increase of carotenoids extracted from wet biomass over dispersion-based methods, regardless of the solvent used (Figure 3.4). On average, THF was also a better solvent for carotenoid extraction than acetone, when dispersion-based homogenization was used. Overall, THF was more efficient than acetone for the extraction of all carotenoids. However, if the biomass was milled with glass beads, THF was found to be a better solvent for the extraction of lutein and *β*-carotene (622 ± 40 and 618 ± 32 µg g^{-1} DW, respectively), but as good as acetone for neoxanthin (38.7-52.3 µg g^{-1} DW) and violaxanthin (123-139 µg g^{-1} DW; Figure 3.4). This is in agreement with the solubility tests made by Craft and Soares (1992), who showed that THF was the best solvent for lutein and *β*-carotene. Furthermore, in a study on *Chlorella sorokiniana*, THF was shown to lead to a higher recovery of lutein than acetone (Chen et al., 2016). However, considering the commercialization of the extracts for food applications, acetone is preferred, as it is both cheaper and listed as a GRAS (Generally Recognized As Safe) solvent. Moreover, acetone does not require the addition of antioxidants with potential for toxicity such as BHT to maintain its stability (EFSA ANS, 2012). The carotenoid concentrations detected in this study are comparable to those found in the literature for other *Tetraselmis* species (Ahmed et al., 2014; Di Lena et al., 2019), although higher contents have been reported in the heat-tolerant microalga *Tetraselmis* sp. DS3 (Tsai et al., 2016). However, carotenoid contents are often dependent on the species and its cultivation conditions, which might explain the differences observed. This is an on-going investigation in this species by our group.

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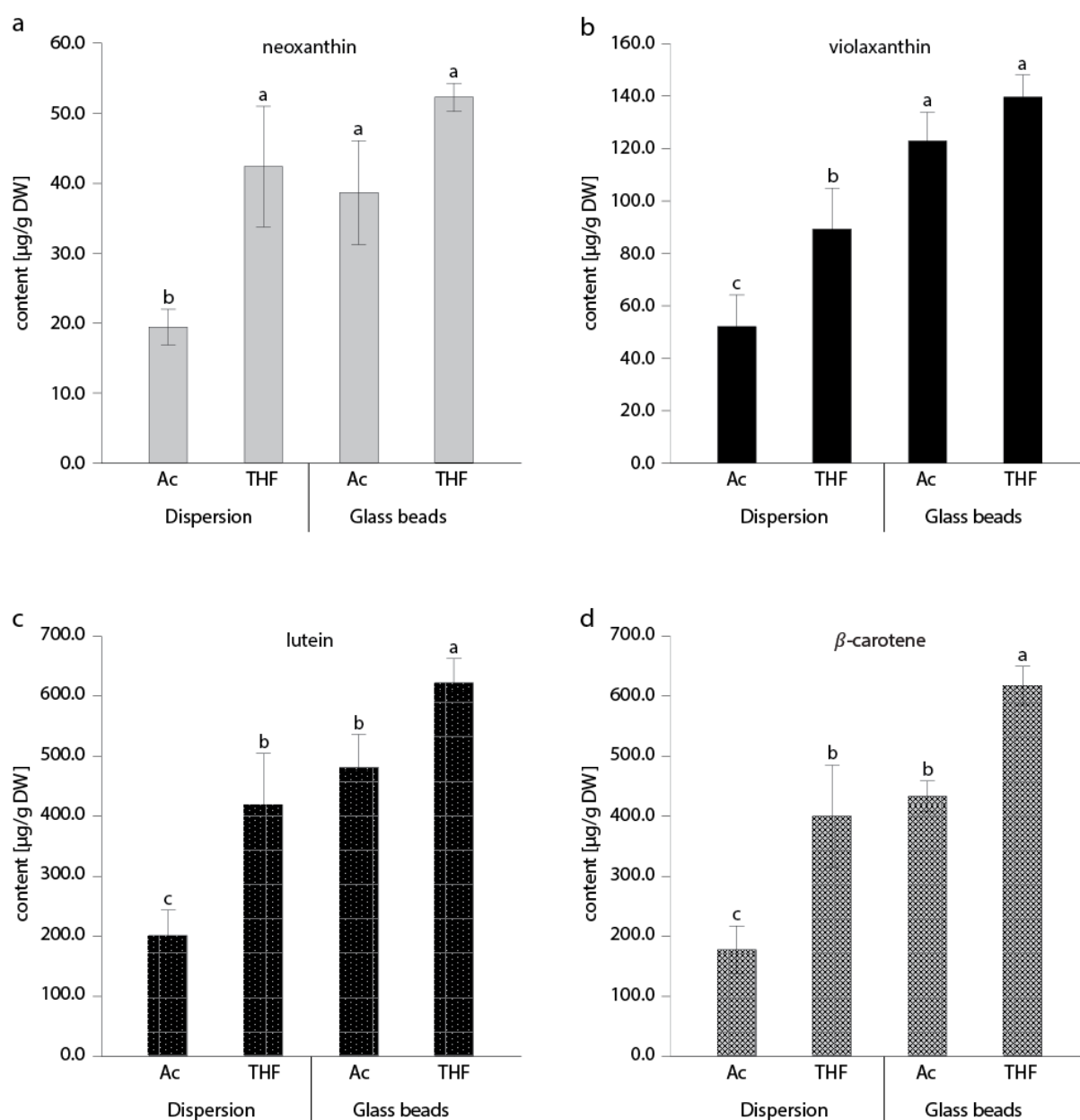
Figure 3.4. Quantification of neoxanthin (a), violaxanthin (b), lutein (c) and β-carotene (d) extracted from wet biomass of *Tetraselmis* sp. CTP4. Carotenoids were extracted using acetone (Ac) or tetrahydrofuran (THF) and glass-bead milled or mechanically dispersed to promote cell disruption. This experiment was performed in triplicate and the means ± standard deviation is shown for all results. For each figure, different letters indicate significant differences ($p < 0.05$) using one-way ANOVA with post hoc Tukey HSD test.

3.4 Conclusions

Tetraselmis sp. CTP4 proved to be a good candidate for the development of a suitable method for carotenoid extraction, as it contained significant amounts of extractable carotenoids, particularly lutein and β-carotene, two pigments with high market value as

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ingredients in food, feed, nutraceutical and cosmeceutical formulations. The best method for carotenoid extraction was a combination of disruption by glass beads using THF applied to wet biomass as storage of freeze-dried biomass might have led to pigment degradation. However, a combination of disruption by glass beads using acetone applied to wet biomass was almost as efficient and is less costly and less time consuming than using freeze-dried biomass or THF as solvent. These characteristics and the scalability of glass bead milling make this method industrially applicable. Furthermore, as it is easy to carry out, it could become a common protocol for carotenoid extraction from mechanically robust microalgae, which would greatly facilitate the comparison between different species.

Funding

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Improved production of lutein and *β*-carotene by thermal and light intensity upshifts in the marine microalga *Tetraselmis* sp. CTP4

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Highlights

- High temperature enhances carotenoid accumulation in *Tetraselmis* sp. CTP4

- Nitrogen repletion conditions are crucial for carotenoid induction

- Lutein accumulation is increased under conditions promoting growth

- *β*-carotene accumulation is increased under low irradiance

- Short-term stress (2 days) increases both lutein and *β*-carotene

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Abstract

The industrial microalga *Tetraselmis* sp. CTP4 is a promising candidate for aquaculture feed, novel food, cosmeceutical and nutraceutical due to its balanced biochemical profile. To further upgrade its biomass value, carotenogenesis was investigated testing four environmental factors, namely temperature, light intensity, salinity and nutrient availability over different growth stages. The most important factor for carotenoid induction in this species is a sufficient supply of nitrates leading to an exponential growth of the cells. Furthermore, high temperatures of over 30 °C compared to lower temperatures (10 and 20 °C) induced the accumulation of carotenoids in this species. Remarkably, the two different branches of carotenoid synthesis were regulated depending on different light intensities. Contents of *β*-carotene were 3-fold higher under low light intensities (33 µmol m^{-2} s^{-1}) while lutein contents increased 1.5-fold under higher light intensities (170 and 280 µmol m^{-2} s^{-1}). Nevertheless, highest contents of carotenoids (8.48 ± 0.47 mg g^{-1} DW) were found upon a thermal upshift from 20 °C to 35 °C after only two days at a light intensity of 170 µmol m^{-2} s^{-1}. Under these conditions, high contents of both lutein and *β*-carotene were reached accounting for 3.17 ± 0.18 and 3.21 ± 0.18 mg g^{-1} DW, respectively. This study indicates that *Tetraselmis* sp. CTP4 could be a sustainable source of lutein and *β*-carotene at locations where a robust, euryhaline, thermotolerant microalgal strain is required.

Keywords: Marine microalgae; carotenoids; short-term stress; RP-HPLC; carotenoid autofluorescence, nitrate repletion

Graphical abstract

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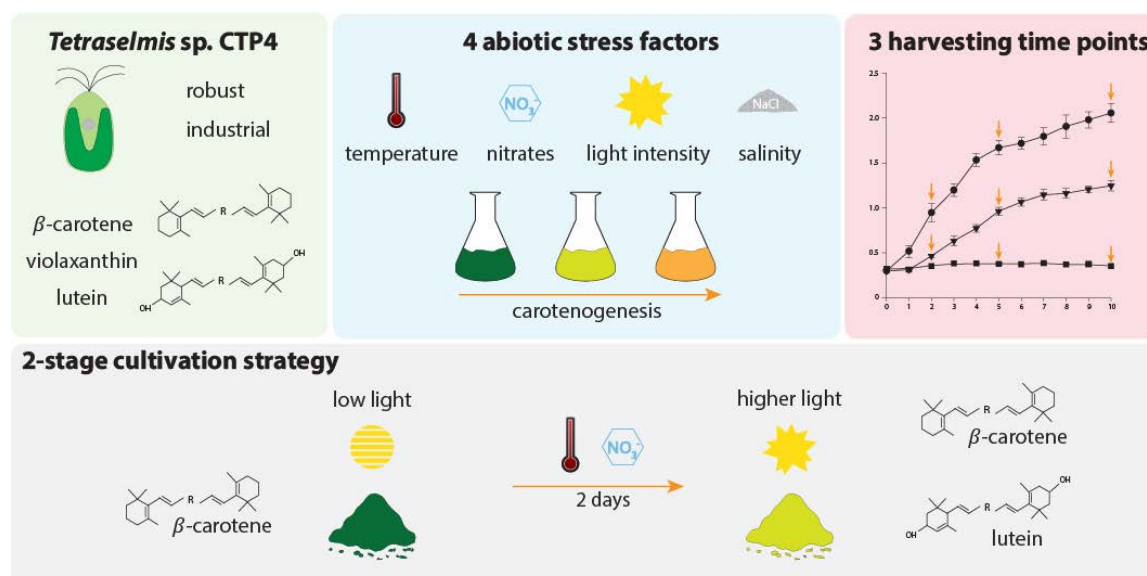
4.1 Introduction

In recent years, consumer interest in healthy food and natural products has been growing due to a rising awareness that a healthy nutrition can increase life expectancy. Evidence has been put forward that ingredients used as nutraceuticals and cosmeceuticals may confer additional health and medical benefits such as a decreased risk for chronic disease or cancer (Matos et al., 2017). In turn, this has led to research efforts for the development of food supplements and cosmetics from natural sources. Microalgae are promising biological resources that meet these market needs due to their well-balanced biochemical profile as well as antioxidant, anticancer and anti-inflammatory activities (Batista et al., 2013). Furthermore, microalgae can be cultivated in bioreactors or ponds placed on non-arable land, for example, deserts or shorelines, thus not competing with plant production. Moreover, they also display higher productivities than land crops (Williams and Laurens, 2010).

One important group of bioactive compounds produced by microalgae are carotenoids. These molecules play essential roles as accessory light harvesting pigments and photoprotective agents, being also structural components of the light harvesting complexes of microalgae (Varela et al., 2015). Their typical polyene structure is responsible for their lipophilic character, and the absence or presence of oxygenated groups defines two different carotenoid sub-classes, namely carotenes (e.g., *α*- and *β*-carotene) and xanthophylls (e.g., lutein and violaxanthin), respectively. Furthermore, because of the presence of conjugated double bonds in their chemical structure, carotenoids not only have different colours, ranging from yellow to red, but also display antioxidant activities (Gouveia et al., 2010). Carotenoids can scavenge reactive oxygen species (ROS), a well-known group of chemicals that can cause oxidative stress (Britton et al., 2008). Because of their diverse roles, and aside from the genetics of a particular strain, carotenogenesis in microalgae is highly influenced by the environmental conditions to which the microalgal cells are exposed. Thus, culture conditions such as light intensity, nutrient availability, temperature and salinity may influence carotenoid contents. In the case of the chlorophytes *Dunaliella salina* and *Haematococcus pluvialis*, carotenogenesis is induced by high light intensity and/or nutrient depletion (Schüler et al., 2017). Under these conditions, the overproduction of these lipophilic carotenoids depends on triacylglycerol synthesis, which form lipid droplets and serve as a sink for carotenoid deposition (Rabbani et al., 1998). However, other microalgae do not accumulate carotenoids in lipid droplets, but in the thylakoid membranes as carotenogenesis usually depends on growth-promoting conditions rather than on abiotic stress.

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Microalgae belonging to the genus *Tetraselmis* are known for their great variety in carotenoids such as *β*-carotene, lutein and violaxanthin (Ahmed et al., 2014). Even though *Tetraselmis* biomass is usually used in aquaculture feeds, recently the European Union approved the human consumption of biomass from a microalga of this genus as "novel food" (AECOSAN, 2014). Moreover, the inclusion of microalgal products in the human diet has been suggested, not only because of their comparable levels of carotenoids with those of vegetables, but also due to their antioxidant and cell-repairing activities against human lung cancer (Di Lena et al., 2019; Sansone et al., 2017).

Recently, a robust marine microalga *Tetraselmis* sp. CTP4 was isolated displaying high growth rates and the ability to outcompete contaminants (Pereira et al., 2016). Remarkably, this microalga can withstand temperatures ranging from 5-40 °C, being able to grow in wastewater as well as seawater with salinities of up to 75‰ (data not shown). All these properties allowed for the successful scale-up to industrial photobioreactors of this robust microalgal strain (Pereira et al., 2018). In addition, because this microalga becomes predominantly unflagellated at later growth stages, a low-cost harvesting step in a settling tank driven by natural sedimentation was also implemented (Pereira et al., 2018a). Furthermore, the wet biomass of this microalga showed significant amounts of extractable carotenoids when using acetone and glass-bead milling (L. Schüler, L. Barreira, J. Varela, unpublished results).

In this study, carotenoid profiles of *Tetraselmis* sp. CTP4 were investigated under four abiotic growth factors, namely light intensity, temperature, salinity, nitrate repletion or depletion during different growth stages. Even though few studies have focused on the induction of carotenoids by abiotic stress factors in microalgae belonging to this genus, this is the first thorough report dissecting the interaction of these growth conditions (Dahmen-Ben Moussa et al., 2017; Dammak et al., 2017; Goiris et al., 2015; Ruivo et al., 2011; Tsai et al., 2016). The aim of this study is to provide knowledge of how carotenoid biosynthesis is regulated in *Tetraselmis* microalgae and where carotenoids accumulate in this chlorophyte. Furthermore, a cultivation strategy is suggested to obtain carotenoid-rich biomass, which could be applicable not only in aquaculture but also as novel food or cosmeceutical.

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4.2   Materials and methods

4.2.1   Organism, standard growth conditions and harvesting

The microalga *Tetraselmis* sp. CTP4 was isolated from Ria Formosa in Portugal as described in Pereira et al. (2016). Standard growth conditions were established in previous reports (Pereira et al., 2016; Schulze et al., 2017). Briefly, initial microalgal cultures were grown in 5-L reactors (Ø = 12 cm) using sterilized Atlantic seawater from the shoreline of Faro, Portugal (salinity ~35 ‰) enriched with Modified Algal Medium (MAM) containing 27.2 mg N-NO_3^- L^{-1} at 20 °C with a photon flux density (PFD) of 100 µmol m^{-2} s^{-1} and aeration of the cultures by 0.2 µm-filtered air for 10 days. After this 10-day growth period, nitrate content was found to be almost depleted (0.75 mg N-NO_3^- L^{-1}) and cultures were used as inoculum for abiotic stress experiments. All experiments were performed using biological triplicates in a growth chamber (Radiber, SA, Spain) to ensure that a stable temperature was achieved. Horizontally mounted LED strips (LEDUP, SMD 5050, 6000K, 10.8 W m^{-1}, Teclusa, Portugal) supplied constant illumination of the cultures and light intensity was measured with a micro quantum sensor (Model US-MQS-B, Walz, Effeltrich, Germany). After the incubation periods, cultures were harvested by centrifugation (5000 *g*, 5 min); pellets and supernatants were kept frozen at -20 °C until further analysis.

4.2.2   Culture conditions for abiotic stress on carotenoid induction

4.2.2.1   Effect of temperature and salinity

Cultures were inoculated in 5-L reactors (Ø = 13 cm) containing different salinities (5, 20 and 35 ‰), which were achieved by diluting the seawater with distilled water. After supplementation with MAM, cultures were grown at 5, 20 or 30 °C with a PFD of 100 µmol m^{-2} s^{-1} until early stationary phase.

4.2.2.2   Effect of light intensity and nitrate availability

This experiment was performed in 100-mL glass photobioreactors (Ø = 3 cm), filled up with culture to a volume of 80 mL. All cultures were inoculated with a biomass concentration of 0.3 g L^{-1} and grown in seawater with a salinity of 35 ‰ supplemented with MAM without nitrate. A solution of $NaNO_3$ was added to half of the cultures to achieve a final concentration of 97 mg N-NO_3^- L^{-1} (N+, nitrogen replete), whereas the other half were incubated without

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additional nitrate (N-, nitrogen depleted). During this growth period, three different PFDs were applied: 33, 170 and 280 µmol m^{-2} s^{-1}. Cultures were incubated at 30 °C and samples were taken after 2, 5 and 10 days.

4.2.2.3   Effect of short-term induction at high temperature

This experiment was performed under the same conditions as the previous experiment with the following modifications. All cultures were inoculated with a biomass concentration of 0.6 g L^{-1} and were incubated at 35 °C using two different PFDs: 33 and 170 µmol m^{-2} s^{-1}. Samples were taken after 2 and 5 days.

4.2.3   Microscopy

For microscopy, samples were taken from cultures grown at high temperature (35 °C) under nitrogen repletion or nitrogen depletions during a period of 5 days. Images were acquired with a Zeiss AXIO IMAGER Z2 microscope, equipped with a coolSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), using a 100x magnification. For fluorescence imaging, a Zeiss 38 HE filter set (ex. 470/40 nm, em. 525/50 nm) was used (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and the transmitted light images were acquired using differential interference contrast (DIC). Images were treated using Image J software (Research Service Branch, NIH, Bethesda, MD).

4.2.4   Analytical methods

4.2.4.1   Determination of growth by optical density and dry weight

To monitor cell growth and biomass concentration, the optical density was measured in 96-well plates at 750 nm (OD$_{750}$) and the dry weight was determined as described previously (Pereira et al., 2016; Schulze et al., 2017). Briefly, dry weight (DW) was determined by filtering a 2-ml sample of each culture through a pre-washed and pre-weighed glass microfiber filter (0.47 µm), washed with 20 mL of ammonium formate (31.5 g L^{-1}) and dried at 60 °C until constant weight. A significant correlation ($r \geq 0.96$, $p < 0.01$) between OD$_{750}$ and DW was found using biomass from different growth conditions ($n = 55$). For the calculation of the DW used in carotenoid extraction, the following equation (1) was used:

$$DW \ [g \ L^{-1}] = 1.6101 * OD_{750} \tag{1}$$

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4.2.4.2 Determination of nitrate concentration

Nitrate concentration was determined by the UV spectrophotometric method as described in Schulze et al. (2017).

4.2.4.3 Carotenoid extraction and quantification

Carotenoids were extracted from wet biomass by resuspending a biomass sample of about 2 mg in 3 mL of acetone under dimmed light to avoid oxidation. After the addition of 0.7 g of glass beads (425-600 µm), the tubes were vortexed using an IKA Vortex Genius 3 at maximum speed for 2 min to lyse the cells. To collect the supernatant, the samples were centrifuged for 8 min at 8000 g. The extraction procedure was repeated until both the pellet and the supernatant became colourless. After evaporation of acetone under a gentle nitrogen flow, the extracts were resuspended in 700 µL of methanol and filtrated through 0.22 µm PTFE filter to remove suspended particles.

The carotenoids present in the extract were identified and quantified by HPLC. The Dionex 580 HPLC System (DIONEX Corporation, United States) consisted of a PDA 100 Photodiode-array detector, P680 Pump, ASI 100 Automated Injector and STH 585 column oven set to 20 °C. Separation of carotenoids was achieved with a LiChroCART RP-18 (5µm, 250x4 mm, LiChrospher) column using a 1 ml min^{-1} flow and a solvent program adapted from Couso et al. (2012). Briefly, the gradient mobile phase composed of solvent A acetonitrile:water (9:1; v/v) and solvent B ethyl acetate was applied as followed: 0–16 min, 0–60% B; 16–30 min, 60% B; 30–32 min 100% B and 32-35 min 100% A. All carotenoids were detected at 450 nm and analysed with Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, US). The quantification was carried out using calibration curves of neoxanthin, violaxanthin, lutein and β-carotene standards (Sigma-Aldrich, Portugal). Injection volume of both extracts and standards was 100 µL.

4.2.5 Data treatment

The carotenoid production was calculated as follows:

Production [mg/L*d] = (content [mg/g] * biomass [g/L])/time[d]   (2)

Results were analysed using SPSS (release 25.0, SPSS Inc., Chicago, IL) software without data treatment. Data was evaluated for normality (Shapiro-Wilk) and homogeneity of

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variances (Levene test). For the comparison of means (ANOVA), the Tukey HSD test for equal variances with a confidence interval of 95% was performed.

4.3 Results and discussion

4.3.1 Carotenoid profile of *Tetraselmis* sp. CTP4

The major carotenoids of the chlorophyte *Tetraselmis* sp. CTP4 were violaxanthin, lutein and *β*-carotene as detected by HPLC analysis of acetone extracts (Figure 4.1A). Under the conditions used, microalgal cells also contained detectable amounts of neoxanthin, zeaxanthin and *α*-carotene.
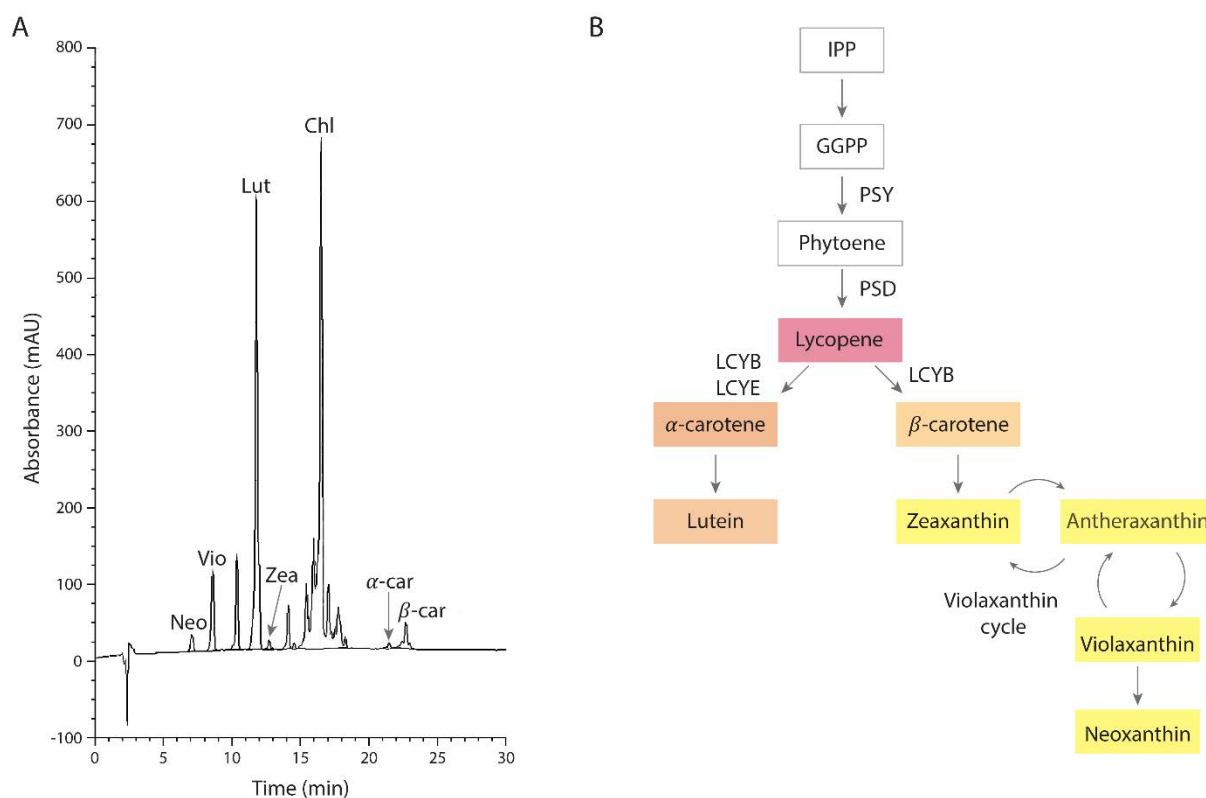
Figure 4.1. Diagram of carotenoids present in the chlorophyte *Tetraselmis* sp. CTP4. (A) Representative pigment acetone extract of CTP4 measured by HPLC (450 nm) revealed the carotenoids: neoxanthin (Neo), violaxanthin (Vio), lutein (Lut), zeaxanthin (Zea), *α*-carotene (*α*-car) and *β*-carotene (*β*-car). Peaks detected at about 18 min were identified as chlorophylls (Chl) by their absorption spectra. (B) A simplified diagram of the carotenoid biosynthetic pathway starting at the conversion of isopentenyl pyrophosphate (IPP) to geranyl-geranyl pyrophosphate (GGPP), highlighting violaxanthin, lutein and *β*-carotene as the carotenoids most abundant. Phytoene synthase (PSY), phytoene desaturase (PDS), lycopene *β*-cyclase (LCYB), lycopene ε-cyclase (LCYE). The aforementioned pathway was adapted from Varela et al. (2015).

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These results thus suggest that this strain has two active branches of the carotenoid biosynthetic pathway (Figure 4.1B): one giving rise to α-carotene and lutein, and a second one leading to the biosynthesis of β-carotene and other high-value xanthophylls (Mulders et al., 2014b). Both branches depend upon different lycopene cyclases that give rise to different intermediates (Ramos et al., 2008). For example, the branch yielding β-carotene is known to generate zeaxanthin via a reaction of hydroxylation. This xanthophyll can be converted to antheraxanthin and then to violaxanthin by different reversible reactions, which are usually referred to as "the violaxanthin cycle" (Varela et al., 2015). Therefore, the detection of violaxanthin in the biomass suggests that this photoprotective pathway may be active in this strain, as appears to be the case for many chlorophytes (Jahns et al., 2009). Interestingly, even though neoxanthin can be synthesized by the direct isomerization of violaxanthin, it was only found at minor concentrations in *Tetraselmis* sp. CTP4.

4.3.2 Effect of temperature and salinity on carotenoid contents

In order to understand what environmental conditions favoured the induction of carotenogenesis in this microalga, the first abiotic factors addressed were temperature and salinity (Figure 4.2). A temperature of 20 °C and a salinity of 35 ‰ corresponded to "standard growth conditions" as used in previous studies on this strain (Pereira et al., 2016; Schulze et al., 2017), under which the cells had contents of neoxanthin, violaxanthin, lutein and β-carotene of 0.5, 0.76, 1.64, and 2.4 mg g^{-1} DW, respectively (Figure 4.2). These amounts are similar or even higher than those reported in other *Tetraselmis* strains (Ahmed et al., 2014; Di Lena et al., 2019), except for one species that was isolated from tropical waters, which had lutein and β-carotene contents of 4.8 and 51.4 mg g^{-1} DW, respectively (Tsai et al., 2016).

At 20 °C, salinity downshifts to as low as 5 ‰ did not significantly affect the carotenoid contents. A decrease in temperature to 10°C, however, resulted in an overall 2-fold decrease in all pigments, regardless of the salinity, as compared to standard growth conditions (Figure 4.2). At low temperatures, the nutrient uptake rate decreases and thus the metabolism slows down leading to lower amounts of carotenoids (Bhosale, 2004). Indeed, lower growth rates of cultures grown at 10°C (data not shown) were observed. Remarkably, when carotenoid contents in cells cultivated at 30 °C are compared, significant differences can be observed between cultures grown at different salinities for carotenoids produced by the β-carotene branch within the carotenoid biosynthetic pathway (Figs. 4.1B and 4.2). Remarkably, at this temperature, neoxanthin and violaxanthin contents increased about 2-fold with increasing

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salinity, reaching a maximum of 1.01 ± 0.06 mg g^{-1} DW and 1.58 ± 0.16 at 35 ‰, respectively (Figure 4.2A, B). In the case of *β*-carotene, cells at a salinity of 5 ‰ showed a 2-fold decrease in the content of this pigment as compared to standard growth conditions. Conversely, at a salinity of 35 ‰, cells increased their *β*-carotene content to a maximum of 3.37 ± 0.36 mg g^{-1} DW (Figure 4.2D).
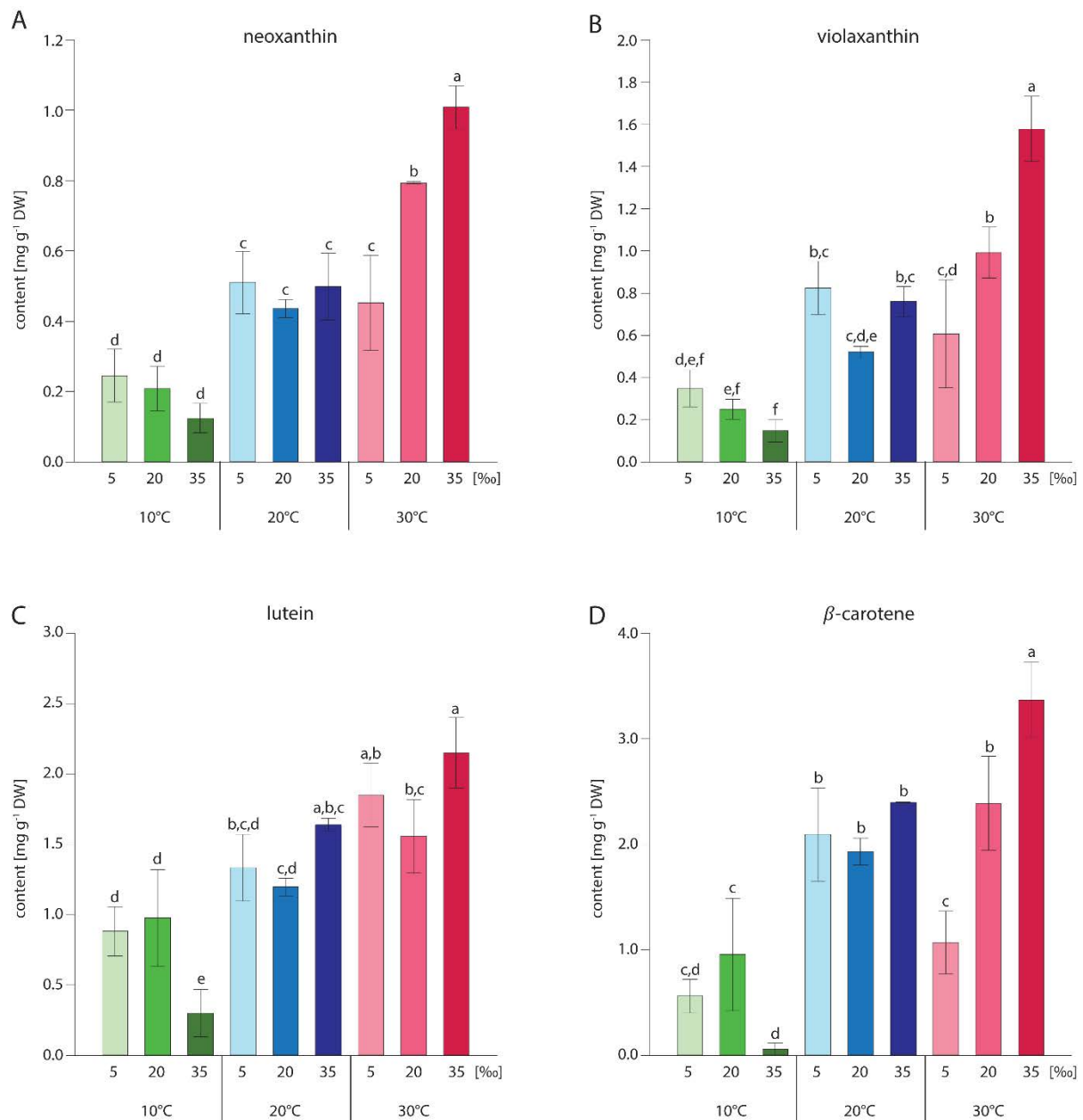
Figure 4.2. Influence of temperature and salinity on carotenoid content of *Tetraselmis* sp. CTP4. Cultures were inoculated in 5-L reactors containing different salinities of 5, 20 and 35‰ and grown at 5, 20 and 30 °C with a light intensity of 100 µmol m^{-2} s^{-1} until stationary phase (*n* = 3, average ± SD). Carotenoid extracts were analyzed by HPLC and neoxanthin (A), violaxanthin (B), lutein (C) and *β*-carotene (D) were quantified. Different letters over the bars indicate significant differences ($p < 0.05$) using two-way ANOVA with post hoc Tukey HSD test.

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The increase in temperature, however, did not result in significant differences in lutein contents (Figure 4.2C). Nevertheless, at a salinity of 35 ‰ and 30°C, lutein content reached the maximum of 2.15 ± 0.25 mg g^{-1} DW. An increase in lutein contents with temperature has been observed in other microalgae such as *Chlorella sorokiniana*, *Muriellopsis* sp. and *Scenedesmus almeriensis* (Cordero et al., 2011b; Del Campo et al., 2000; Sánchez et al., 2008). Xanthophylls are structural components in the thylakoid membrane and may help to stabilize the membranes upon thermal upshifts to maintain membrane fluidity, which is important for molecule exchange or the function of light harvesting complexes (Havaux, 1998). Furthermore, high salinity and heat stress are responsible for the accumulation of ROS, which can be scavenged by *β*-carotene and lutein (Britton et al., 2008). In this study, the combined application of a temperature and salinity upshift led to the highest total carotenoid contents in *Tetraselmis* sp. CTP4 (8.11 ± 0.61 mg g^{-1} DW).

4.3.3 Effect of light intensity and nitrogen availability on carotenoid contents

As previous experiments have shown that a temperature of 30 °C and a salinity of 35 ‰ are optimal for carotenoid induction, these parameters were maintained constant. Cultures under nitrogen depletion (N-, 0.75 mg N-NO$_3^-$ L^{-1}) remained at a plateau of about 0.4 g L^{-1} regardless of the light intensity over the incubation period of 10 days whereas cultures under nitrogen repletion (N+) showed exponential and/or linear growth (Figure 4.3). At light intensities of 170 and 280 µmol m^{-2} s^{-1}, cells grew exponentially with a very short lag phase during the first day. After 10 days, the highest biomass of 2.1 g L^{-1} was reached under both light intensities. At this time point, nitrates were completely consumed and cells entered into nitrogen starvation (Figure 4.3B). At a lower light intensity (33 µmol m^{-2} s^{-1}), cells grew slower with a longer lag phase in the first two days. After 10 days, the biomass rose up to 1.25 g L^{-1} and a nitrate concentration of 21.75 mg N-NO$_3^-$ L^{-1} was still present in the media.

Regarding carotenoid contents, the most decisive factor was nitrogen availability, as cells under nitrogen repletion showed on average total carotenoid contents 2.5-fold higher than those of cells under nutrient depletion (Figure 4.4). Enhanced carotenoid contents under nitrogen repletion is in agreement with studies on other *Tetraselmis* species (Dammak et al., 2017; Goiris et al., 2015; Tsai et al., 2016). The importance of, for example, nitrogen availability for carotenogenesis may be due to the connection with the synthesis of proteins of the light harvesting complex that bind, among others, to carotenoids (Jahns et al., 2009). Even though this microalga accumulated high lipid contents of up to 30% of its DW under nitrogen

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depletion, this condition did not promote the accumulation of carotenoids (Pereira et al., 2016). On the contrary, the observed decreased contents of carotenoids under nitrogen depletion may be the result of reduced metabolic and photosynthetic activity of the cells. Interestingly, the contents of lutein in N-starving cells levelled at about 0.95 mg g^{-1} DW throughout the experiment (Figure 4.4C). This result suggests that this xanthophyll is perhaps one of the most important carotenoids, particularly when this alga is under stress. Lutein has been proposed to be an important photoprotective pigment with a scavenging role in cells under these conditions (Couso et al., 2012). When comparing different microalgal cultures grown at different light intensities, total carotenoid contents were 1.5-fold higher in cells at a low light intensity (33 μmol m^{-2} s^{-1}) compared to those under a higher light intensity (170 μmol m^{-2} s^{-1}). The induction of carotenoids under low light conditions could be due to a larger chloroplast and the need for a more efficient light utilization (Berner et al., 1989).
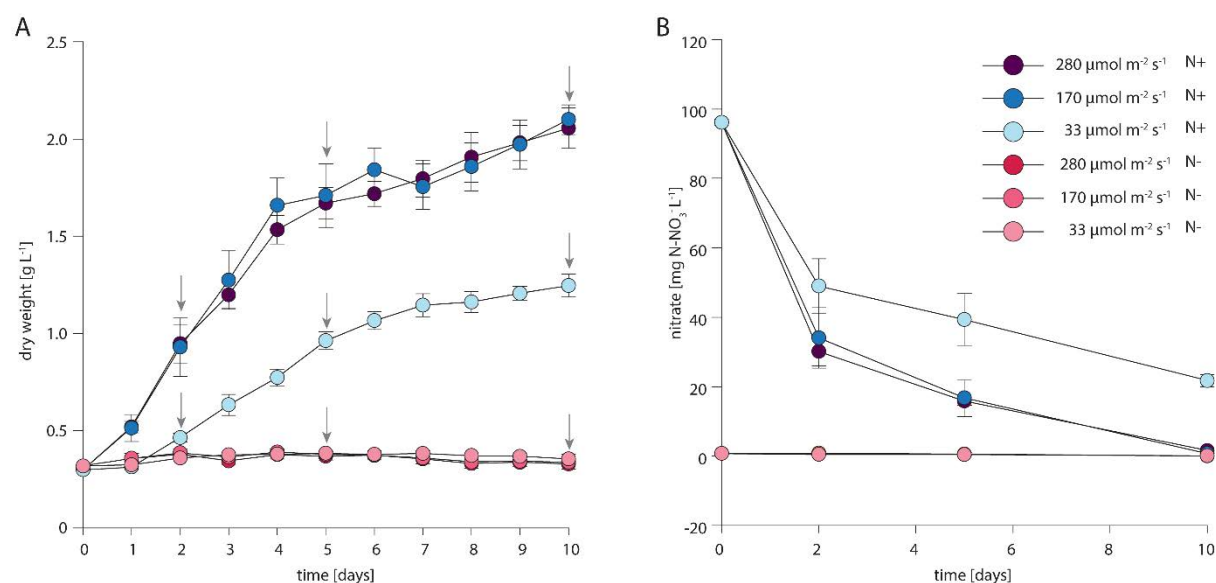
Figure 4.3. Cultures of *Tetraselmis* sp. CTP4 under nitrogen repletion (N+) and depletion (N-) at different light intensities. Both growth curves (A) and nitrate availability (B) are shown (*n* = 3, average ± SD). Arrows on the growth curves indicate the harvesting time points of the biomass for carotenoid and nitrate analysis.

The xanthophylls neoxanthin and violaxanthin were found to be up to 3-fold higher in cells grown under these lower light conditions than under higher light (Figure 4.4B). Furthermore, the content of *β*-carotene was up to 3-fold higher in cells at low light (33 μmol m^{-2} s^{-1}) as compared to those under higher light intensities, most probably due to its role as a light harvesting pigment under this lower light intensity (Figure 4.4D). Lutein contents, however, were 1.5-fold higher under higher light intensities (170 and 280 μmol m^{-2} s^{-1}) than under lower

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light (Figure 4.4C) in cells under nutrient repletion. Conversely, this was not observed in cells under nutrient starvation.
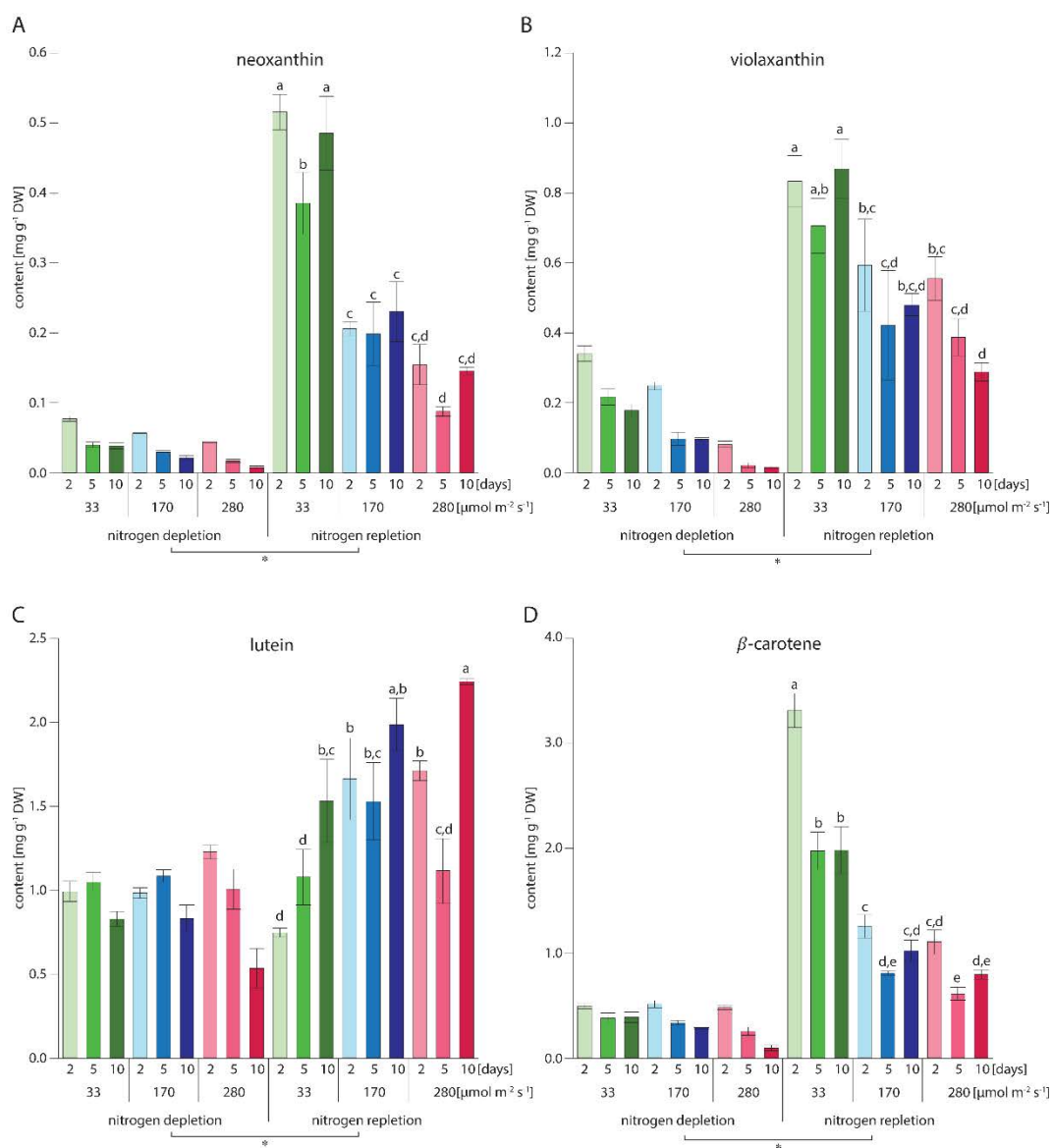
Figure 4.4. Effect of light intensity and nutrient availability on the carotenoid contents of *Tetraselmis* sp. CTP4 at 30 °C. Cultures were supplemented with MAM without (nutrient depletion) or with (nutrient repletion) nitrate to a final concentration of 97 mg N-NO$_3$ L^{-1} and grown at light intensities of 33, 170 and 280 μmol m^{-2} s^{-1} (n = 3, average ± SD). Contents of neoxanthin (A), violaxanthin (B), lutein (C) and *β*-carotene (D) were quantified in the biomass harvested after 2, 5 and 10 days of growth using RP-HPLC. Significant differences between carotenoid contents of cells under nitrogen depletion or repletion are indicated with an asterisk. For neoxanthin, violaxanthin, lutein and *β*-carotene a two-way ANOVA with post hoc Tukey HSD test was performed. Different letters over the bars indicate significant differences (p < 0.05).

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Taken together, these results seem to highlight once more the role of lutein as a photoprotective pigment in actively growing *Tetraselmis* cells. However, the observed increase in the intracellular lutein levels is apparently inhibited in starved microalgal cultures. These observations are in agreement with studies on *C. sorokiniana* (3.1 mg lutein g^{-1} DW at 690 μmol $m^{-2} s^{-1}$) and *Muriellopsis* sp. (0.51 pg lutein $cell^{-1}$ at 460 μmol $m^{-2} s^{-1}$), where lutein contents increased with increasing light intensity in actively growing cultures (Cordero et al., 2011b; Del Campo et al., 2000).

Furthermore, the incubation period influenced the carotenoid contents. Already after 2 days of incubation at a light intensity of 33 μmol $m^{-2} s^{-1}$ *β*-carotene content reached a maximum of 3.31 ± 0.16 mg g^{-1} DW, followed by a significant decrease after 5 and 10 days of incubation to 1.98 ± 0.2 mg g^{-1} DW (Figure 4.4D). A similar response was observed for violaxanthin and neoxanthin contents, which were highest 2 days after a downshift of the light intensity to 33 μmol $m^{-2} s^{-1}$ (0.87 ± 0.09 mg g^{-1} DW and 0.52 ± 0.03 mg g^{-1} DW, respectively). Lutein contents at low light intensities of 33 μmol $m^{-2} s^{-1}$ increased with increasing incubation time reaching 1.53 ± 0.25 mg g^{-1} DW after 10 days (Figure 4.4C). These contents at low light intensities are similar to those obtained previously (Figure 4.2), which leads to the assumption that the actual light intensity in the larger photobioreactors (5 L) was sensed as low light by the cells. The observed increase in carotenoid levels after 2 days may be a short-term acclimation response to the new growth conditions. At low light, *β*-carotene is apparently the most important pigment due to its double role in light harvesting and $^1O_2$ quenching, while at higher light intensities lutein becomes the main carotenoid. After 5 days of incubation, the cells seemed to have become acclimated to the new environment, ceasing carotenoid biosynthesis, so that most of the carbon (and energy) is used in growth and cell division, resulting in the observed drop in carotenoid contents. After 10 days, however, when the cultures reach high cell concentrations and nitrates became limiting (Figure 4.3), carotenoid contents tend to rebound, in particular at the highest light intensity used, probably due to their role in photoprotection. Highest contents of lutein were observed at this time point, reaching a maximum of 2.24 ± 0.02 mg g^{-1} DW at higher light intensities of 280 μmol $m^{-2} s^{-1}$. In a previous study on lutein production by *Scenedesmus obliquus*, the highest contents of this xanthophyll (4.75± 1.69 mg g^{-1} DW) were also observed at the beginning of nitrogen depletion at a light intensity of 150 μmol $m^{-2} s^{-1}$ (Ho et al., 2014).

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4.3.4   Short-term induction of lutein and *β*-carotene upon a thermal upshift

As previous experiments showed that high temperature (30 °C), nutrient repletion and an incubation period of only 2 days were sufficient to achieve high contents of neoxanthin, violaxanthin, lutein and *β*-carotene, cells were further exposed to an even higher temperature (35 °C) for a period of only 2 days (Figure 4.5). Interestingly, carotenoid contents (8.48 ± 0.47 mg g^{-1} DW) increased under these conditions compared with previous experiments (Figure 4.4). Indeed, the contents of neoxanthin and *β*-carotene were highest at a light intensity of 33 µmol m^{-2} s^{-1} (0.82 ± 0.04 and 4.41 ± 0.12 mg g^{-1} DW, respectively, Figure 4.5A). Conversely, the lutein content (3.17 ± 0.18 mg g^{-1} DW) was highest at the highest light intensity (170 µmol m^{-2} s^{-1}) tested (Figure 4.5A). Contents of violaxanthin (1.67 ± 0.04 mg g^{-1} DW) did not show significant differences between the treatments. These results confirm the trends observed earlier (Figure 4.2 and 4.4). However, when productions are compared, no significant difference for neoxanthin and *β*-carotene can be found between cells at both light intensities (0.34 ± 0.01 and 1.85 ± 0.12 mg L^{-1} d^{-1}, respectively; Figure 4.5B). Conversely, violaxanthin (0.95 ± 0.05 mg L^{-1} d^{-1}) and lutein (1.83 ± 0.12 mg L^{-1} d^{-1}) productions are highest at 170 µmol m^{-2} s^{-1} (Figure 4.5B).
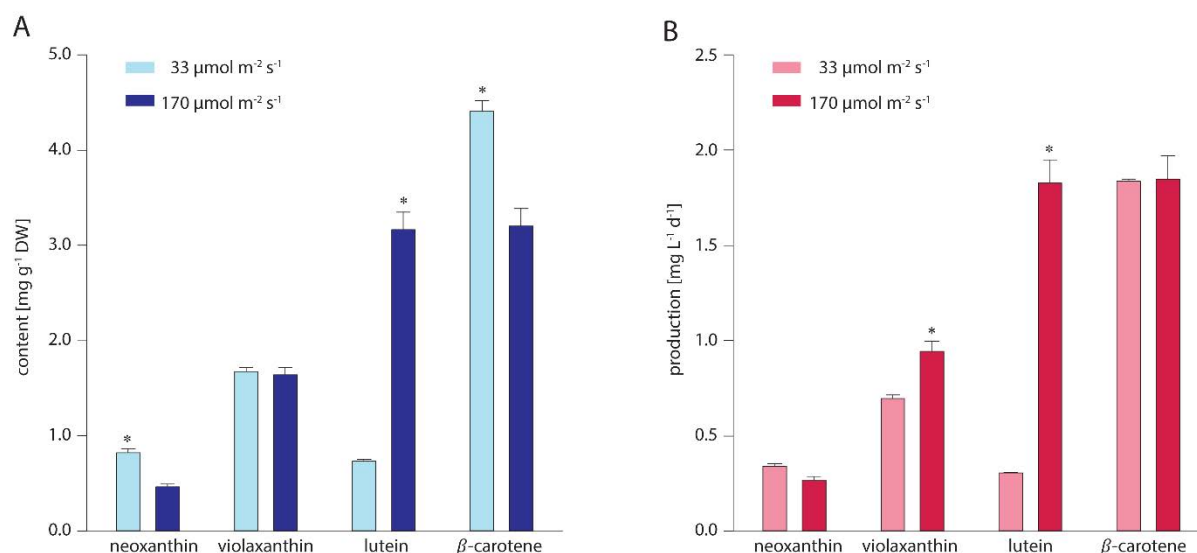
Figure 4.5. Carotenoid contents and productions of *Tetraselmis* sp. CTP4 after 2 days of cultivation under nitrogen repletion at 35 °C. Cultures were grown at light intensities of 33 µmol m^{-2} s^{-1} and 170 µmol photons m^{-2} s^{-1} and the carotenoids neoxanthin, violaxanthin, lutein and *β*-carotene were analysed (*n* = 3, average ± SD). Significant differences (p < 0.05) between carotenoid contents (A) and productivities (B) are indicated with an asterisk.

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Taken together, these results point out a possible combination of high temperature of 35 °C and a light intensity of 170 µmol m^{-2} s^{-1} under which *Tetraselmis sp.* CTP4 can simultaneously produce high amounts of lutein and *β*-carotene. Remarkably, this was possible despite their biosynthesis being differently regulated in response to a changing environment in the previous experiments. Furthermore, these higher contents of carotenoids were achieved in a very short period of only two days. Thus, in combination with the easy harvesting step of this microalga, this process becomes more economical and *Tetraselmis sp.* CTP4 may be considered a novel source of carotenoids. However, further studies are necessary to examine the production of carotenoids in industrial photobioreactors.
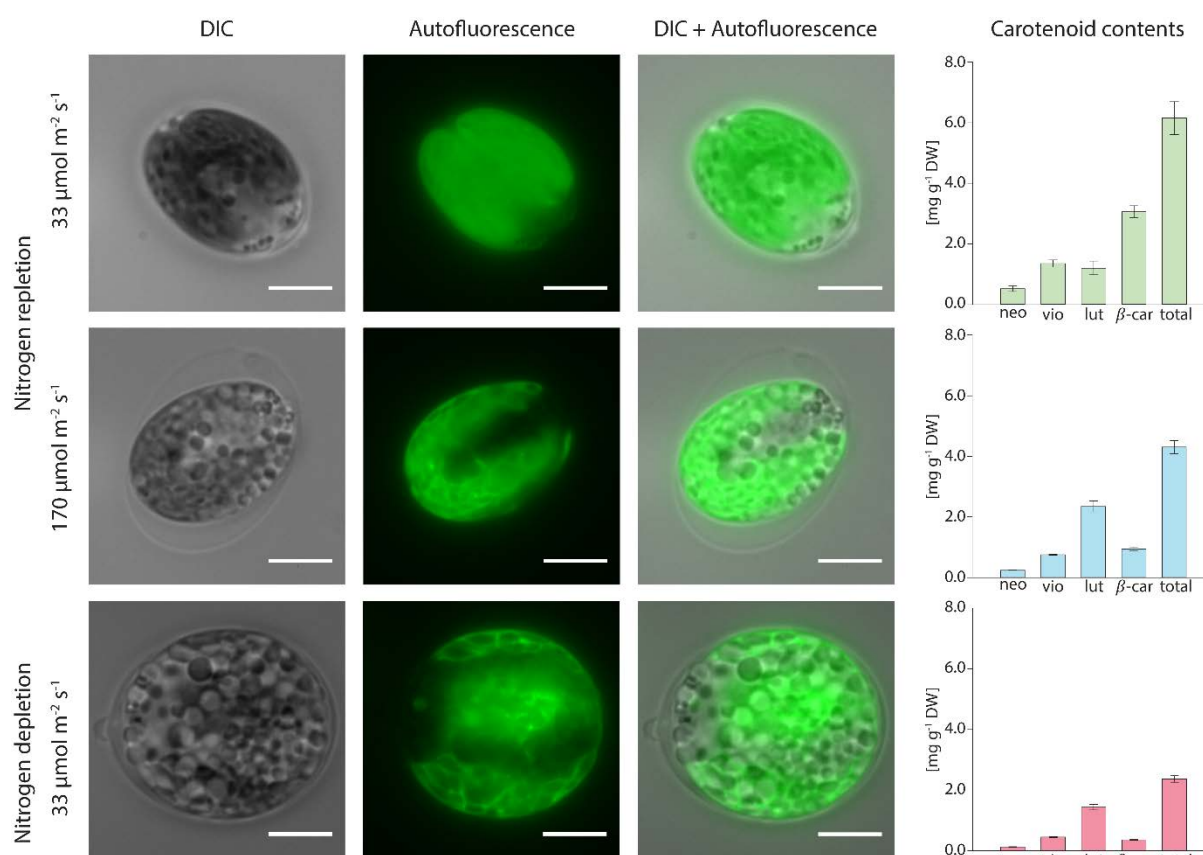
Figure 4.6. Autofluorescence and carotenoid contents of *Tetraselmis* sp. CTP4 cells under different abiotic stressors**.** After incubation of the cultures for 5 days at 35 °C, neoxanthin (neo), violaxanthin (vio), lutein (lut) and *β*-carotene (*β*-car) were analysed. Furthermore, of the same samples, images were taken using differential interference contrast (DIC) and filter set 38 HE (ex. 470/40 nm, em. 525/50 nm) for autofluorescence. Scale bar = 5 µm.

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Microscopic observation of the cultures grown at a PFD of 33 µmol m^{-2} s^{-1} and 170 µmol m^{-2} s^{-1} under nitrogen repletion and depletion sampled on day 5 revealed the localization of carotenoids and lipids inside cells (Figure 4.6). Cells under the higher light intensity and under nitrate depletion contained round intracellular structures corresponding to lipid droplets as reported previously (Pereira et al., 2018, 2016). As expected, total carotenoid content was low (2.37 ± 0.11 mg g^{-1} DW), of which lutein accounted for more than 50% (1.44 ± 0.09 mg g^{-1} DW). Under nitrate repletion conditions, however, almost no lipid droplets are visible; the carotenoid autofluorescence is diffuse throughout the cell. The total carotenoid content was 2.6-fold higher than under depletion conditions (6.15 ± 0.54 mg g^{-1} DW, Figure 4.6), of which $β$-carotene accounted for more than 50% (3.07 ± 0.2 mg g^{-1} DW). Conversely, at a PFD of 170 µmol m^{-2} s^{-1}, the autofluorescence becomes more intense at specific spots inside the cell, becoming less diffuse. Furthermore, the carotenoids seem to be present only in the chloroplast, following its typical U-shape (Pereira et al., 2018). Compared with the lower light intensity, the total carotenoid content was lower (4.31 ± 0.54 mg g^{-1} DW) while lutein content increased 2-fold (2.35 ± 0.18 mg g^{-1} DW). On closer examination of the overlay of DIC and autofluorescence micrographs, cells under nitrogen depletion appear to accumulate carotenoids outside of the lipid bodies, unlike *Haematococcus pluvialis* and *Dunaliella salina*, which accumulate astaxanthin and $β$-carotene in lipid bodies (Davidi et al., 2014; Ota et al., 2018). These findings strongly suggest that lutein and $β$-carotene are present in the thylakoid membrane rather than in lipid droplets in this species (Schüler et al., 2017), which may explain why *Tetraselmis* cells do not accumulate carotenoids under the exact same conditions that favour lipid production. Therefore, it would be important to improve this microalga by selection of carotenoid-hyperproducing mutants, which may be able to accumulate carotenoids inside the lipid bodies.

4.4   Conclusion

This study evaluated the carotenoid profiles of *Tetraselmis* sp. CTP4 under a wide range of abiotic stress factors. A two-stage cultivation strategy could maximize the production of lutein, violaxanthin and $β$-carotene; during the first stage high biomass concentrations would be attained, and in the second stage, pigments would be induced by thermal and light upshifts. Although *Tetraselmis* microalgae are mainly used in aquaculture as feed, *Tetraselmis* sp. CTP4 could be an interesting candidate for the production of violaxanthin, lutein and $β$-

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carotene for nutraceutical and/or cosmeceutical applications, in particular when thermotolerant, robust microalgae are needed, such as the southern part of Portugal.

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Funding

CHAPTER 5

Carotenoid biosynthetic gene expression, pigment and *n*-3 fatty acid contents in carotenoid-rich *Tetraselmis striata* CTP4 strains under heat stress combined with high light

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Carotenoid biosynthetic gene expression, pigment and *n*-3 fatty acid contents in carotenoid-rich *Tetraselmis striata* CTP4 strains under heat stress combined with high light

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Highlights

- Random mutagenesis coupled with FACS as powerful tool for strain improvement

- Norflurazon-resistant mutants showed increase in carotenoids over WT

- Expression of six carotenogenic genes was upregulated in mutants

- Norflurazon-resistant mutants showed fatty acid profile enriched in EPA

- Increase in carotenoids and EPA might have improved survival under stress

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Abstract

In this study, two carotenoid-rich strains of the euryhaline microalga *Tetraselmis striata* CTP4 were isolated by random mutagenesis combined with selection via fluorescence activated cell sorting and growth on norflurazon. Both strains, ED5 and B11, showed an up to 1.5-fold increase in carotenoid contents as compared with the wildtype, independent of the growth conditions. More specifically, violaxanthin, *β*-carotene and lutein contents reached as high as 1.63, 4.20 and 3.81 mg g^{-1} DW, respectively. Several genes encoding enzymes involved in carotenoid biosynthesis were found to be upregulated in ED5 microalgae under stress conditions. Conversely, in B11 cells, upregulation was mainly observed under mesophilic growth. Both strains showed an enrichment in eicosapentaenoic acid in their fatty acid profiles as compared with those of the wildtype, reaching 5.8 and 8.7% of total fatty acids, respectively, suggesting they are a promising feedstock for high-value compounds for future applications in the nutraceutical or cosmeceutical industries.

Keywords: Microalgae; random mutagenesis; flow cytometry; lutein; EPA

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5.1   Introduction

Microalgae are alternative biological feedstocks rich in proteins, carbohydrates and lipids, containing bioactive high-value compounds such as carotenoids and essential fatty acids. Unlike traditional food crops, microalgae display higher productivities and can grow on non-arable land using non-potable water. Recently, high attention has been paid to microalgae rich in carotenoids and *n*-3 fatty acids, due to their applications in the food, feed, pharmaceutic, nutraceutical and cosmetic industries (M. U. et al., 2019). The simultaneous production of several high-value compounds has the potential to decrease production costs and to facilitate the application of the biorefinery concept to microalgal biomass for the manufacture of products with enhanced appeal to stakeholders and consumers alike.

Fatty acids are the major components of lipids, which can be divided into polar and non-polar lipids (Guschina and Harwood, 2013). Non-polar lipids such as triacyclglycerols (TAGs) are storage lipids and provide energy to the cells. Polar lipids, however, are composed of phospho- and glycolipids, which are important structural components of membranes. While TAGs are predominantly composed of saturated fatty acids, membrane lipids usually contain high amounts of polyunsaturated fatty acids (PUFA), which play an important role in several defence and signalling mechanisms (Okuyama et al., 2008). For example, under stress conditions, PUFA, e.g., eicosapentaenoic acid (EPA), remove reactive oxygen species (ROS) and thus prevent oxidative stress-induced cell damage. Carotenoids are lipophilic isoprenoid biomolecules and can be divided into two classes, namely hydrocarbon carotenoids, referred to as carotenes, and their oxygenated derivates, the xanthophylls. The carotenoid biosynthesis in microalgae occurs in the chloroplast and has been explained in detail previously (Mulders et al., 2014b; Varela et al., 2015). Briefly, the first carotenoid phytoene results from the condensation of two geranylgeranyl pyrophosphates (GGPP) catalysed by phytoene synthase (PSY). The following reaction is catalysed by phytoene desaturase (PDS) leading to lycopene, the branch point of the synthesis. Either *β*-carotene or *α*-carotene are synthesized from lycopene depending on the activities of lycopene *β*- and *ε*-cyclases (LCYB and LCYE, respectively). The hydroxylation of *β*-carotene catalysed by the *β*-carotene hydroxylase (CHYB) gives rise to the xanthophylls of the violaxanthin cycle. Different types of hydroxylases, namely *β*-ring hydroxylase CYP97A5 and *ε*-ring hydroxylase CYP97C3 catalyse the stepwise synthesis of lutein from *α*-carotene (Kim and DellaPenna, 2006). In the microalgal cell, carotenoids play essential roles in photosynthesis, in light harvesting and energy transfer, as well as in photoprotection by dissipating excess energy as heat, thus preventing photooxidative damage (Mulders et al., 2014b). Moreover, xanthophylls such as

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zeaxanthin are structural components of membranes and may affect the fluidity of the lipid bilayer (Varela et al., 2015). The production of PUFA and carotenoids is not only highly species-dependent but also depends on environmental conditions such as light intensity and temperature. These conditions are known to influence the carotenoid and fatty acid composition in the cell due to changes in enzyme activity, gene expression and other physiological processes (Zhao et al., 2019).

In several attempts at genetic engineering of microalgae to enhance carotenoid and lipid production, one or several biosynthetic genes have been overexpressed, leading to inconsistent results concerning higher carotenoid or lipid levels (Gimpel et al., 2015). This lack of success is most probably due to the existence of several interdependent rate-limiting steps in these complex multienzyme pathways. Furthermore, specific transformation tools need to be generated for each particular strain, limiting the application of genetic engineering to microalgae. Alternatively, random mutagenesis has been widely used to generate improved strains, a method that does not require prior knowledge of biosynthetic pathways, because no heterologous DNA is introduced in the cell to target specific sequences of the wildtype genome. The combination of mutagenesis with a selection by visual observation or by the use of herbicides has been performed to obtain carotenoid or EPA-rich mutants of several microalgae (Cordero et al., 2011a; Huang et al., 2018; Moha-León et al., 2019). Herbicides inhibit enzymes of desired pathways (e.g., carotenoid or fatty acid biosynthesis) and an inhibitor-based screening facilitates the selection of strains expressing a mutation in the corresponding enzyme which may be related to higher compound accumulation. The herbicide norflurazon has been shown to not only inhibit PDS but also Δ6 desaturases in *Arthrospira* (Spirulina) *platensis* and *Monodus subterraneus* leading to changes in carotenoid and fatty acid composition, respectively (Breitenbach et al., 2001; Cohen and Heimer, 1990). Moreover, fluorescence activated cell sorting (FACS) is a powerful tool to select single cells with desired traits and has been used to enriched strains with higher carotenoid or EPA/lipid contents (Lim et al., 2015; Mendoza et al., 2008; Pereira et al., 2018b). Nevertheless, to the best of our knowledge, a combination of mutagenesis with mutant selection by herbicides followed by enrichment using FACS has never been used for the improvement of high-value compounds in microalgae.

In this study, mutants of a robust and industrially interesting euryhaline microalga *Tetraselmis striata* CTP4 were developed. To this end, random chemical mutagenesis using ethyl methane sulfonate was applied and mutants with improved carotenoid contents were selected using a two-step strategy: i) resistance to the herbicide norflurazon and ii) enrichment

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of improved strains by FACS. The carotenoid contents and fatty acid profiles of wildtype and mutants were studied under two different growth conditions, which have been previously shown to influence carotenoids in this species (Schüler et al., 2020b). Moreover, for the first time in this microalga, the expression profiles of six carotenogenic genes under different abiotic stresses were analysed. This study provides important information on the regulation of the carotenoid biosynthetic pathway in wildtype and mutant cells of *T. striata*. Furthermore, protein and lipid contents as well as fatty acid profiles were analysed to better understand the potential of these mutants in future products.

5.2 Methods

5.2.1 Strain and mesophilic growth conditions

The euryhaline microalga *Tetraselmis striata* CTP4 was isolated from the Ria Formosa, Algarve, Portugal using fluorescence activated cell sorting (FACS) by a selection procedure for lipid-rich strains (Pereira et al., 2016). Culture scale-up was performed at mesophilic growth conditions at 20 °C and a light intensity of 100 µmol m^{-2} s^{-1} as reported previously (Pereira et al., 2016; Schulze et al., 2017). Briefly, 50-mL Erlenmeyer flasks containing diluted seawater (20 ppt) enriched with Modified Algal Medium (MAM, (Pereira et al., 2016; Schulze et al., 2017)) were inoculated with colonies from plates and scaled up to 100-mL test tubes. Growth was followed by optical density measurements at 750 nm and cell count using a Neubauer chamber. Dry weight (DW) was calculated using the following equation (1):

$$DW [g\ L^{-1}] = 1.6101 * OD_{750} \tag{1}$$

5.2.2 Random mutagenesis and mutant selection

Exponentially growing cells of *T. striata* (2×10^6 cells mL^{-1}) were subjected to random mutagenesis by ethyl methane sulfonate (EMS, Merck, USA) as described in (Schüler et al., 2020a) with slight modifications. After 10-fold concentration upon centrifugation (3000 *g*, 3 min) and resuspension in the same media, cells were treated with 100, 150, 200, 250 and 300 mM of EMS for 1 h under mild agitation in the dark. The action of EMS was stopped by addition of sodium thiosulfate to a final concentration of 5% (m/v). Cells were pelleted by centrifugation at 3000 *g* for 3 min, washed thrice with sterile seawater (20 ppt) and incubated for 24 h in the dark in sterile seawater enriched with MAM. Afterwards, cells were plated onto solid (agar)

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MAM plates in serial dilutions and incubated at room temperature (RT) and a light intensity of 100 µmol m^{-2} s^{-1} for 2-3 weeks. Colony forming units were counted to determine cell viability. Mutant selection was carried out by plating at different norflurazon concentrations that were otherwise lethal to the wildtype (WT) cells. Herbicide-resistant colonies were sub-cultured several times on plates containing the herbicide to confirm their resistance. Afterwards, mutants were grown in liquid media under mesophilic growth conditions to compare growth and pigment contents with those of the WT strain.

WT and mutant strains with highest carotenoid contents were used for FACS to enrich for high carotenoid producing strains. Samples were acquired in a Becton Dickinson FACS Aria II (BD Biosciences, Belgium) equipped with a blue, red and violet laser (488, 633 and 407 nm, respectively) and FACSDiva (version 6.1.3) software. Two filters were used to record the fluorescence signal, namely FL1 and FL2 centred respectively at 695/40 and 530/30 nm after excitation with the blue laser. Cells emitting higher levels of fluorescence due to chlorophyll (FL1) and carotenoids (FL2) were sorted directly onto 96-well microplates containing 250 µL of solid MAM. Plates were incubated at RT with a photon flux density of 45 µmol m^{-2} s^{-1} for 3-4 weeks until colonies emerged. Mutants were plated against the herbicide to confirm their resistant phenotype and scaled up for further experiments.

5.2.3   Growth experiment to compare gene expression and biochemical profiles of WT and selected mutants

Pre-cultures of WT and two selected mutants, here named ED5 and B11, were grown under mesophilic conditions in 1-L photobioreactors with constant renewal of media to maintain cultures in the exponential growth phase. Afterwards, WT and mutant strains were inoculated at a biomass concentration of 0.24 g L^{-1} in 1-L bioreactors in triplicate and supplemented with MAM. Cultures were grown under mesophilic growth (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) for three days. Samples for RNA extraction were taken from the inoculum and after one day by centrifugation at 3000 g for 3 min at the corresponding growth temperature; biomass pellets were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Biomass for biochemical analysis was harvested from the inoculum and after two and three days of growth by centrifugation, lyophilized and stored at -20°C.

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5.2.4   Cell viability staining with SYTOX Green and cell count by flow cytometry

Cells obtained from the inoculum and after one and two days of growth were stained with the fluorescent dye SYTOX Green at a final concentration of 5 nM and incubated in the dark on ice for 10 min. For cell counts, samples were analysed in a Becton Dickinson FACS Calibur (BD Biosciences, Belgium) equipped with a blue and red laser (488 and 633 nm, respectively) and CellQuest Pro (version 6.0) software. The fluorescence signals of chlorophyll and SYTOX Green were obtained using the respective filters FL1 (630/30 nm) and FL2 (585/42 nm) upon excitation with the blue laser.

SYTOX Green binds to the nucleic acids of cells with permeabilized membranes, while intact cells remain unstained (Sato et al., 2004). In control experiments, the use of this method in flow cytometry (FC) was validated by comparing cell counts obtained by microscopy with those of the flow cytometer. To this end, exponentially growing cells and heat inactivated cells were stained with SYTOX Green and the cell viability was analysed. In a first step, microalgal cells emitting chlorophyll autofluorescence were counted in the microscope or gated by FC. Out of these cells, SYTOX Green emitting cells were counted to obtain the percentage of damaged or permeabilized cells. Moreover, in a recent study on *Tetradesmus obliquus*, SYTOX Green stained cells were successfully analysed by FC to measure cell viability (Dias et al., 2020).

5.2.5   Microscopy

For microscopy, WT and mutant ED5 and B11 cultures were sampled after a growth period of three days. Images were acquired with an Axio Imager Z2 microscope coupled to a Zeiss-Hrm camera (Carl Zeiss Microscopy, Germany) as described previously (Schüler et al., 2020b). For fluorescence imaging, the Zeiss filter sets 38 HE (ex. 470/40 nm, em. 525/50 nm) and 50 (ex. 640/30 nm, em. 690/50) were used and the transmitted light images were acquired using differential interference contrast (DIC). Images were treated using Zeiss Zen lite microscope software (BLUE edition 3.3).

5.2.6   RNA extraction and cDNA synthesis

RNA was extracted using the NZYol protocol (NZYTech, Portugal) in combination with RNeasy Mini kit (Qiagen, Germany). To this end, the frozen biomass was resuspended in 1

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mL of NZYol and 0.25 mL of glass beads (450-600 µm) were added. Cells were disrupted in a MM400 mixer mill (Retsch, Germany) by three cycles at 30 Hz for 1 min with 1 min intervals on ice. The supernatant was collected after centrifugation at 12,000 g for 10 min at 4 °C, and incubated at RT for 7 min, after which 0.2 volumes of chloroform were added, followed by another incubation at RT for 3 min. Phase separation was achieved by centrifugation at 12,000 g for 15 min at 4°C and the aqueous upper phase was collected. After addition of 1 volume of 70% ethanol to the aqueous phase, the solution was transferred to the RNeasy Mini spin column and manufacturer's instructions were followed. To remove all traces of genomic DNA, on-column DNase digestions using RNase-free DNase sets (Qiagen, Germany) were performed. Extracted RNA was quantified by NanoDrop One spectrophotometry (Thermo Fisher Scientific, USA) and integrity was confirmed by denaturing gel analysis. First strand cDNA synthesis was performed using superscript IV reverse transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. A mixture of random hexamers and oligod(T)$_{18}$ primers (1:10) was used and cDNA was synthesized with a prolonged protocol as follows: 23 °C for 10 min, 50 °C for 20 min, 55 °C for 20 min and 65 °C for 10 min. Enzymatic activity was inhibited by an incubation at 80 °C for 10 min. The obtained cDNA was diluted 1:50, stored at -20°C until use in the subsequent reactions.

5.2.7   Primer design and real-time PCR

To identify the sequences of genes involved in carotenoid biosynthesis, namely *PSY*, *PDS*, *LCYB*, *LCYE*, *CHYB* and *CYP97C3*, mRNA sequences of chlorophytes were aligned to the published genome of a related *Tetraselmis striata* strain (AccNo. GCA_006384855.1) using CLC Genomics Workbench 20 (Qiagen, Germany). Upon intron removal, the exon sequences of the aforementioned genes were obtained. Afterwards, primers for real-time PCR were designed using primer-BLAST (NCBI) (Table 5.1). The primer sequences for the *PDS* and *LCYB* genes were partially adapted from a previous report on *Chlamydomonas* sp., while the primers for *LCYE* were modified from primers targeting the same gene in *Chlorella vulgaris* (Kim et al., 2020; Ma et al., 2019).

Transcript expression levels of genes involved in carotenoid biosynthesis were evaluated by amplification using NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech, Portugal) and a CFX 96-well Real-Time PCR system (BioRad, USA) with the following protocol: initial heating to 95 °C for 2 min, 45 cycles of 95 °C, 5 s, and 62 °C, 20 s (64 °C were used for the primers *CHYB* and *CYP97C3*). Small subunit (*SSU*) 18S ribosomal RNA was

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used as internal control and raw data were analysed by the $2^{-\Delta\Delta Ct}$ method based on cycle threshold (Ct) values.

Table 5.1. Nucleotide sequences of primers designed for carotenoid gene expression analysis

Gene name	forward primer (5´→3´)	reverse primer (5´→3´)
PSY	ACTCGTCCCGCATTACCCCT	GCATGCCGTCGATCATGTCG
PDS	TGAGGATGGGGACTGGATCGAG	GCATGGCGAAGATCATCTGGTG
LCYB	GCAGCAGCGCGAGTTCTTCTG	GCGCGAGGCCAAACTTGATGAG
LCYE	GTTCCACGTGTTTGGCATGGAGC	AAAGAAGAGCCCCGCAAACAGC
CHYB	AGATGACCTATCAAGTCTCCGCCA	AGCGTAGCGGGCATACATCTC
CYP97C3	ACGTGCTGCCCTACTGGAAG	GCCTCCACCATCTGCTTGCA
SSU (18S)	GCCCGTCGCTCCTACCGATT	TGGGGCGGTTTGGAGAACTT

5.2.8   Pigment analysis

Pigment extraction and analysis was performed using samples obtained after two and three days of growth as described in (Schüler et al., 2020a). Briefly, about 5 mg of freeze-dried biomass were extracted with 1 mL methanol using glass bead-based cell disruption. After the recovery of the supernatant, the remaining biomass was reextracted until both the pellet and the supernatant became colourless. The extracts were dried by a gentle nitrogen flow, resuspended in 1 mL of HPLC grade methanol and filtered (0.22 μm).

Chlorophyll *a* and *b* contents were determined spectrophotometrically using the following formulas (Lichtenthaler and Wellburn, 1983):

Chl *a* = 15.65 A_{666} – 7.34 A_{653}

Chl *b* = 27.05 A_{653} – 11.21 A_{666}

Carotenoid profiles were analysed by a HPLC system equipped with a LiChroCART RP-18 column (5 μm, 250 × 4 mm, LiChrospher) and photodiode-array detector set to 450 nm. Carotenoid elution was performed by a gradient consisting of: 0–16 min, 0-60% B; 16–30 min, 60% B; 30–32 min 60–100% B and 32–35 min 100% A, with solvent A and B representing acetonitrile:water (90:10) and ethyl acetate, respectively. Identification and quantification of neoxanthin, violaxanthin, lutein, zeaxanthin and *β*-carotene were performed through calibration curves of corresponding pigment standards (Sigma-Aldrich, Portugal).

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5.2.9 Biochemical analysis

Biochemical analysis was performed on samples obtained from the inoculum and after three days of growth.

The protein content was determined by CHN elemental analysis using a Vario el III (Vario EL, Elemental Analyzer system, Germany). The final protein content was calculated by multiplying the percentage of nitrogen by 4.78 (Lourenço et al., 2004).

Lipids were extracted using a modified Bligh and Dyer method as described previously (Pereira et al., 2011). Briefly, about 10 mg of freeze-dried biomass were dispersed using an Ultra-Turrax T10B disperser (IKA-Werke, Germany) in a mixture of chloroform, methanol and water (2:1:1). After phase separation by centrifugation, the chloroform phase containing the lipids was transferred to a new tube. A known volume of chloroform was pipetted to a pre-weighed glass vial and evaporated overnight. The weight of the dried residues was divided by the initial DW to determine the lipid fraction.

5.2.10 Fatty acids

The profile of fatty acid methyl esters (FAMEs) of samples from the inoculum and harvested after three days were analysed following a protocol described in (Pereira et al., 2012) with slight modifications. About 20 mg of freeze-dried biomass were homogenized by an Ultra-Turrax T10B disperser (IKA-Werke, Germany) in a solution of methanol and acetyl chloride (20:1, v/v) for 1.5 min. After the addition of 1 mL of n-hexane, the fatty acids were derivatised for 60 min at 70 °C. FAMEs were then sequentially extracted two times from the reaction mixture using n-hexane. Hexane extracts were dried with anhydrous sodium sulphate, filtered (0.2 μm) and evaporated under a gentle nitrogen flow. The dried extracts were resuspended in 500 μL of HPLC grade hexane and stored at −20 °C until GC-MS analysis. FAME profiles were analysed by a Scion 456/GC Scion TQ MS (Bruker, USA) equipped with a 30-m ZB-5MS capillary column (30×0.25 mm of internal diameter with 0.25 μm film thickness; Phenomenex) using helium as a carrier gas. Elution was carried at 1 mL min^{-1} using an injection temperature of 300 ºC in split-less mode with the following temperature settings: 60 ºC for 1 min, 30 ºC min^{-1} to 120 ºC, 4 ºC min^{-1} to 250 ºC and 20 ºC min^{-1} to 300 ºC, hold for 4 min. Identification of FAME was performed using calibration curves of the 37-component standard (Supelco, Sigma-Aldrich, Portugal). Results are presented as percentage of total fatty acid (TFA) content.

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5.2.11 Statistical analysis

Data were tested for normality using the Shapiro-Wilk test (XLStat software, Vers. 2016.02.27444, Addinsoft, USA). ANOVA and Tukey's HSD post hoc test were performed for the comparison of means of treatments with a confidence interval of 95% if not otherwise indicated.

5.3   Results and discussion

5.3.1   Mutant isolation

To obtain carotenoid-rich mutants of *T. striata*, random mutagenesis using the alkylating agent EMS with subsequent selection of mutants resistant to the herbicide norflurazon was performed. Mutant selection on the herbicide norflurazon was used to enhance the selective pressure to find cells mutated in genes involved in the carotenoid biosynthetic pathway, in particular the phytoene desaturase (*PDS*) gene (Breitenbach et al., 2001). Mutants resistant to norflurazon are likely to display also higher carotenoid contents. The minimal lethal concentration of norflurazon for the WT strain was 2.5 µM. Afterwards, a killing curve to the mutagenic agent EMS was established to find a concentration leading to a 5-10% survival, which usually decreases the occurrence of multiple secondary mutations that lead to detrimental growth. The killing curve of the WT strain displayed the expected sigmoidal course and a survival rate of 8.68% was obtained by using an EMS concentration of 250 mM (Figure 5.1A). The subsequent selection on norflurazon led to the isolation of 20 herbicide-resistant colonies, of which one colony, here named ED5, revealed a 24% carotenoid content increase as compared with the WT. This mutant strain was therefore subjected to further selection by fluorescence activated cell sorting (FACS; Pereira et al., 2018). As cultures were not axenic, a first gate (Chl) was set to select only for chlorophyll positive cells using the red fluorescence (695 nm) emitted by chlorophyll *a* (FL1, Figure 5.1B and 1C). This first gated subpopulation was then sorted for cells with higher carotenoid fluorescence (Car) at the emission wavelength of 530 nm (FL2, Figure 5.1D and 1E). The emission in the green range of the electromagnetic spectrum has been related with carotenoid contents previously (Chen et al., 2017; Kleinegris et al., 2010; Schüler et al., 2020b). The ED5 strain displayed six times more cells in this gate as compared with the WT, representing 12.9 and 2.08% of total carotenoid positive cells, respectively (Figure 5.1D and 1E). After cell sorting of the carotenoid gate of ED5, 48 colonies were able to grow on 96-well plates. Eight mutant strains were

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selected for analysis of carotenoid profiles by HPLC upon testing their resistance to norflurazon and growth performance. The mutants B11 and G8 showed significant higher carotenoid contents as compared with the WT, 4.8 and 4.0 mg g^{-1} DW, respectively (Figure 5.1F). However, only B11 showed a significantly different carotenoid content compared to ED5 and was therefore selected for the following growth experiment and gene expression analysis.
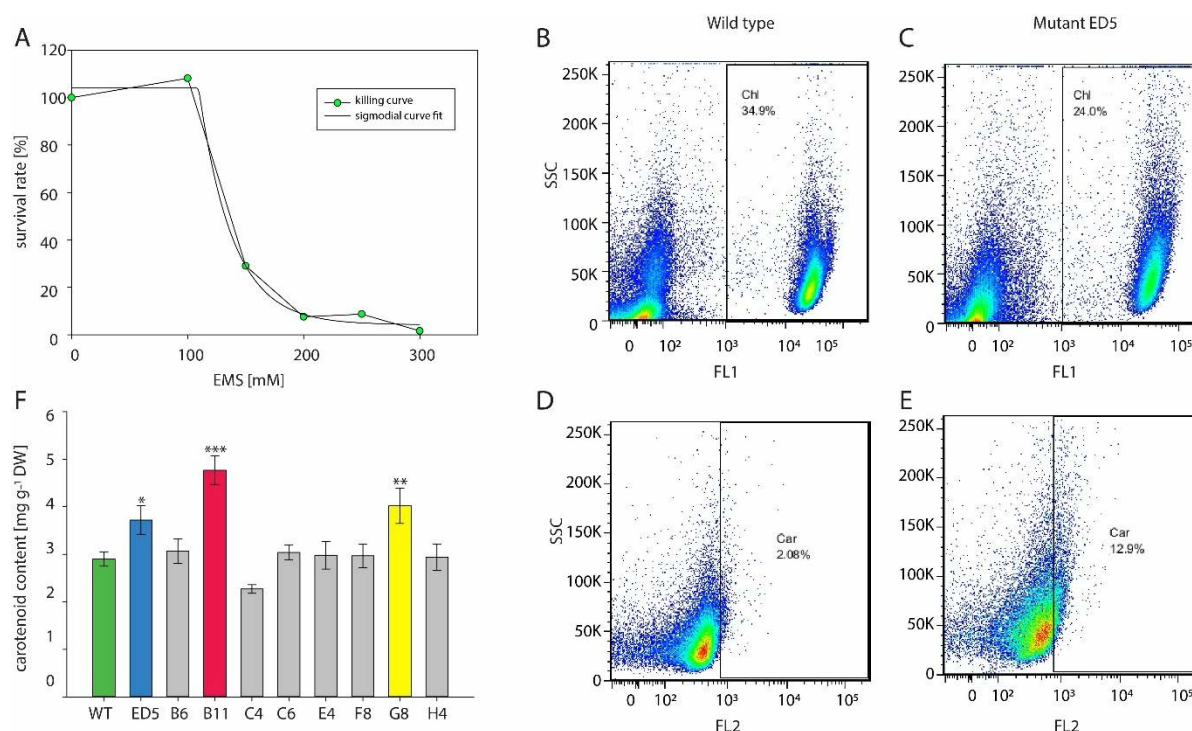
Figure 5.1. Isolation of carotenoid-rich mutants of *T. striata*. A killing curve of the WT using different concentrations of EMS was established (A). Fluorescence activated cell sorting (FACS) of WT and mutant ED5 strains showing the gates for chlorophyll (Chl) positive cells (B, C) and the gates for sorting of carotenoid (Car) positive cells (D, E). Carotenoid contents of the WT, ED5 and other mutants isolated by FACS determined by HPLC (*n*=3, mean ± SD). Significant differences (Dunnett´s test) to the WT are represented by one (*p* < 0.05), two (*p* < 0.01) or three (p < 0.001) asterisks (F).

5.3.2 Growth performance of WT and mutants under different growth conditions

The growth performance of the WT and two of the most promising mutants, ED5 and B11, was followed under mesophilic (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) that have been shown previously to enhance carotenoid contents in this microalga (Schüler et al., 2020b). The growth curves of all three strains showed the beginning of exponential phase with a lag phase in the first two days (Figure 5.2A and 2B). Upon three days of growth, no significant differences between the WT and mutant microalgae could be

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observed under all growth conditions. However, the WT culture reached a slightly higher biomass concentration under stress conditions than ED5 and B11 cultures: 0.74 ± 0.07, 0.53 ± 0.07 and 0.58 ± 0.08 g L^{-1}, respectively (Figure 5.2B).
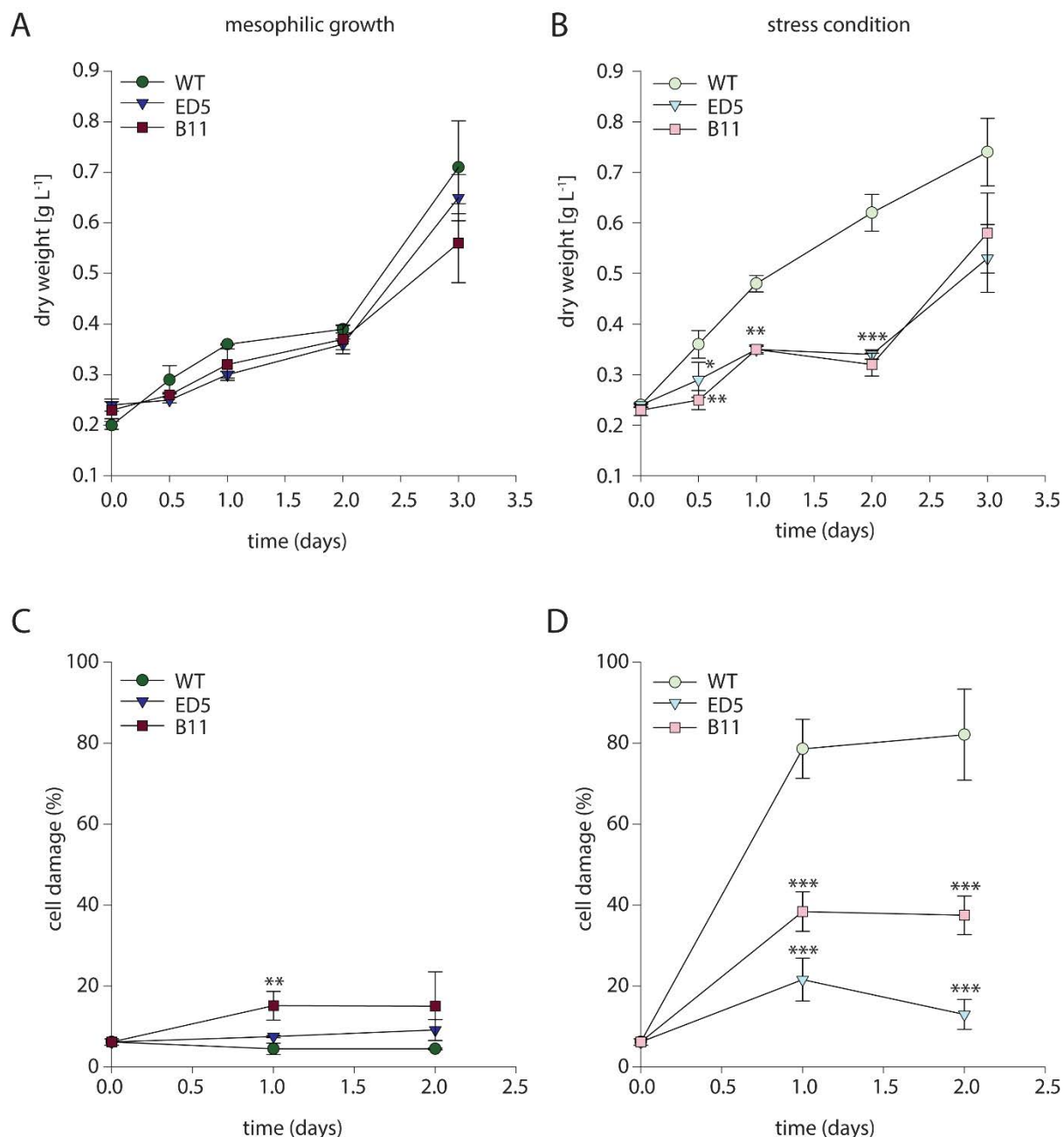
Figure 5.2. Growth analysis of *T. striata* WT and mutants ED5 and B11 under different growth conditions. Biomass concentrations and cell damage are represented under mesophilic (20 °C, 100 µmol m^{-2} s^{-1}, A and C) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}, B and D) of all cultures in triplicate (mean ± SD). Cell damage was estimated by flow cytometry counts using the fluorescent dye SYTOX Green. Significant differences (Dunnett´s test) to the WT at each timepoint are represented by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) asterisks.

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Additionally, cell damage during the experiment was measured by means of flow cytometry cell count using SYTOX Green staining. No significant difference of damaged cells between WT and mutants was observed under mesophilic growth conditions, showing more than 85% of viable cells throughout the experiment (Figure 5.2C). However, stress conditions led to a significant increase in permeabilized cells in WT and B11 microalgae, peaking at 80% and 38% after two days of growth, respectively. In ED5 microalgae the number of damaged cells increased to 20% after one day of growth, however, it decreased to 13% after two days of growth under stress conditions. Under heat and high light stress an increased production of ROS may lead to oxidative stress resulting into the observed damage to cell membranes (Apel and Hirt, 2004). However, the lower damage in mutant cells suggests that these cells were less sensitive to high light intensities and heat stress or were at least less permeable to SYTOX Green as compared with the WT. The presence of compounds with antioxidant activity such as carotenoids and *n*-3 fatty acids might be the reason for the lower cell damage in mutant cells (Goiris et al., 2012; Okuyama et al., 2008). Nevertheless, as under stress conditions a faster growth of the WT cells was observed during the first two days as compared with the mutants, it seems as though overall cell viability might not have been affected. One possible explanation for this result might be the occurrence of membrane repair mechanisms which together with the presence of membrane-stabilizing xanthophylls would allow the WT cells to recover rapidly (Cooper and McNeil, 2015; Schapire et al., 2009). If this is the case, caution must be taken not to directly infer cell viability of *T. striata* cultivated under stress conditions using this dye.

5.3.3   Pigment profiles of WT and mutants under different growth conditions

Remarkably, the chlorophyll contents of the WT were significantly higher than those of the mutants under both conditions tested (Figure 5.3). At mesophilic conditions, after three days of growth, chlorophyll reached the highest content in the WT followed by ED5 and B11: 40.5 ± 2.6, 33.8 ± 1.1 and 25.5 ± 2.1 mg g^{-1} DW, respectively. Chlorophyll contents of 35 mg g^{-1} DW have been previously reported in this species (Pereira et al., 2019). Conversely, stress (heat stress and higher light) conditions led to lower chlorophyll contents in all three strains, 18.7 ± 1.6, 7.96 ± 0.95 and 13.9 ± 0.4 mg g^{-1} DW, respectively. A higher chlorophyll content under lower (mesophilic) light conditions is a common phenomenon which can be explained by the need for better light utilization and therefore the increase in chlorophyll molecules (da Silva Ferreira and Sant'Anna, 2017).

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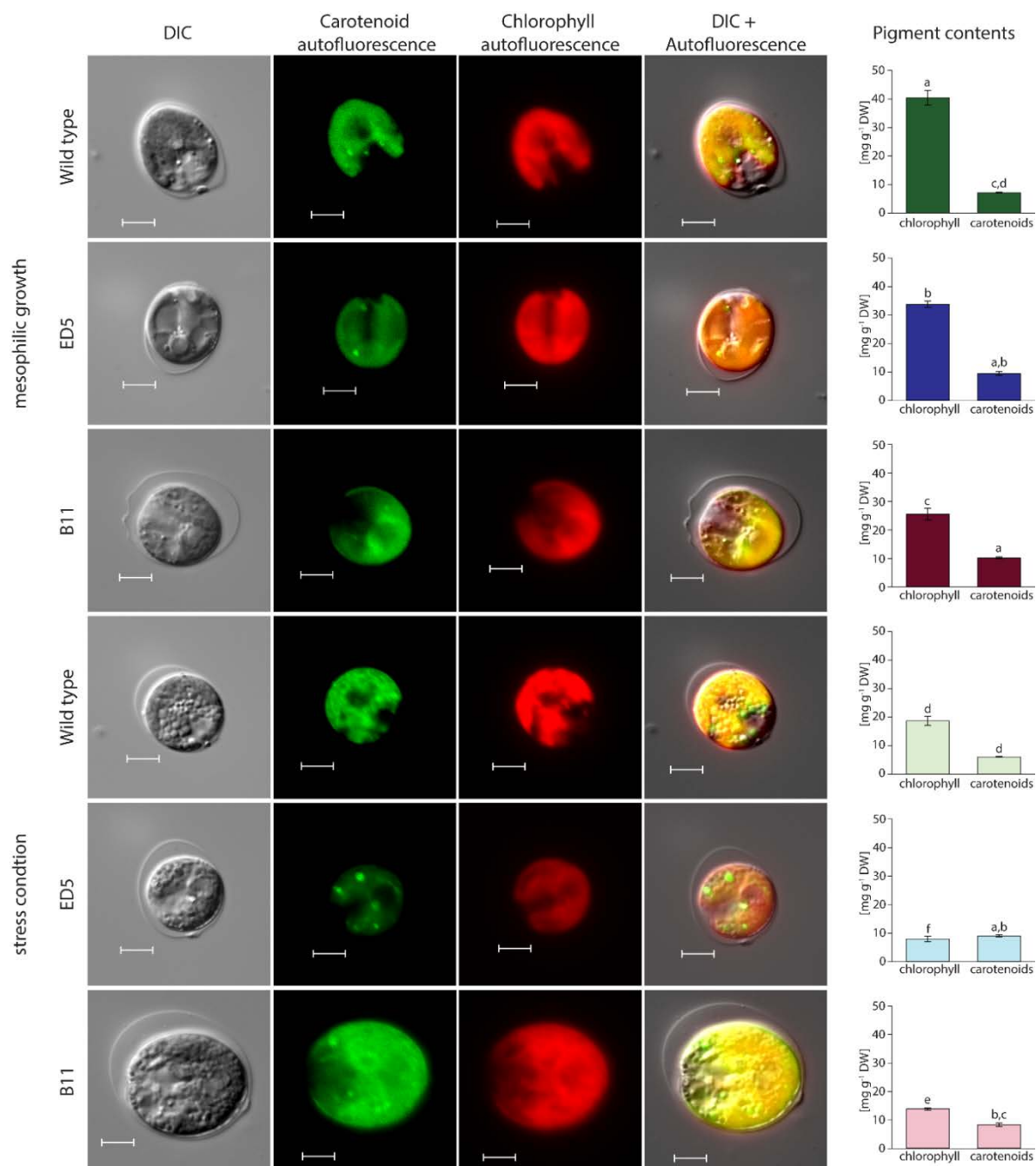
Figure 5.3. Microscopic observations and pigment contents of *T. striata* WT and mutants under different growth conditions. Chlorophyll and carotenoid contents were analysed of the cultures grown for three days under mesophilic (20 °C, 100 μmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 μmol m^{-2} s^{-1}) in triplicate (mean ± SD). Different letters over the bars indicate significant differences between samples of the same pigment ($p < 0.05$). Furthermore, using the same samples, images were taken using differential interference contrast (DIC) and the autofluorescence of carotenoid and chlorophyll pigments (38 HE and 50 filter sets, respectively). Scale bar = 5 μm.

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When comparing carotenoid contents, the WT showed significantly higher contents than both ED5 and B11 mutant strains at the beginning of the experiment: 6.69 ± 0.12, 5.04 ± 0.41 and 4.63 ± 0.13 mg g^{-1} DW, respectively. However, upon three days of growth, carotenoid contents in both mutants increased significantly, overtaking the WT by up to 47%. The highest carotenoid contents (10.2 ± 0.4, 9.48 ± 0.67 and 7.17 ± 0.20 mg g^{-1} DW) could be found under mesophilic growth conditions, while stress conditions led to slightly lower carotenoid contents: 8.36 ± 0.59, 9.01 ± 0.41 and 6.12 ± 0.14 mg g^{-1} DW for B11, ED5 and WT, respectively (Figure 5.3).

Microscopic observations revealed that WT and mutant cells under mesophilic growth conditions displayed similar cell sizes (≈15 μm). When comparing chlorophyll autofluorescence, the WT showed a chloroplast with its typical U-shape in these cells; however, the plastid autofluorescence in the mutant cells seemed to be more diffuse. Furthermore, the autofluorescence in B11 microalgae appeared less intense than those of the other two strains, which is in agreement with the lower chlorophyll contents under mesophilic growth conditions. The carotenoid (38 HE) fluorescence in all three strains has the same shape as the chlorophyll (50 HE) fluorescence, confirming the assumption of a previous study on this species that the carotenoids seem to accumulate in the chloroplast (Schüler et al., 2020b). However, the carotenoid to chlorophyll fluorescence ratio in both mutants is more intense than that of the WT, which becomes evident in the DIC + autofluorescence overlay; in this case, mutant cells display a more intense yellowish hue due to higher carotenoid fluorescence and lower chlorophyll fluorescence. Interestingly, under stress conditions, B11 microalgae are on average 50% larger as compared with WT and ED5 cells. The chlorophyll autofluorescence appears less intense in both mutants than WT under these conditions. Conversely, carotenoid autofluorescence is more intense in B11 microalgae, while ED5 cells show several intense spots of fluorescence against a less intense fluorescent background. These observations confirm the differences measured in pigment contents. Indeed, a larger cell and/or spots emitting intense carotenoid autofluorescence could be the reason for increased carotenoid contents in the mutants.

5.3.4   Carotenoid profile of WT and mutant strains under different growth conditions

The carotenoid profile of the WT under mesophilic growth conditions did not change significantly during the experiment, showing mainly *β*-carotene (3.65 ± 0.20 mg g^{-1} DW) and lower amounts of violaxanthin (1.14 ± 0.06 mg g^{-1} DW), neoxanthin (1.08 ± 0.07 mg g^{-1} DW)

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and lutein (1.31 ± 0.10 mg g^{-1} DW) after three days of growth (Figure 5.4). Only zeaxanthin contents increased, reaching their highest level (0.05 ± 0.003 mg g^{-1} DW) after three days of growth. However, under stress conditions, zeaxanthin and lutein contents increased significantly to respectively 0.07 ± 0.003 and 2.65 ± 0.13 mg g^{-1} DW after three days, being accompanied by decreased β-carotene (1.48 ± 0.10 mg g^{-1} DW) and neoxanthin contents (0.74 ± 0.04 mg g^{-1} DW). These observations are in agreement with a previous study on this strain (Schüler et al., 2020b); lower light intensities at mesophilic growth conditions (100 µmol m^{-2} s^{-1}) led to an increase in light harvesting pigments, while photoprotective pigment levels rose under heat and high light stress. Moreover, the violaxanthin cycle is an important photoprotective mechanism for cells to adapt to changing environmental conditions; under lower light, violaxanthin is usually increased while WT cells show enhanced zeaxanthin levels under high light (Jahns et al., 2009). The pigment profile of ED5 cells was similar to that of the WT under mesophilic growth conditions, though with significantly higher contents of β-carotene (4.87 ± 0.44 mg g^{-1} DW) and violaxanthin (1.43 ± 0.07 mg g^{-1} DW), but equivalent neoxanthin contents (1.15 ± 0.03 mg g^{-1} DW) at day 3 (Figure 5.4). Moreover, up to 1.6-fold higher lutein and 2.1-fold higher zeaxanthin contents than those in WT cells were observed under mesophilic growth conditions, reaching their highest values after three days (2.03 ± 0.17 mg g^{-1} DW and 0.10 ± 0.004 mg g^{-1} DW, respectively). Under stress conditions, zeaxanthin content remained relatively high (0.09 ± 0.01 mg g^{-1} DW). However, lutein content rose to 3.81 ± 0.25 mg g^{-1} DW on the third day of growth, which corresponded to a 1.4-fold increase compared to WT levels (Figure 5.4B). Moreover, ED5 microalgae showed a 2.3-fold increase in β-carotene content (3.32 ± 0.19 mg g^{-1} DW) when compared to that in WT cells under stress conditions (Figure 5.4A). Lutein and β-carotene are important photoprotective pigments that may contribute to the lower cell damage observed in ED5 cells under stress conditions (Figure 5.2D). Taken together, these observations suggest that in this mutant both branches of the carotenoid pathway are more active than in the WT, regardless of the growth conditions. The B11 mutant strain is a derivate of ED5 cells and was isolated under low light conditions (45 µmol m^{-2} s^{-1}). Remarkably, after three days of growth under mesophilic conditions, B11 microalgae displayed not only the expected high β-carotene (4.20 ± 0.27 mg g^{-1} DW), violaxanthin (1.63 ± 0.11 mg g^{-1} DW) and neoxanthin (1.28 ± 0.02 mg g^{-1} DW) contents but also relatively high zeaxanthin levels (0.09 ± 0.005 mg g^{-1} DW). Moreover, the lutein contents showed a 2.4-fold increase as compared to the WT, i.e., 3.09 ± 0.23 mg g^{-1} DW (Figure 5.4B). Such increase in photoprotective pigments by B11 microalgae may be linked to a higher light sensibility and the exposure to 100 µmol m^{-2} s^{-1} under mesophilic growth was perceived by the cells as high light. Moreover, this mutant displayed the lowest chlorophyll contents as

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compared with those of other strains under mesophilic growth (Figure 5.3), which might be also related to the stress sensed under this condition. Under stress conditions, lutein (3.07 ± 0.22 mg g^{-1} DW) and zeaxanthin (0.09 ± 0.005 mg g^{-1} DW) contents did not change significantly as compared with those found under mesophilic growth conditions (Figure 5.4). However, β-carotene, violaxanthin and neoxanthin contents decreased to 3.66 ± 0.32, 1.09 ± 0.07 and 0.54 ± 0.02 mg g^{-1} DW, respectively. Nevertheless, β-carotene content in B11 cells was the highest observed under stress conditions after three days, representing a 2.5-fold increase as compared with WT levels.
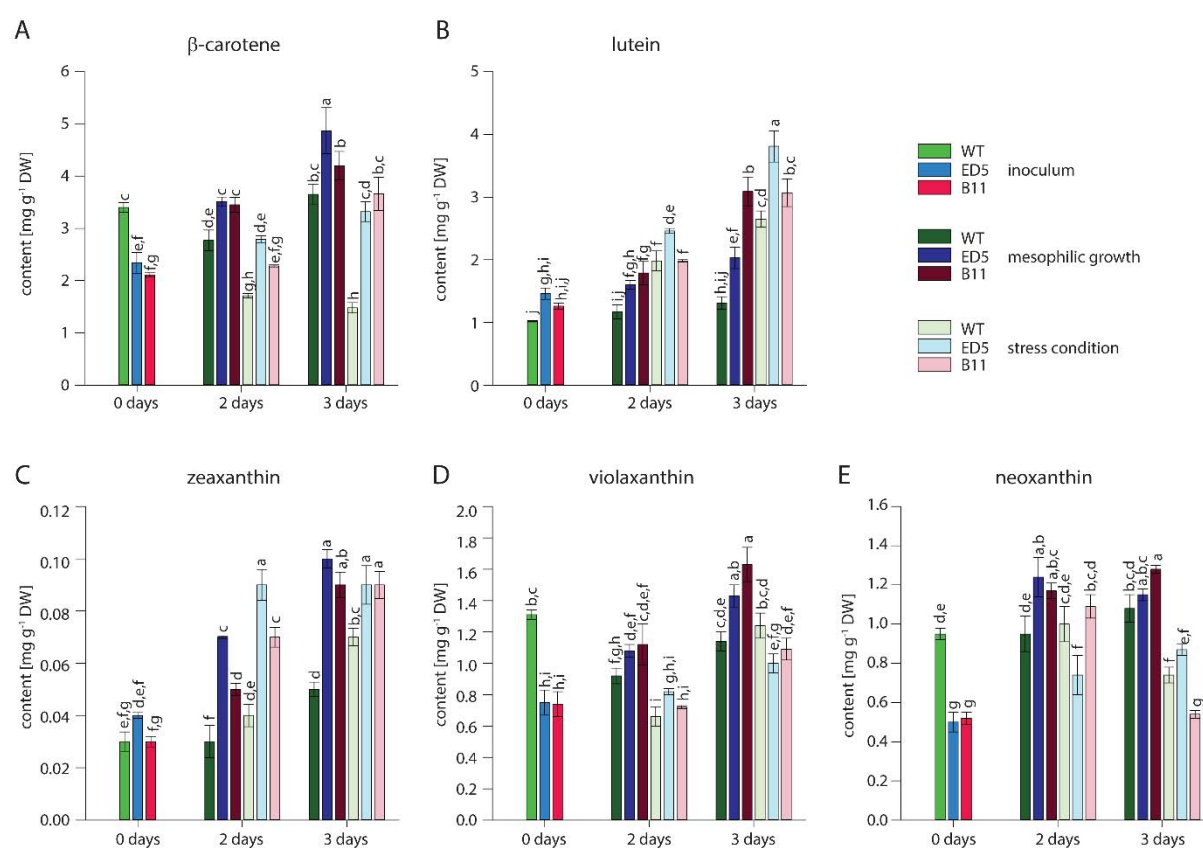
Figure 5.4. Carotenoid contents of WT and mutant strains ED5 and B11 under different growth conditions. Cultures were grown in triplicate for three days under mesophilic growth conditions (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}). Contents of β-carotene (A), lutein (B), zeaxanthin (C), violaxanthin (D) and neoxanthin (E) were quantified using RP-HPLC in the biomass harvested from the inoculum and after two and three days (mean ± SD). Different letters over the bars indicate significant differences between samples for each compound ($p < 0.05$).

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5.3.4.1 Transcript expression analysis of carotenogenic genes in WT and mutants

For a better understanding of the above-mentioned carotenoid contents in WT and mutant strains ED5 and B11, the gene expression analysis of the carotenoid genes *PSY*, *PDS*, *LCYB*, *LCYE*, *CHYB* and *CYP97C3* was performed at the beginning and after one day of growth under either mesophilic or stress conditions (Figure 5.5). Phytoene synthase (PSY) catalyses the first step of the carotenoid pathway leading to phytoene (Figure 5.6), which is often considered to be a rate-limiting step of the pathway (Cordero et al., 2011a). The transcript levels of this enzyme did not change significantly in the WT cells throughout the experiment. Conversely, in both mutants ED5 and B11 under stress conditions, the *PSY* transcript levels were significantly upregulated, 1.9- and 1.5-fold, respectively, compared to the levels of WT cells (Figure 5.5A). Down in the pathway, phytoene is then converted to ζ-carotene by phytoene desaturase (PDS), which has been identified as a rate-limiting enzyme in lutein biosynthesis (Li et al., 2013). Interestingly, *PDS* transcript levels were increased 2.0-fold in ED5 cells under stress conditions as compared with the WT (Figure 5.5B), matching the higher lutein contents observed in ED5 cells (Figure 5.4B). For the WT and B11 and under mesophilic growth conditions, no significant changes in the levels of transcripts coding for this enzyme were observed throughout the experiment. Nevertheless, when comparing transcript levels between B11 cells, *PDS* underwent a 3.7-fold upregulation under stress conditions as compared with the beginning of the experiment (Figure 5.5B). The higher expression of *PSY* and *PDS* genes in the mutant cells under high light (stress conditions, 30 °C, 380 µmol m^{-2} s^{-1}) is in accordance with previous studies on *Haematococcus pluvialis*, *Dunaliella salina*, *Chlamydomonas* spp. and *Chlorella* (*Chromochloris*) *zofingiensis* (Coesel et al., 2008; Cordero et al., 2012; Couso et al., 2012; Ma et al., 2019; Steinbrenner and Linden, 2003). After the synthesis of lycopene, the carotenoid pathway splits into two branches (Figure 5.6). In one branch, lycopene-*β*-cyclase (LCYB) catalyses the formation of *β*-carotene, which is further converted by *β*-carotene hydroxylase (CHYB) to zeaxanthin, followed by the biosynthesis of violaxanthin and neoxanthin. In the other branch, the activity of both lycopene-*β*- and *ε*-cyclases (LCYE) lead to the biosynthesis of *α*-carotene, which is hydroxylated to lutein by the stepwise reaction of two heme-containing cytochrome P450 monooxygenases, namely *β*-ring hydroxylase CYP97A5 and *ε*-ring hydroxylase CYP97C3. In ED5 and B11 cells, the *CYP97C3* transcript levels were upregulated under stress conditions, rising 2.7- and 1.8-fold when compared to the levels found in WT cells (Figure 5.5F). Together with the higher expression of the upstream genes involved in the carotenoid biosynthetic pathway, this observation agrees with the higher lutein contents observed in these strains as compared with

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the WT (Figure 5.4B). Moreover, high light intensity has previously been shown to increase the expression of *CYP97C3* in *Chlamydomonas* sp. (Cordero et al., 2012). As has already been assumed from the carotenoid profile of B11 microalgae under mesophilic growth conditions, the light intensity of 100 µmol m^{-2} s^{-1} seemed to be sensed as high light by these mutant cells. The 1.7-fold upregulation of *CYP97C3* transcript levels, when compared with those of the WT, provides another evidence for this assumption (Figure 5.5F).
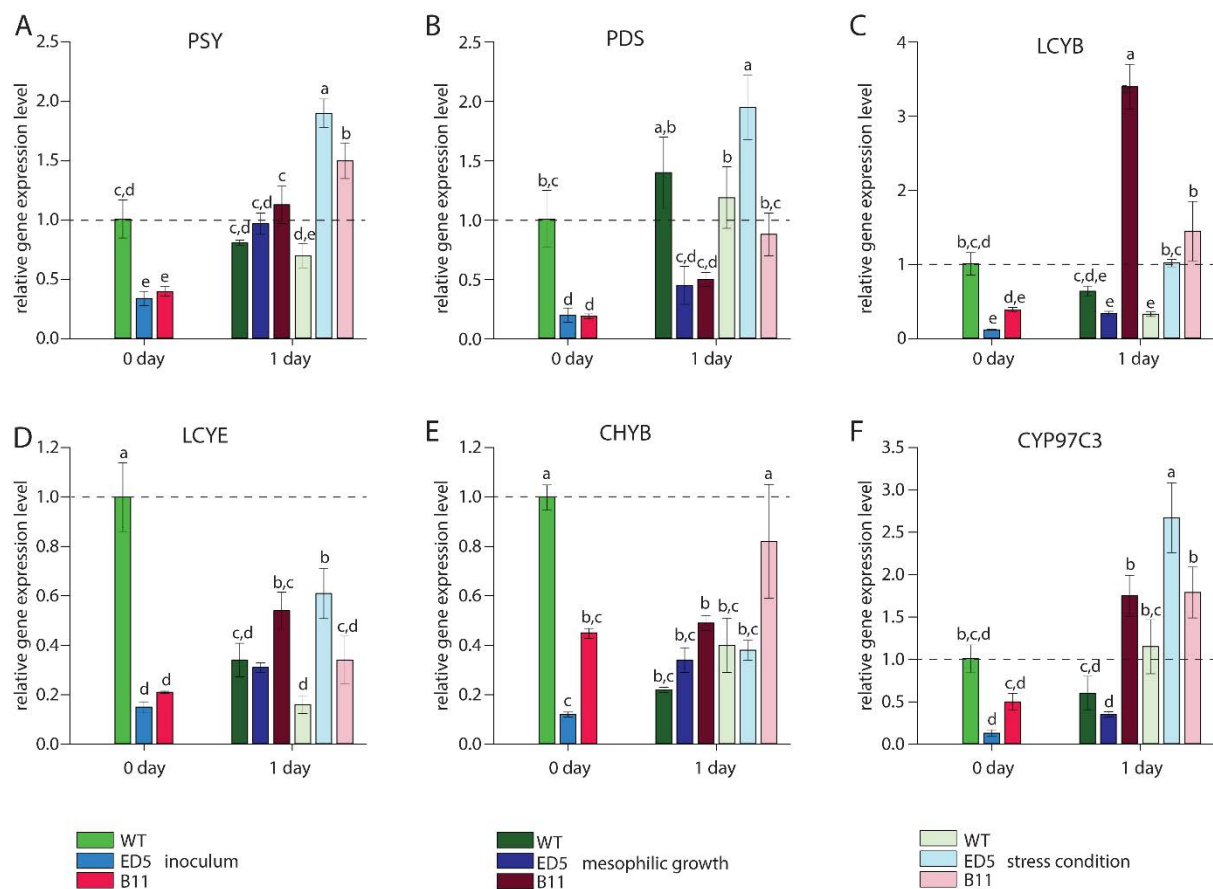
Figure 5.5. Relative transcript steady-state levels of genes involved in carotenoid biosynthesis in *T. striata* WT and mutant cells under different growth conditions. Cultures were grown in triplicate under mesophilic growth conditions (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) and RNA was extracted from the inoculum and after one day of growth. Relative gene expression levels of phytoene synthase (PSY) (A), phytoene desaturase (PDS) (B), lycopene-*β*-cyclase (LCYB) (C), lycopene-*ε*-cyclase (LCYE) (D), *β*-carotene hydroxylase (CHYB) and cytochrome P450 *ε*-ring hydroxylase (CYP97C3) (E) were analysed by the 2$^{-\Delta\Delta Ct}$ method based on cycle threshold (Ct) values using 18S ribosomal RNA as internal control (mean ± SD). Different letters over the bars indicate significant differences between samples of the same gene (*p* < 0.05).

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Remarkably, these conditions led to the highest *LCYB* gene expression in B11 cells, resulting in a 3.4-fold increase compared to the levels found in WT cells. Low temperature (20 °C), as compared with high temperature (35 °C), has previously been reported to increase the *LCYB* gene expression in *Chlamydomonas* sp. (Ma et al., 2020). Therefore, temperature rather than light intensity might have been the most important factor for the observed higher expression levels of *LCYB* under mesophilic growth than under stress, representing only a 1.4-fold increase in B11 cells as compared with the WT levels.

When comparing transcript levels between the beginning and after one day of the experiment, the expression levels of all investigated genes were upregulated in both mutants under both mesophilic and stress conditions. This observation matches the observed ≈2-fold increase in carotenoid contents in these strains after three days of growth as compared with the beginning of the experiment. However, carotenoid contents did not increase with cultivation time in WT cells, which can be explained by the absence of any observable increase in the expression of the corresponding genes (Figure 5.5). The observed changes in carotenoid contents might be due to shifts from one metabolite to the other, most probably controlled post-transcriptionally by changing the activities of the corresponding enzymes under different environmental conditions.

To sum up, the 1.5-fold increased carotenoid content of ED5 microalgae under stress conditions as compared with the WT can be correlated with the higher *PSY* and *PDS* gene expression, affecting the initial enzymes of the pathway (Figure 5.6). Moreover, the higher lutein contents observed in this mutant are probably a result of a higher expression of the *CYP97C3* gene. Conversely, in B11 microalgae, the observed 1.4-fold increase in carotenoid contents as compared with the WT took place under both growth conditions tested. However, under mesophilic growth conditions, higher *LCYB* and *CYP97C3* transcript levels seemed to have been the most decisive factors for the 2.4-fold increase in lutein found in B11 cells when compared with WT microalgae (Figure 5.6). Conversely, under stress conditions, high *PSY* and *PDS* gene expression apparently led to the observed 2.5-fold increase in *β*-carotene contents. Interestingly, the *CYP97C3* transcript levels remained high under these conditions, agreeing with the high lutein levels found in this strain, regardless of the growth conditions. Interestingly, in the norflurazon-resistant mutants, not only the *PDS* transcript levels were affected but also those of other carotenogenic genes. These observations provide further evidence that this pathway possesses multiple regulatory mechanisms, which are difficult to target in a metabolic engineering approach.

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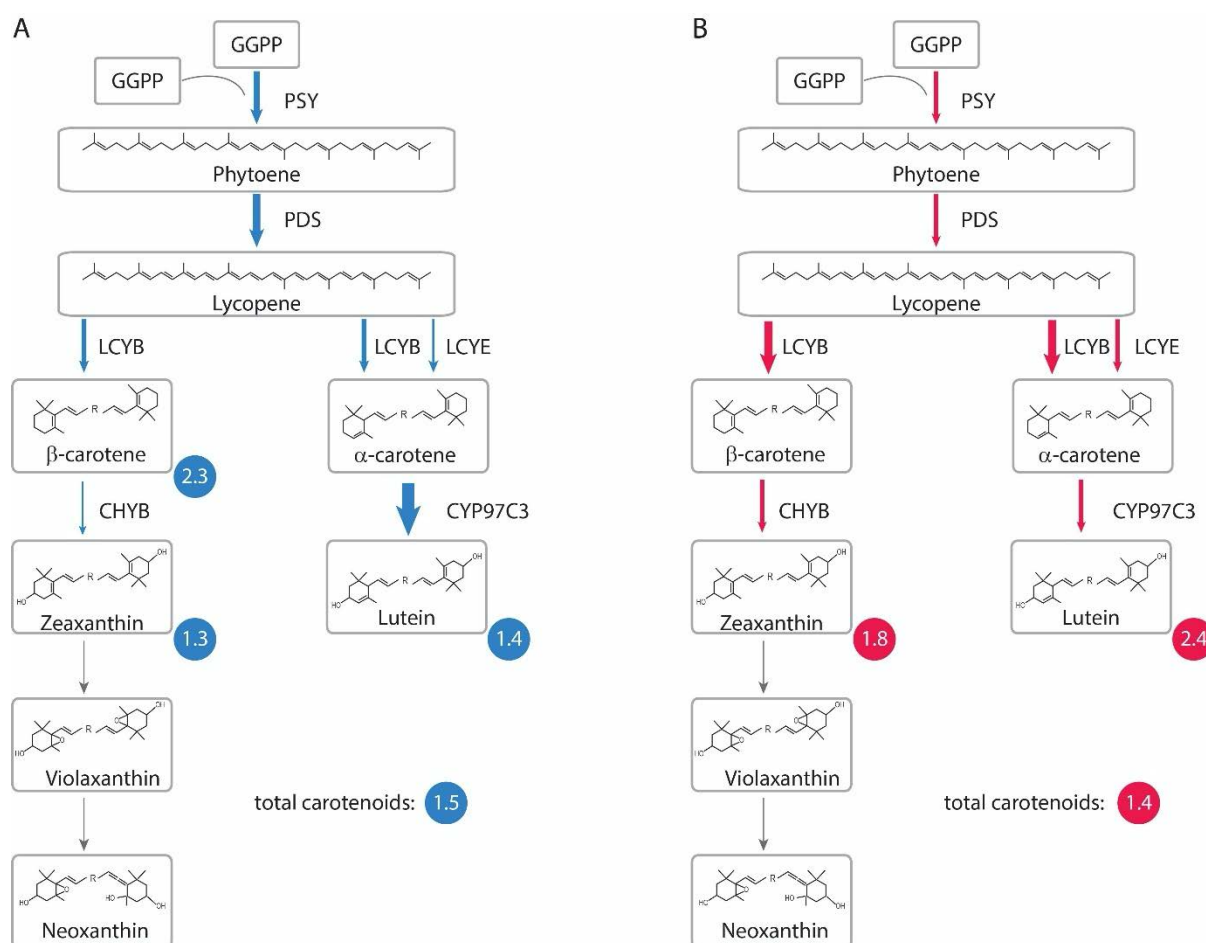
Figure 5.6. Simplified carotenoid biosynthetic pathway in *Tetraselmis striata* mutant strains ED5 (A, blue) and B11 (B, red). The numbers to the right of each metabolite correlate with the fold increase in carotenoid levels as compared with the WT. The thickness of the arrows indicates the relative gene expression of transcripts coding for the corresponding enzyme as compared to the WT. The fold increases shown for ED5 and B11 are those of cells under stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) and mesophilic growth conditions (20 °C, 100 µmol m^{-2} s^{-1}), respectively. PSY, phytoene synthase; PDS, phytoene desaturase, LCYB, lycopene-β-cyclase; LCYE, lycopene-ε-cyclase, CHYB, β-carotene hydroxylase; and CYP97C3, heme-containing cytochrome P450 ε-ring hydroxylase.

5.3.5   Biochemical composition and fatty acid profile of WT and mutants under different growth conditions

To elucidate possible changes and better understand the potential of the novel strains ED5 and B11 in future products, the protein and lipid contents as well as fatty acid profiles were compared to those of the WT under mesophilic growth and stress conditions.

The protein contents of the inoculum of WT and both mutants ED5 and B11 did not show any significant differences. Under mesophilic growth conditions, a maximum protein content was reached in all three strains, which was on average 24.7 ± 1.7% of DW (Table

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5.2). Protein contents in this range have been reported previously in microalgae of the genus *Tetraselmis* under these growth conditions (G. Kim et al., 2016). However, under stress conditions, protein contents decreased in all three strains, particularly in B11 cells (13.3 ± 0.6% DW). Reduced protein contents under heat stress have been reported earlier in other marine microalgae and can be related to a breakdown of housekeeping proteins in order to have enough amino acids for a more reduced proteome geared to protect the cell against heat-induced denaturation (Renaud et al., 2002).

The lipid content of all the three strains in the beginning of the experiment and at mesophilic growth conditions was between 8-10% of DW (Table 5.2). This is in agreement with previous reports on this species, presenting lipid contents of about 10% of DW under mesophilic growth conditions (Pereira et al., 2016; Schulze et al., 2017). Under stress conditions, the lipid content of WT and B11 increased significantly to 17.1 ± 0.3 and 14.0 ± 2.7% of DW, respectively. This increase is most probably related to the heat stress and higher light intensity, conditions known to increase lipid contents in marine microalgae (Nogueira et al., 2015). Under unfavourable conditions, lipids in the form of triacylglycerols serve as storage compounds for carbon and energy. Conversely, lipid contents in the mutant ED5 did not change significantly under stress conditions. One explanation could be that instead of being used for lipid biosynthesis, carbon is being diverted to carotenoid production.

Table 5.2. Protein and lipid contents in WT, ED5 and B11 strains under different growth conditions. Cultures were grown in triplicate under mesophilic growth conditions (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) for three days (mean ± SD). Significant differences between samples of the same compound are indicated by letters ($p < 0.05$).

conditions	strain	proteins (% DW)	lipids (% DW)
inoculum	WT	23.7 ± 0.3[a]	8.26 ± 0.08[b]
	ED5	24.8 ± 1.2[a]	7.90 ± 0.11[b]
	B11	21.8 ± 0.3[a,b]	7.31 ± 0.28[b]
mesophilic	WT	25.7 ± 5.1[a]	8.64 ± 0.27[b]
	ED5	25.7 ± 3.6[a]	9.19 ± 1.01[b]
	B11	22.7 ± 0.5[a,b]	9.78 ± 0.12[b]
stress	WT	17.5 ± 3.6[a,b]	17.1 ± 0.3[a]
	ED5	17.1 ± 1.5[a,b]	9.26 ± 1.46[b]
	B11	13.3 ± 0.6[b]	14.0 ± 2.7[a]

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The fatty acid composition, regardless of the microalgal strain, changed under the different growth conditions tested (Table 5.3). On average, the polyunsaturated fatty acids (PUFA) fraction increased significantly from 51 to 56% of TFA under mesophilic growth accompanied by a significant decrease from 24 to 21% of TFA in saturated fatty acids (SFA). Both low light and low temperature, which were used to grow *T. striata* under mesophilic conditions have been shown previously to increase PUFA contents in marine microalgae (Boelen et al., 2013; Mitra et al., 2015b). Under stress conditions, the opposite trend was observed: SFA contents increased on average to 35% of TFA while PUFA significantly decreased to 40% of TFA. The better packing effect of SFA in lipid bodies is usually the reason for an increase in this fraction with increased lipid contents and has been reported previously under heat stress and high light intensities (He et al., 2015; Renaud et al., 2002). The fraction of monosaturated fatty acids (MUFA), however, did not change significantly under the growth conditions assayed.

Table 5.3. Fatty acid profile of WT and mutants under different growth conditions. Cultures were grown in triplicate under mesophilic (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) for three days (mean ± SD). Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids as well linoleic (LA), stearidonic (SDA) and eicosapentaenoic (EPA) acid are shown in percentage of total fatty acids (TFA). Significant differences between samples of the same compounds are indicated by letters ($p < 0.05$). The complete profile is presented in Table S5.1 (Supplemental data).

conditions	strain	SFA (% TFA)	MUFA (% TFA)	PUFA (% TFA)	LA C18:2 (% TFA)	SDA C18:4 (% TFA)	EPA C20:5 (% TFA)
inoculum	WT	23.4 ± 1.1[c,d]	24.1 ± 1.0[a]	52.5 ± 2.8[a]	17.2 ± 0.6[a]	5.59 ± 0.32[d]	2.79 ± 0.51[d]
	ED5	23.7 ± 0.2[c,d]	23.9 ± 0.3[a]	52.4 ± 3.3[a]	**5.38 ± 1.04[c]**	**14.8 ± 0.7[b]**	**8.67 ± 0.49[a]**
	B11	25.3 ± 2.0[c]	25.7 ± 0.5[a]	48.9 ± 3.0[a,b]	**7.46 ± 0.50[c]**	12.2 ± 0.6[c]	5.81 ± 0.43[b,c]
mesophilic	WT	19.5 ± 1.1[d]	24.4 ± 0.9[a]	56.1 ± 2.4[a]	9.27 ± 0.88[b,c]	14.5 ± 0.4[b,c]	3.83 ± 0.21[c,d]
	ED5	21.1 ± 0.7[c,d]	21.9 ± 1.4[a]	57.0 ± 2.8[a]	7.63 ± 0.67[c]	**17.8 ± 0.6[a]**	5.69 ± 0.31[b,c]
	B11	21.9 ± 1.7[c,d]	23.6 ± 1.0[a]	54.6 ± 5.2[a]	7.75 ± 2.12[c]	14.4 ± 0.4[b,c]	5.51 ± 1.02[b,c]
stress	WT	32.3 ± 1.4[b]	25.9 ± 7.6[a]	41.8 ± 7.2[b,c]	12.3 ± 1.5[b]	4.71 ± 1.24[d]	2.92 ± 0.49[d]
	ED5	36.4 ± 2.2[a]	24.0 ± 0.7[a]	39.6 ± 6.5[c]	9.01 ± 1.47[b,c]	**5.78 ± 0.90[d]**	**6.67 ± 1.01[a,b]**
	B11	35.6 ± 1.3[a,b]	26.3 ± 1.2[a]	38.1 ± 4.0[c]	12.3 ± 1.1[b]	4.27 ± 0.53[d]	4.20 ± 0.80[c,d]

The fatty acid profile of WT and mutant cells contained palmitic (C16:0), hexadecatetraenoic (C16:4), oleic (18:1) and linoleic (LA, C18:2*n-6*) acids, which corresponded to 70% of TFA. The remaining 20 to 30% of TFA were composed of palmitoleic (C16:1), hexadecatrienoic (C16:3*n-3*), stearidonic (SDA, C18:4*n-3*) and eicosapentaenoic (EPA, C20:5*n-3*) acids (Table S5.1). This fatty acid profile is comparable to those of previous

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studies on this strain (Pereira et al., 2019; Schulze et al., 2017). Remarkably, under all tested conditions, the fatty acid profile in ED5 microalgae showed an enrichment of the *n*-3 fatty acids SDA and EPA as compared to the WT, representing 14.8 ± 0.7 and 8.67 ± 0.49% of TFA at the beginning of the experiment, respectively (Table 5.3). These fatty acids are also increased in the profile of B11 microalgae as compared with the WT corresponding to 12.2 ± 0.6 and 5.81 ± 0.43% of TFA in the inoculum, respectively. Furthermore, the precursor LA of these *n*-3 fatty acids was significantly decreased in both mutants as compared with the WT at the beginning of the experiment and remained at a lower level throughout the experiment. These observations may be explained by the resistance of the strains to the herbicide norflurazon and therefore a possible higher activity of *Δ*6 desaturases. However, further studies of the expression profiles of fatty acid biosynthesis enzymes are needed to confirm this assumption. Moreover, *n*-3 fatty acids such as EPA can act as antioxidants protecting the cells from oxidative damage caused by ROS (Okuyama et al., 2008). The increased fraction of EPA in both mutants ED5 and B11 as compared with the WT could be the reason for a lower number of cells damaged by heat combined with high light as observed in Figure 5.2.

Because changes were observed in different classes of metabolites, it seems as though that the mutations generated have a pleiotropic effect on the overall metabolism of the microalgal cell. These results highlight the fact that mutations might affect the expression of several different genes even in diverse metabolic pathways. These changes can be brought about by a mutation in a gene coding for a regulatory factor (e.g., transcription factor) or a single biosynthetic enzyme. Although the first possibility has an immediate pleiotropic potential, the second scenario might affect the metabolism via multiple metabolite feedback loops, resulting also in changes in the expression of multiple genes. In any case, these results underline the complexity of biological systems and the inherent difficulty of targeting a specific gene to obtain the desired phenotype.

Moreover, the protein and lipid contents of WT and mutant strains under mesophilic conditions are similar to those reported in a recent study of this strain produced industrially and could therefore find their application in food or feed products (Pereira et al., 2019). Additionally, the presence of antioxidant compounds such as carotenoids and EPA could provide further health-promoting effects on both animals and humans (M. U. et al., 2019). However, the consumption of the whole biomass by humans is hampered because this species has not yet been approved by the European Food Safety Agency (EFSA). Different applications to the pharmaceutical, nutraceutical or cosmetic industry are thus conceivable

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upon setting up a biorefinery to yield the most value from a robust industrial microalga such as *T. striata* CTP4 and its derivatives.

5.4   Conclusions

We successfully isolated two strains of the euryhaline microalga *Tetraselmis striata* CTP4 with improved carotenoid contents and fatty acid profiles enriched with EPA. The creation of non-GMO strains of biotechnologically relevant microalgae by random mutagenesis represents, thus, a powerful tool to gather knowledge of metabolic pathways and provides a cost-effective way of isolating improved strains with a promising future in the food, feed and pharmaceutical industries. Moreover, as *T. striata* has already been grown at large-scale, these non-GMO mutants can be considered as promising sources of carotenoids and EPA using processes that can be easily scaled up at industrial facilities.

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Supplementary material

Table S5.1. Fatty acid profile of WT and mutant under different growth conditions. Cultures were grown in triplicate under mesophilic (20 °C, 100 µmol m^{-2} s^{-1}) and stress conditions (30 °C, 380 µmol m^{-2} s^{-1}) for three days and fatty acids are represented as % of total fatty acids (mean ± SD). Significant differences between samples of the same compounds are indicated by letters ($p < 0.05$).

FAME	Inoculum			Mesophilic growth			Stress condition		
	WT	ED5	B11	WT	ED5	B11	WT	ED5	B11
C14:0	0.63 ± 0.16^{b,c,d}	0.54 ± 0.02^{d}	0.59 ± 0.02^{c,d}	0.47 ± 0.04^{d}	0.44 ± 0.05^{d}	0.39 ± 0.04^{d}	0.91 ± 0.22^{a,b,c}	0.97 ± 0.11^{a,b}	1.00 ± 0.04^{a}
C16:0	22.0 ± 0.3^{c,d,e}	22.6 ± 0.1^{c,d}	24.3 ± 1.9^{c}	18.7 ± 0.7^{e}	20.1 ± 0.3^{d,e}	21.1 ± 1.4^{c,d,e}	30.6 ± 1.0^{b}	34.5 ± 1.9^{a}	33.5 ± 1.1^{a,b}
C18:0	0.78 ± 0.67^{a}	0.47±0.03^{a}	0.42±0.07^{a}	0.37 ± 0.32^{a}	0.60 ± 0.34^{a}	0.42 ± 0.21^{a}	0.74 ± 0.19^{a}	1.02 ± 0.23^{a}	1.11±0.16^{a}
∑ SFA	**23.4 ± 1.1^{c,d}**	**23.7 ± 0.2^{c,d}**	**25.3 ± 2.0^{c}**	**19.5 ± 1.1^{d}**	**21.1 ± 0.7^{c,d}**	**21.9 ± 1.7^{c,d}**	**32.3 ± 1.4^{b}**	**36.4 ± 2.2^{a}**	**35.6 ± 1.3^{a,b}**
C16:1	5.36 ± 0.61^{a}	4.58 ± 0.07^{a,b}	5.03 ± 0.37^{a}	4.85 ± 0.28^{a}	4.50 ± 0.42^{a,b}	4.30 ± 0.31^{a,b}	3.84 ± 0.21^{b}	2.20 ± 0.37^{c}	2.33 ± 0.10^{c}
C18:1	18.8 ± 0.4^{a,b}	19.4 ± 0.2^{a,b}	20.7 ±0.2^{a,b}	19.6 ± 0.6^{a,b}	17.4 ± 1.0^{b}	19.3 ± 0.6^{a,b}	22.1 ± 7.4^{a,b}	21.8 ± 0.4^{a,b}	24.0 ± 1.13^{a}
∑ MUFA	**24.1 ± 1.0^{a}**	**23.9 ± 0.3^{a}**	**25.7 ± 0.5^{a}**	**24.4 ± 0.9^{a}**	**21.9 ± 1.4^{a}**	**23.6 ± 1.0a**	**25.9 ± 7.60^{a}**	**24.0 ± 0.7^{a}**	**26.3 ± 1.2^{a}**
C16:2 n-6	2.30 ± 0.14^{a}	0.56 ± 0.12^{d,e}	0.75 ± 0.10^{d,e}	0.72 ± 0.06^{d,e}	0.37 ± 0.10^{e}	0.80 ± 0.06^{c,d}	1.53 ± 0.27^{b}	0.78 ± 0.08^{d}	1.20 ± 0.16^{b,c}
C16:3 n-3	5.91 ± 0.37^{a}	1.07 ± 0.41^{c,d}	1.47 ± 0.11^{b,c,d}	2.46 ± 0.26^{b}	1.76 ± 0.19^{b,c}	1.85 ± 0.23^{b,c}	0.84 ± 0.14^{c,d}	0.91 ± 0.77^{c,d}	0.40±0.04^{d}
C16:4 n-3	14.1 ± 0.7^{b,c,d}	19.0 ± 0.3^{a,b}	17.7 ± 1.1^{a,b,c}	22.1 ± 0.4^{a}	21.6 ± 0.7^{a}	21.8 ± 1.0^{a}	15.9 ± 3.1^{b,c,d}	13.1 ± 1.6^{c,d}	11.8 ± 0.9^{d}
C18:2 n-6	17.2 ± 0.6^{a}	5.38 ± 1.04^{c}	7.46 ± 0.50^{c}	9.27 ± 0.88^{b,c}	7.63 ± 0.67^{c}	7.75 ± 2.12^{c}	12.3 ± 1.5^{b}	9.01 ± 1.47^{b,c}	12.3 ± 1.1^{b}
C18:3 n-3	1.12 ± 0.04^{a,b}	0.80 ± 0.06^{a,b}	1.34 ± 0.02^{a,b}	0.82 ± 0.05^{a,b}	0.62 ± 0.15^{b}	0.71 ± 0.18^{b}	1.15 ± 0.29^{a,b}	0.90 ± 0.62^{a,b}	1.56 ± 0.14^{a}
C18:4 n-3	5.59 ± 0.32^{d}	14.8 ± 0.7^{b}	12.2 ± 0.6^{c}	14.5 ± 0.4^{b,c}	17.8 ± 0.6^{a}	14.4 ± 0.4^{b,c}	4.71 ± 1.24^{d}	5.78 ± 0.90^{d}	4.27 ± 0.53^{d}
C20:3 n-3	1.27 ± 0.04^{a}	0.45 ± 0.07^{d,e}	0.56 ± 0.03^{b,c,d}	0.70 ± 0.05^{b,c}	0.47 ± 0.03^{d,e}	0.45 ± 0.02^{d,e}	0.72 ± 0.07^{b}	0.36 ± 0.08^{e}	0.55 ± 0.06^{c,d}
C20:4 n-6	2.31 ± 0.07^{a}	1.64 ± 0.08^{b,c,d}	1.58 ± 0.07^{c,d}	1.69 ± 0.09^{b,c,d}	1.04 ± 0.04^{e}	1.29 ± 0.14^{d,e}	1.81 ± 0.19^{b,c}	2.09 ± 0.05^{a,b}	1.87 ± 0.30^{a,b,c}
C20:5 n-3	2.79 ± 0.51^{d}	8.67 ± 0.49^{a}	5.81 ± 0.43^{b,c}	3.83 ± 0.21^{c,d}	5.69 ± 0.31^{b,c}	5.51 ± 1.02^{b,c}	2.92 ± 0.49^{d}	6.67 ± 1.01^{a,b}	4.20 ± 0.80^{c,d}
∑ PUFA	**52.5 ± 2.8^{a}**	**52.4 ± 3.3^{a}**	**48.9 ± 3.0^{a,b}**	**56.1 ± 2.4^{a}**	**57.0 ± 2.8^{a}**	**54.6 ± 5.2^{a}**	**41.8 ± 7.2^{b,c}**	**39.6 ± 6.5^{c}**	**38.1 ± 4.0^{c}**

CHAPTER 6

Isolation and characterization of novel *Chlorella vulgaris* mutants with low chlorophyll and improved protein contents for food applications

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Isolation and characterization of novel *Chlorella vulgaris* mutants with low chlorophyll and improved protein contents for food applications

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Abstract

Microalgae are widely used as food supplements due to their high protein content, essential fatty acids and amino acids as well as carotenoids. The addition of microalgal biomass to food products (e.g., baked confectioneries) is a common strategy to attract novel consumers. However, organoleptic factors such as colour, taste and smell can be decisive for the acceptability of foods supplemented with microalgae. The aim of this work was to develop chlorophyll-deficient mutants of *Chlorella vulgaris* by chemically induced random mutagenesis to obtain biomass with different pigmentations for nutritional applications. Using this strategy, two *C. vulgaris* mutants with yellow (MT01) and white (MT02) colour were successfully isolated, scaled up and characterized. The changes in colour of MT01 and MT02 mutant strains were due to an 80% and 99% decrease in their chlorophyll contents, respectively, as compared to the original wild type (WT) strain. Under heterotrophic growth, MT01 showed a growth performance similar to that of the WT, reaching a concentration of 5.84 and 6.06 g L^{-1}, respectively, whereas MT02 displayed slightly lower growth (4.59 g L^{-1}). When grown under a light intensity of 100 µmol m^{-2} s^{-1}, the pigment content in MT01 increased without compromising growth, while MT02 was not able to grow under this light intensity, a strong indication that it became light-sensitive. The yellow colour of MT01 in the dark was mainly due to the presence of the xanthophyll lutein. On the other hand, phytoene was the only carotenoid detected in MT02, which is known to be colourless. Concomitantly, MT02 contained the highest protein content, reaching 48.7% of DW, a 60% increase as compared to the WT. MT01 exhibited a 30% increase when compared to that of the WT, reaching a protein content of 39.5% of DW. Taken together, the results strongly suggest that the partial abrogation of pigment biosynthesis is a factor that might promote higher protein contents in this species. Moreover, because of their higher protein and lower chlorophyll contents, the MT01 and MT02 strains are likely candidates to be feedstocks for the development of novel, innovative food supplements and foods.

Keywords: heterotrophic cultivation; microalgae; nutritional applications; pigments; protein; random mutagenesis; scale-up

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6.1   Introduction

The consumer demand for health-promoting and nutritional-rich foods has been increasing over the last few years. Microalgae are a sustainable biological resource with a well-balanced biochemical profile, rich in protein and bioactive compounds such as carotenoids and essential fatty acids that provide potential benefits for human health (Lucas et al., 2018). Nevertheless, from the thousands of microalgal strains currently described and identified, only a narrow number of strains are currently approved for human consumption. In the EU, *Arthrospira platensis* ("*Spirulina*") and *Chlorella vulgaris* are approved for human consumption due to a long history of safe use, being well established in the market, while *Odontella aurita* and *Tetraselmis chui* were recently approved as novel foods by the European Food Safety Authority (EU, 2017/2470).

Microalgal biomass is widely commercialised worldwide in the nutraceutical sector as food supplements (e.g., tablets and capsules), while in the food market they are normally incorporated as a natural food colorant or as a healthy supplement, able to enhance the nutritional value of conventional food products (e.g., bars, pasta and cookies; Sahni et al., 2019). Nevertheless, the incorporation of microalgae in food products face challenges mainly due to their organoleptic characteristics, including a strong colour, taste and odour (Lafarga, 2019). The sensory attributes of foods are directly linked to the consumer acceptance whereby the colour is the first parameter observed by the consumer and can be decisive for whether or not to include the food in their diet (Delwiche, 2012). Therefore, microalgal-based food products that are usually green in colour comes with very low sensorial acceptance by the consumer. Moreover, chlorophyll, the pigment responsible for the green colour of microalgae and higher plants, usually imparts a grassy taste to tea (van Lelyveld and Smith, 1989). Therefore, these less favourable organoleptic characteristics of microalgal biomass need to be modified in order to improve its acceptance in food products.

Alternative strategies to improve the organoleptic qualities of food containing microalgal biomass have included the extraction of the target compounds with the concomitant removal of chlorophyll or the addition of ingredients such as chocolate to improve the final flavour and colour (Lucas et al., 2018). Another option could be isolation of novel microalgal strains with improved organoleptic characteristics. Random mutagenesis is an interesting cell modification tool for food applications, as it is not considered a method that generates genetic modified organisms (GMOs), because it does not introduce any foreign genetic material into the target cell (Zimny et al., 2019, DIRECTIVE 2001/18/EC). By exposure of the target cells

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to physical (e.g., UV light) or chemical mutagenic agents (e.g., ethyl methane sulfonate), strains with improved characteristics are generated. Upon mutagenesis, it is important to apply a selection procedure to screen for the desired mutants, e.g., abiotic stress factors such as light intensity. Furthermore, when the genes of the carotenoid biosynthetic pathway are targeted, specific inhibitors can be used such as compactin, diphenylamine, nicotine or norflurazon (J.-H. Chen et al., 2017; Cordero et al., 2011b; Huang et al., 2018).

Accordingly, the aim of this work was to develop chlorophyll-deficient mutants of *C. vulgaris* by chemically induced random mutagenesis in order to obtain biomass with different pigmentations for nutritional applications. The heterotrophic growth performance under light and dark conditions of wild type (WT) and established mutants was evaluated as well as their proximate biochemical composition and pigment profile. One of the mutants was scaled up to evaluate the growth performance in 5-L and 200-L fermenters and determine their feasibility as future feedstocks for the food industry.

6.2   Materials and Methods

6.2.1   Wild type inoculum and growth

Chlorella vulgaris was obtained from Allmicroalgae Natural Products S.A. culture collection. The cryopreserved cultures stored in liquid nitrogen were transported to the Centre of Marine Sciences (University of Algarve) on dry ice. The inoculum was transferred to a 50 mL centrifuge tube containing 20 mL of culture medium, comprising 0.1% glucose, 0.25% yeast extract and 0.5% peptone. The culture was later divided into several 250-mL Erlenmeyer flasks with a working volume of 50 mL containing the same medium and incubated in an orbital shaker at 28 ± 2 °C under constant shaking (100 rpm).

6.2.2   Random mutagenesis and selection of chlorophyll-deficient mutants

Cells of *C. vulgaris* growing exponentially (3.2×10^6 cells mL^{-1}) were concentrated 10-fold by centrifugation (3000 g, 3 min) and treated with 150, 200, 250, 300, 350 and 400 mM of ethyl methane sulfonate (EMS, Merck, USA) for 1 h under mild agitation in the dark (FAO/IAEA, 2018). By addition of sodium thiosulfate to a final concentration of 5%, the reaction of EMS was stopped, and cells were pelleted by centrifugation at 3000 g for 3 min. Cells were washed thrice with sterile distilled water and incubated for 24 h in the dark to prevent light-

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dependent DNA repair. For the determination of the cell survival rate, cells were plated onto Plate Count Agar (PCA; VWR, Portugal) in serial dilutions and incubated at 30 °C for 72 h in the dark. The mutant selection was carried out by visual observation of the plates in dim light. A colony with yellow colour was picked, sub-cultured several times on PCA plates and subsequently transferred into liquid media. This yellow mutant was grown to exponential phase and subjected to a second round of random mutagenesis using 300 mM EMS. This time, mutant selection was performed on PCA plates with the carotenoid biosynthesis inhibitor norflurazon, which blocks phytoene desaturase (Breitenbach et al., 2001; Koschmieder et al., 2017). To choose the lowest concentration that inhibits cell growth of the mutant, cells were previously spread onto 2, 4, 8 and 10 µM of norflurazon plates. Upon mutagenesis, cultures were plated onto PCA plates containing 10 µM of norflurazon and incubated at 30 °C in the dark for one week. Herbicide-resistant white colonies were sub-cultured several times, first on plates containing norflurazon and afterwards on plates without herbicide to confirm the phenotypic stability of the mutants.

6.2.3 Experimental trials in Erlenmeyer flasks

Experimental trials were conducted to evaluate the heterotrophic growth performance and biochemical composition of WT and established mutants under dark and light conditions. The trial was conducted in 250-mL Erlenmeyer flasks, with a final working volume of 50 mL, using a heterotrophic basal medium (HM; Barros et al., 2019) supplemented with glucose (20 g L^{-1}). Cultures were then placed in two orbital shakers at 30 °C and 200 rpm. A spotlight was kept on top of one orbital shaker using a photon flux density of 100 µmol m^{-2} s^{-1} (light condition), while the other orbital shaker was covered with aluminium foil (dark condition). All experimental trials were carried out in triplicate.

6.2.4 Growth comparison of wild type vs. mutant in 5 L and 200 L fermenters

The seed for heterotrophic growth was obtained sequentially in 50- and 250-mL Erlenmeyer flasks, in order to reach a volume of 5 L in a bench-top fermenter (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany), which was later used to inoculate a 200-L fermenter, developed and assembled in-house. Temperature in both fermenters was maintained at 30 °C and pH at 6.5 by addition of ammonia solution (24% m m^{-1}). As in the Erlenmeyer flask tests, HM medium was used (Barros et al., 2019), but glucose was added in

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fed batch so that a non-limiting concentration of 1 to 20 g L^{-1} was kept. Samples were collected aseptically for supernatant analysis or biomass concentration analysis. Throughout the growing period the air inlet flowrate was adjusted to maintain approximately 1 vvm. Accordingly, the agitation rate ranged from 100 to 1200 rpm, so that the dissolved oxygen in the medium was not a limiting factor for culture growth.

6.2.5   Sampling and growth assessment

Sampling of each culture was done twice a day in order to analyse growth parameters, namely optical density (OD) at 600 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), pH and optical microscopy (Zeiss Axio Scope A1, Oberkochen, Germany).

Dry weight (DW) determination was only possible for the samples of cultures grown in fermenters. Culture samples were filtered using pre-weighed 0.7 µm GF/C 698 filters (VWR, Pennsylvania, USA) and dried at 120 °C until constant mass was obtained using a DBS 60-30 electronic moisture analyser (KERN & SOHN GmbH, Balingen - Germany). All dry weight samples were washed with demineralized water to remove growth medium salts. Whenever the previous procedure could not be carried out, a DW vs. optical density correlation developed in-house for this strain was used. Biomass productivity was obtained by equation 1 and growth rate by equation 2.

$$P \left(g \, L^{-1} \, d^{-1} \right) = \frac{DW_f - DW_i}{t_f - t_i} \tag{1}$$

$$\mu \left(d^{-1} \right) = \frac{ln \left(DW_f / DW_i \right)}{t_f - t_i} \tag{2}$$

6.2.6   Proximate composition

The protein content was determined by CHN elemental analysis, according to the procedure provided by the manufacturer using a Vario el III (Vario EL, Elemental Analyzer system, GmbH, Hanau, Germany). The final protein content was calculated by multiplying the percentage of nitrogen by 6.25.

The lipid content was determined using the Bligh and Dyer (1959) method described in Pereira et al. (2011) with minor modifications. Briefly, freeze dried samples were extracted

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with methanol through bead-milling with glass beads, using a Retsch MM 400 mixer mill at 30 Hz for 3 min to ensure effective cell disruption. The tubes were centrifuged (10000 g) and the supernatants were collected to new vials. The pellets suffered a second extraction and both methanol supernatants were pooled. Chloroform and water were added to the methanol (2:1:2) and the tubes were vortexed for 5 min. Afterwards, the samples were centrifuged to obtain a biphasic system and the lipid extract was separated. A known volume of the extracts was transferred to pre-weighed tubes, evaporated and weighted in order to determine the lipids gravimetrically.

The ash content was determined by burning the freeze-dried biomass in a furnace (J. P. Selecta, Sel horn R9-L, Barcelona, Spain) at 550 °C for 6 h. The carbohydrate content was determined by difference of the remaining macronutrients.

6.2.7   Chlorophyll content

A culture volume corresponding to 10 mg of biomass was taken from each sample and centrifuged for 15 min, at 2547 g (HERMLE Labortechnik GmbH, Wehingen – Germany). Chlorophyll extraction was performed in acetone by successive zirconia bead milling. The supernatant was collected by centrifugation and re-extraction of the biomass was performed until colourless. The absorbance of the supernatant was measured in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, EUA) at 630, 647, 664 and 691 nm. The chlorophyll a content was then estimated according to the following equation by (Ritchie, 2008):

$$Chl_a = -0.3319\,Abs_{630} - 1.7485\,Abs_{647} + 11.9442\,Abs_{664} - 1.4306\,Abs_{691} \tag{3}$$

6.2.8   Carotenoid profile

The extraction of carotenoids was carried out on ice and under dim light to avoid oxidation. Approximately 5 mg of freeze-dried biomass was weighed in a glass vial, ~0.6 g of glass beads (425-600 µm) and 1 mL of ice-cold methanol containing 0.03% butylhydroxytoluene (BHT) were added. Cells were disrupted using a Retsch MM 400 mixer mill at 30 Hz for 3 min. To collect the supernatant, the samples were centrifuged for 3 min at 21000 g. The remaining biomass was extracted repeatedly with 1 mL of methanol/BHT by vortexing for 10 s, followed by centrifugation until both the pellet and the supernatant became

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colourless. The extracts were combined, and methanol was evaporated under a gentle nitrogen flow. Prior to HPLC analysis, the extracts were resuspended in 1 mL of methanol and filtrated through 0.22 µm PTFE filter to remove suspended particles.

Carotenoid analysis was performed by HPLC as described previously (Schüler et al., 2020b). Briefly, a Dionex 580 HPLC System (DIONEX Corporation, United States) consisting of a PDA 100 Photodiode-array detector, STH 585 column oven set to 20°C and a LiChroCART RP-18 (5 µm, 250x4 mm, LiChrospher) column was used. Carotenoid separation was achieved using a mobile phase composed of solvent A acetonitrile:water (9:1; v v^{-1}) and solvent B ethyl acetate with the following gradient: 0 - 16 min, 0 - 60% B; 16 - 30 min, 60% B; 30 - 32 min 100% B and 32-35 min 100% A. All carotenoids were detected at 450 nm and 280 nm and analysed with Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, US). The quantification was carried out using calibration curves of neoxanthin, violaxanthin, lutein, zeaxanthin and *β*-carotene standards (Sigma-Aldrich, Portugal). Phytoene was identified by its specific absorbance profile at 280 nm and only quantified as equivalent to lutein. Injection volume of both extracts and standards was 100 µL.

6.2.9   Statistical analysis

Statistical analyses were performed with R software (version 3.6.1). Statistical significance was tested using analysis of variance (one-way ANOVA) and Tukey HSD post-hoc at a 0.05 probability level.

6.3   Results and Discussion

6.3.1   Development of mutants

In the first stage of this work, chlorophyll-deficient mutants of *C. vulgaris* were obtained by random mutagenesis using the alkylating agent ethyl methane sulfonate (EMS). Different concentrations of EMS were tested on the WT to find the concentration, which resulted into a survival rate between 5-10% (Figure 6.1). The selection of the correct survival rate is critical to increase the likelihood that the survivors contain at least one mutation, but also to avoid the selection of cells containing multiple mutations, which are often detrimental to growth. The selection and further scale up of the mutants were carried out in the absence of light and with

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glucose as carbon source to suppress the need for energy supply via photosynthesis, and thus promoting the growth of chlorophyll-deficient mutants. After treatment of the cells with a concentration of 300 mM of EMS, a yellow colony indicating the absence of chlorophyll emerged onto the plate. The repeated sub-cultivation on solid media of this mutant, MT01, confirmed the stability of the yellow colour throughout 10 generations. Most probably, a mutation in the photosynthetic machinery is the reason for the reduction of chlorophyll in this mutant (Tiwari et al., 2019).
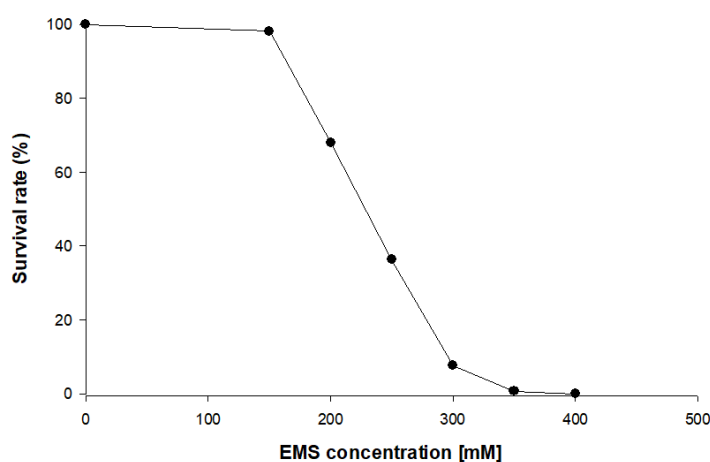

Figure 6.1. Survival rate of heterotrophic *Chlorella vulgaris* upon exposure to different ethyl methane sulfonate (EMS) concentrations on plate count agar (PCA) plates.

Thereafter, a second random mutagenesis was conducted on MT01, with subsequent selection of mutants by their resistance to the carotenogenic pathway inhibitor norflurazon. A wide range of concentrations of norflurazon was tested to find out that 10 µM was the minimal lethal concentration to MT01. This selection procedure gave rise to white colonies with resistance to the bleaching herbicide norflurazon. After sub-cultivation, only one mutant maintained the white colour when the herbicide was removed from the media over 10 generations. This mutant, MT02, most probably contains an irreversible mutation in the phytoene desaturase gene leading to the inhibition of the following steps within the carotenoid and/or plastoquinone biosynthetic pathways (McCarthy et al., 2004; Qin et al., 2007). Other studies on *Chlorella zofingiensis* and *Chlorella sorokiniana* used a similar approach to obtain mutants with accumulation of zeaxanthin or lutein, respectively (J.-H. Chen et al., 2017; Huang

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et al., 2018). In those cases, the inhibitors diphenylamine or nicotine were used to select for mutations in genes coding for enzymes involved in carotenoid biosynthesis.

6.3.2 Wild type vs. mutants in dark and light conditions

6.3.2.1 Growth performance

C. vulgaris WT and mutants were grown in 250-mL Erlenmeyer flasks under light and dark conditions, to assess the effect of light on growth performance and biomass colour (Figure 6.2). After a lag phase of about 20 h the cultures grew exponentially until the depletion of glucose, which led to cell death after 48 h.
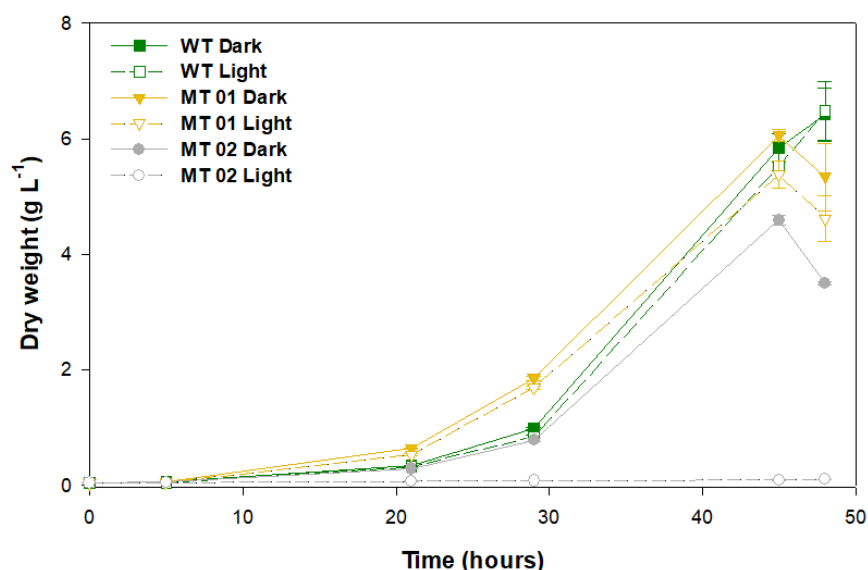

Figure 6.2. Growth curves of wild type and mutants, under light and dark conditions grown in 250-mL Erlenmeyer flasks for 48 h.

The WT along with the yellow mutant MT01, both in the dark, reached the highest DW after 45 h of growth, 5.84 g L^{-1} and 6.06 g L^{-1}, respectively. Under light conditions, the WT and MT01 achieved a similar DW ($p > 0.05$), 5.52 g L^{-1} and 5.38 g L^{-1}, respectively, but significantly lower than that obtained under dark conditions (Figure 6.2). Several pale-green *C. vulgaris* mutants reported in literature also showed biomass productivity similar to that of the WT strain used, however, under autotrophic conditions (Dall'Osto et al., 2019; Shin et al., 2016). Furthermore, those mutants showed with increasing light intensity higher biomass productivities (up to a 68% increase) than that of the WT. Those studies further showed that

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the changes observed not only improved growth performance, but also the pigment profile, at the cost of higher sensitivity to light. Interestingly, all these phenotypes were associated to smaller antenna sizes in the photosynthetic machinery of the mutants (Dall'Osto et al., 2019; Shin et al., 2016).

The white mutant MT02 displayed a significantly lower biomass concentration in the dark compared to the WT and MT01 ($p < 0.05$), attaining a maximum DW of 4.59 g L^{-1} after 45 h of growth. Moreover, MT02 was not able to grow under light conditions, achieving only 0.08 g L^{-1} of DW at the end of the assay. Similarly, Kamiya (1985) also described light, particularly blue light, as inhibitory for growth, cell division and glucose uptake for colourless *Chlorella* mutants. Nonetheless, in the dark, MT02 displayed a promising growth performance, which was statistically indistinguishable from that of the WT ($p > 0.05$). In spite of enhancing pigment content, exposure to excess light might lead to a more or less noticeable growth inhibition, which in this case was observed not only in the white MT02 mutant growth, but also in the WT and yellow MT01 mutant cultures exposed continuously to light.

6.3.2.2   Pigment profile

Macroscopically, WT cultures displayed a green colour and acquired a more intense green colour when grown under a spotlight (Figure 6.3). MT01 cultures presented an intense yellow colour under dark conditions, which was reversed back to green when cultures were exposed to light conditions. On the other hand, MT02 cultures exhibited a white tonality and absence of any other colour under dark conditions, while no biomass was produced under light conditions.

In order to characterize the colour of WT and mutant strains under light and dark conditions, the chlorophyll and carotenoid content of the cultures were analysed. It is evident that light significantly increased the chlorophyll content of WT and mutant cultures ($p < 0.05$) (Figure 6.4). Although, MT01 and WT exhibited equivalent growth performances ($p > 0.05$), MT01 contained significantly lower chlorophyll content than the WT ($p < 0.05$) under both light and dark conditions. WT cultures displayed the highest chlorophyll content, 9.16 mg g^{-1} under dark conditions, which was enhanced to 14.1 mg g^{-1} in the presence of light. MT01 cultures grown in the dark registered 1.69 mg g^{-1} of chlorophyll, while under light exposure 8.02 mg g^{-1} of chlorophyll was detected, which granted them the green coloration. In fact, no significant differences were observed between the chlorophyll content of WT grown in the dark and the light grown MT01 displaying a pale green colour ($p > 0.05$). This is in agreement with studies

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of *C. vulgaris*, where EMS-induced light green mutants with a 50% reduced chlorophyll content compared to the WT were selected (Dall'Osto et al., 2019; Shin et al., 2016). However, cultures in those studies were grown under autotrophic conditions as the objective was to enhance biomass productivities and photosynthetic efficiencies.
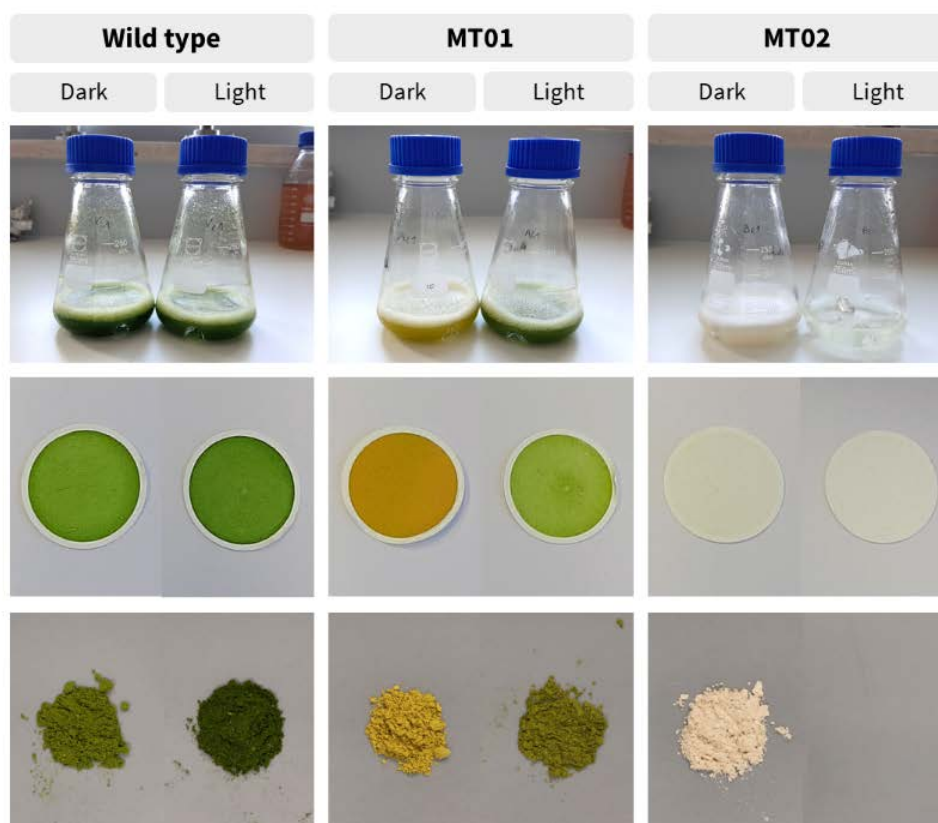

Figure 6.3. Different coloration of wild type and mutant cultures, dry weight filters and freeze-dried biomass, grown under light and dark conditions in 250-mL Erlenmeyer flasks, after 42 h.

The MT02 mutant, however, displayed only residual chlorophyll contents grown in the dark (0.045 mg g^{-1}). Although not easily visible, after some days of light exposure, MT02 started to acquire a pale green tonality, which was evidenced by the detection of an increased chlorophyll content in the biomass as compared with the dark cultured biomass (0.25 mg g^{-1}; $p < 0.05$). This is in accordance with a study of EMS-induced white mutants of *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, which showed a pale green colour due to a 40-fold decrease in chlorophyll content compared to the WT (Kamiya, 1985; McCarthy et al., 2004). However, with the mutants developed in this work, which are heterotrophically cultivated, it is possible to maintain a stable non-green colour under dark conditions.

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The carotenoid profile of *C. vulgaris* WT was mainly composed of lutein and *β*-carotene, while neoxanthin, violaxanthin and zeaxanthin were only detected in minor quantities (Table 6.1). The carotenoid profile of MT01 showed the same characteristics as compared with the WT, however, with lower contents of 0.93 ± 0.01 mg g^{-1} and 1.70 ± 0.13 mg g^{-1} DW in the dark, respectively. As lutein is the major carotenoid, this can explain the yellow colour of MT01 under dark conditions (Figure 6.3). Huang et al. (2018) also obtained a yellow *Chlorella* mutant by random mutagenesis with similar growth performances of the wild type strain. That mutant strain displayed a dysfunction in carotenoid ketolase enzyme, which prompted zeaxanthin accumulation (up to 7.00 mg g^{-1}) enhanced by high-light irradiation, nitrogen depletion and glucose feeding. Those treatments also led to the accumulation of other carotenoids, such as *β*-carotene (7.18 mg g^{-1}) and lutein (13.81 mg g^{-1}), which together imparted their yellowish hues to the biomass. In addition, Dresbach and Kowallik (1974), which also established a chlorophyll-free *C. vulgaris* mutant pointed out that carotenoid biosynthesis might be enhanced by permanent irradiation with blue light. Moreover, several positive effects on human health such as the reduced risk for cardiovascular disease and age-related macular degeneration as well as cancer prevention have been attributed to lutein (Astorg, 1997; Han et al., 2015; Ma et al., 2012). Therefore, it could be interesting to study the accumulation of this pigment in MT01 by testing other stressing or stimulating factors, such as nitrogen depletion, glucose feeding and other light wavelengths or intensities.
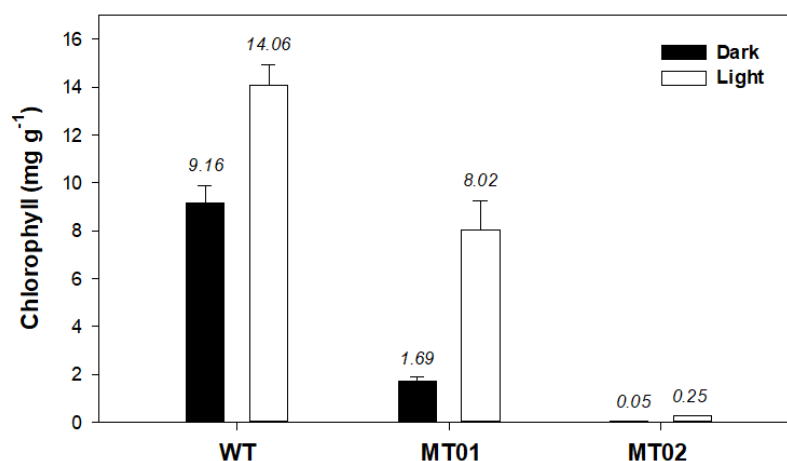

Figure 6.4. Chlorophyll content (mg g^{-1}) of *Chlorella vulgaris* wild type and mutants grown in 250-mL Erlenmeyer flasks under dark and light conditions. Values are given as means ± standard deviation of three biological replicates (*n* = 3).

Increased light intensity seems to promote the induction of carotenoids in both WT and MT01 by about 1.6-fold (Table 6.1). This is most probably related with the function of

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carotenoids, as they are important pigments involved not only in light harvesting, but also in the protection of the photosynthetic apparatus from excess light (Mulders et al., 2014b). As expected, the content of violaxanthin decreased with the concomitant increase of the photoprotective xanthophyll zeaxanthin (Table 6.1). Remarkably, the content of β-carotene in MT01 cultivated under light conditions increased 10-fold compared with cells under dark conditions, confirming the importance of this carotenoid as photoprotective pigment in this microalga. Conversely, as its white colour indicated already, all coloured carotenoids were absent in the MT02 mutant; the only carotenoid detected was the colourless phytoene with 2-fold higher concentrations as compared with the WT under dark conditions (Table 6.1). Phytoene is a linear carotenoid without a conjugated system of double bonds, which has already been reported to be ineffective in photoprotection (León et al., 2005). This is most probably the reason why MT02 was not able to grow under light conditions. Phytoene, however, has gained interest in the cosmetic industries due to its absorption of UV radiation, anti-inflammatory and anti-oxidant effects (Meléndez-Martínez et al., 2018). Therefore, it would be interesting to study the accumulation of this carotenoid in the *C. vulgaris* MT02 strain.

Table 6.1. Carotenoid content of *Chlorella vulgaris* WT and chlorophyll-deficient mutants MT01 and MT02 grown in 250 mL Erlenmeyer flasks under light and dark conditions. Different letters indicate significant differences ($p < 0.05$) between strains and treatments. Values are given as means ± standard deviation of three biological replicates ($n = 3$).

Culture	Condition	Neoxanthin (μg g^{-1} DW)	Violaxanthin (μg g^{-1} DW)	Lutein (μg g^{-1} DW)	Zeaxanthin (μg g^{-1} DW)	β-carotene (μg g^{-1} DW)	Phytoene (μg g^{-1} DW)*
WT	Dark	85.2 ± 8.1[b]	42.7 ± 7.3[a]	1280 ± 77[b]	7.22 ± 0.66[b]	284 ± 36[b]	194 ± 10[e]
	Light	181 ± 12[a]	33.2 ± 7.0[ab]	1853 ± 60[a]	9.61 ± 0.46[a]	585 ± 47[a]	252 ± 12[d]
MT01	Dark	4.7 ± 0.9[d]	33.0 ± 10.5[ab]	**858 ± 3[c]**	3.34 ± 0.66[c]	34.1 ± 1.3[c]	320 ± 4[c]
	Light	37.9 ± 8.9[c]	15.6 ± 5.7[b]	1167 ± 79[b]	8.69 ± 0.92[ab]	322 ± 26[b]	363 ± 8[b]
MT02	Dark	0	0	0	0	0	**414 ± 11[a]**
	Light	n.a.	n.a.	n.a.	n.a.	n.a.	

n.a. = not analysed due to insufficient biomass sample. *due to the lack of standard this carotenoid was analysed as equivalent of lutein

6.3.2.3 Proximate composition of main macronutrients

The comparison of the composition of main macronutrients revealed significant differences between WT, MT01 and MT02 in terms of protein, ash, and carbohydrate contents (Table 6.2). MT02 grown in the dark displayed the highest protein content, 48.7% of DW,

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followed by MT02 grown in the light and dark conditions, 45.5 and 39.5% of DW, respectively ($p < 0.05$). The WT displayed the lowest protein content under light and dark conditions, 35.3 and 30.5% of DW, respectively ($p < 0.05$). On the other hand, the highest carbohydrate content (48.8 and 42.2% of DW, in the dark and in the light, respectively) was achieved by the WT ($p < 0.05$), while MT01 and MT02 presented similar carbohydrate contents (27.1-32.0% of DW; $p > 0.05$). Interestingly, despite the great variations found in chlorophyll content between cultures and conditions, no significant differences in total lipid content were detected, which ranged from 14.3 to 18.4% of DW in all cultures and conditions ($p > 0.05$). The WT revealed the lowest ash content (5.4 and 6.6 % of DW in the dark and in the light, respectively), followed by MT01 and MT02 grown in the dark (9.3-10.1% of DW), whereas MT02 grown under light conditions displayed the highest ash content (12.7% of DW; $p < 0.05$). The conditions (light vs. dark) affected protein, carbohydrate and ash significantly, resulting in higher content of both protein and ash, and lower content of carbohydrates, when cells were exposed to light ($p < 0.05$).

Table 6.2. Proximate composition of macronutrients of *Chlorella vulgaris* WT and mutants presented as percentage of dry weight. Different letters indicate significant differences ($p < 0.05$) between strains and treatments. Values are given as means ± standard deviation of three biological replicates ($n = 3$).

Culture	Condition	Proteins (% DW)	Lipids (% DW)	Carbohydrates (% DW)	Ashes (% DW)
WT	Dark	30.5 ± 0.8[e]	15.4 ± 1.9[a]	48.8 ± 2.9[a]	5.4 ± 0.5[d]
	Light	35.3 ± 0.4[d]	15.8 ± 1.5[a]	42.2 ± 1.8[b]	6.6 ± 0.7[c]
MT01	Dark	**39.5 ± 0.9[c]**	18.4 ± 1.8[a]	32.0 ± 1.1[c]	10.1 ± 0.2[b]
	Light	45.5 ± 0.8[b]	14.3 ± 2.3[a]	27.5 ± 3.3[c]	12.7 ± 0.4[a]
MT02	Dark	**48.7 ± 1.3[a]**	14.9 ± 2.4[a]	27.1 ± 2.1[c]	9.3 ± 0.2[b]
	Light	n.a.	n.a.	n.a.	n.a.

n.a. = not analysed due to insufficient biomass sample.

Both higher amounts of proteins and lower amounts of chlorophyll detected in both mutants may suggest a truncated chlorophyll antenna size of the photosystems as reported in other chlorophyll-deficient mutants (Dall'Osto et al., 2019; Polle et al., 2002; Shin et al., 2016). Those chlorophyll-deficient mutants have been characterized with similar or even higher protein levels, namely chlorophyll-binding proteins and thylakoid membrane proteins (Gu et al., 2017; Polle et al., 2002). Furthermore, a previous report revealed that higher light

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exposure induces the accumulation of proteins; thus, in this case, low light might have induced the synthesis of larger photosynthetic units, resulting in higher protein content in the light (Seyfabadi et al., 2011). While the higher content of carbohydrate found in the dark conditions was probably due to the accumulation of polysaccharides such as starch. In addition, increased ash content in chlorophyll-free biomass has also been previously reported (T. Li et al., 2016), suggesting that the mineral metabolism might have also been affected in the mutants. Overall, WT cultures revealed proximate composition values within those previously reported for *C. vulgaris* grown in heterotrophic conditions (Canelli et al., 2020; Kim et al., 2019), while MT01 and MT02 displayed significantly higher protein contents. Therefore, the low ash associated with high protein contents of mutants, adds to these cultures improved nutritional profiles with commercial interest for their application as feedstocks for food products.

6.3.3 Scale-up case study: MT01 growth validation in 5-L and 200-L fermenters

In order to validate the previous results, the WT and MT01 growth performance was compared at a larger scale in 5-L and 200-L fermenters (Figure 6.5).
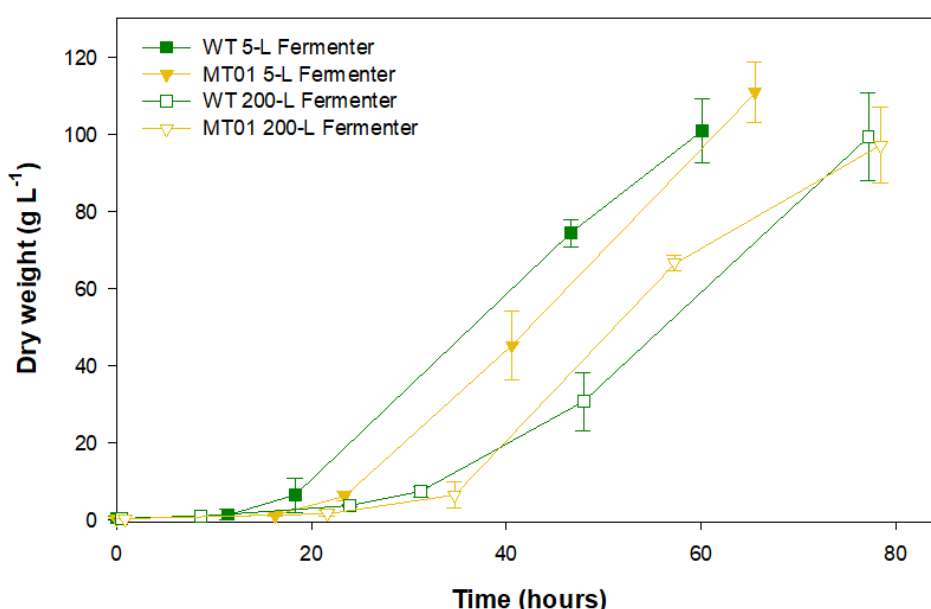

Figure 6.5. Growth curves of wild type vs. MT01 mutant in 5-L and 200-L fermenters. Values are given as means ± standard deviation of three biological replicates (*n* = 3).

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In the 5-L fermenters, growth was similar for both strains ($p > 0.05$) reaching a maximum DW of 100.9 and 110.9 g L^{-1} for the WT and MT01 cells, respectively, approximately 60 h upon inoculation. Similarly, no significant differences ($p > 0.05$) were observed in the growth of MT01 and WT in the 200-L fermenters as shown by the key process indicators (KPI; Table 6.3). Final DW here obtained was of 99.4 g L^{-1} and 97.1 g L^{-1} for WT and MT01 strains, respectively, after approximately 75 h. These values are below those previously reported for the WT strain of 174.5 g L^{-1} (Barros et al., 2019). Nevertheless, the aforementioned dry weight was obtained after 7 days of growth, whereas in this run only 3 days are considered. A similar scale-up case study for a mutant of *Chlorella pyrenoidosa* was obtained by Song et al., (2018). In this case, the mutant obtained yielded 81.9 and 84.9 g L^{-1} of biomass in the 5-L and 2000-L fermenters, respectively. As in this study, the authors point out to the homogeneity and growth patterns of their mutant upon scale-up as a strong indicator of the suitability of the mutant strain for industrial biomass production.

Table 6.3. Mean and maximum biomass productivities and growth rates of *Chlorella vulgaris* WT and mutant MT01 in 5 L and 200 L fermenters. Same letters in superscript after the values denote significant statistical differences (*p* < 0.05) between values on the same column. Values are given as means ± standard deviation of three biological replicates (*n* = 3).

Strain/Fermenter	Mean productivity (g L^{-1} d^{-1})	Maximum productivity (g L^{-1} d^{-1})	Mean specific growth rate (d^{-1})	Maximum specific growth rate (d^{-1})
WT 5-L Ferm	42.44 ± 5.31[a]	48.22	2.67 ± 0.32[ab]	2.92
MT01 5-L Ferm	41.03 ± 1.56[a]	42.11	2.98 ± 0.04[a]	3.01
WT 200-L Ferm	30.98 ± 2.25[b]	33.06	2.38 ± 0.08[b]	2.47
MT01 200-L Ferm	30.07 ± 1.47[b]	31.73	2.46 ± 0.26[ab]	2.64

Concurrently, there were no statistical differences ($p > 0.05$) in the specific growth rate of WT and MT01 growth in the scales tested: 5 L and 200 L. This is an excellent indicator of the robustness of this mutant for industrial scale heterotrophic production. On the other hand, the productivity was higher ($p < 0.05$) for both strains in the 5-L fermenter compared to the 200-L, given the shorter lag phase observed in these growth curves. In fact, the KPI for the WT and MT01 strains in the 200-L fermenter are well in accordance with the previously reported for the WT grown in the same 200-L fermenter – productivity of 27.54 ± 5.07 g L^{-1} d^{-1} and mean growth rate of 0.92 ± 0.11 d^{-1} (Barros et al., 2019). Furthermore, the biomass

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productivity and specific growth rate obtained for the MT01 strain were higher than those previously obtained for a *C. pyrenoidosa* mutant (19.68 g L^{-1} d^{-1} and 1.44 d^{-1}, respectively) using a reactor with a volume of 2000 L (Song et al., 2018).

6.4 Conclusions

The established *Chlorella vulgaris* strains with yellow (MT01) and white (MT02) colours showed high biomass productivities comparable to the wild type. The colour change in MT01 and MT02 cells were due to a 5- and 180-fold decrease in chlorophyll contents and the presence of lutein and phytoene, respectively, when the cells were grown heterotrophically in the dark. Both mutants displayed improved protein contents compared to that of the WT with a 60% increase under heterotrophic growth. MT01 was successfully scaled up to industrial 200-L fermenters, reaching a concentration of about 100 g DW L^{-1}. Because of this growth performance as well as improved organoleptic and nutritional characteristics, both new strains MT01 and MT02 show a high potential for applications in the food and nutraceutical industries for novel products based on microalgal biomass.

CHAPTER 7

General discussion, conclusions and future perspectives

7.1   General discussion

Microalgae are a renewable, sustainable and rich source of protein, carbohydrates and lipids containing high-value compounds such as polyunsaturated fatty acids and pigments. Compared with traditional sources of these compounds (e.g., plants, fruits and animals), microalgae are advantageous because they do not require arable land, present higher productivities and many species can be grown all year using sea- or wastewater. Despite these advantages and the increasing demand for natural products, only few microalgal species have been exploited industrially for high-value compounds, such as carotenoids and EPA (Ruiz et al., 2016; Silva et al., 2020). Therefore, the overall aim of this thesis was to provide new insights into the metabolism of high-value compounds and the generation of mutants to facilitate future applications of microalgal biomass to the feed, food and pharmaceutical markets.

To this end, two industrial relevant and robust strains were studied in this thesis, namely the obligate photoautotroph, euryhaline microalga *Tetraselmis striata* CTP4 and the non-obligate photoautotrophic freshwater species *Chlorella vulgaris* (Figure 7.1). The former is a representative microalga with a strong cell covering and was therefore used to optimize carotenoid extraction (Chapter 3). Afterwards, carotenoid and EPA production of this species was improved by using a physiological and genetic approach (Chapters 4 and 5). Finally, pigment mutants of heterotrophically grown *C. vulgaris* were generated to improve food applications (Chapter 6). The present Chapter aims to connect the subjects addressed during the thesis, comparing the two different species selected and discussing the overall outcome.
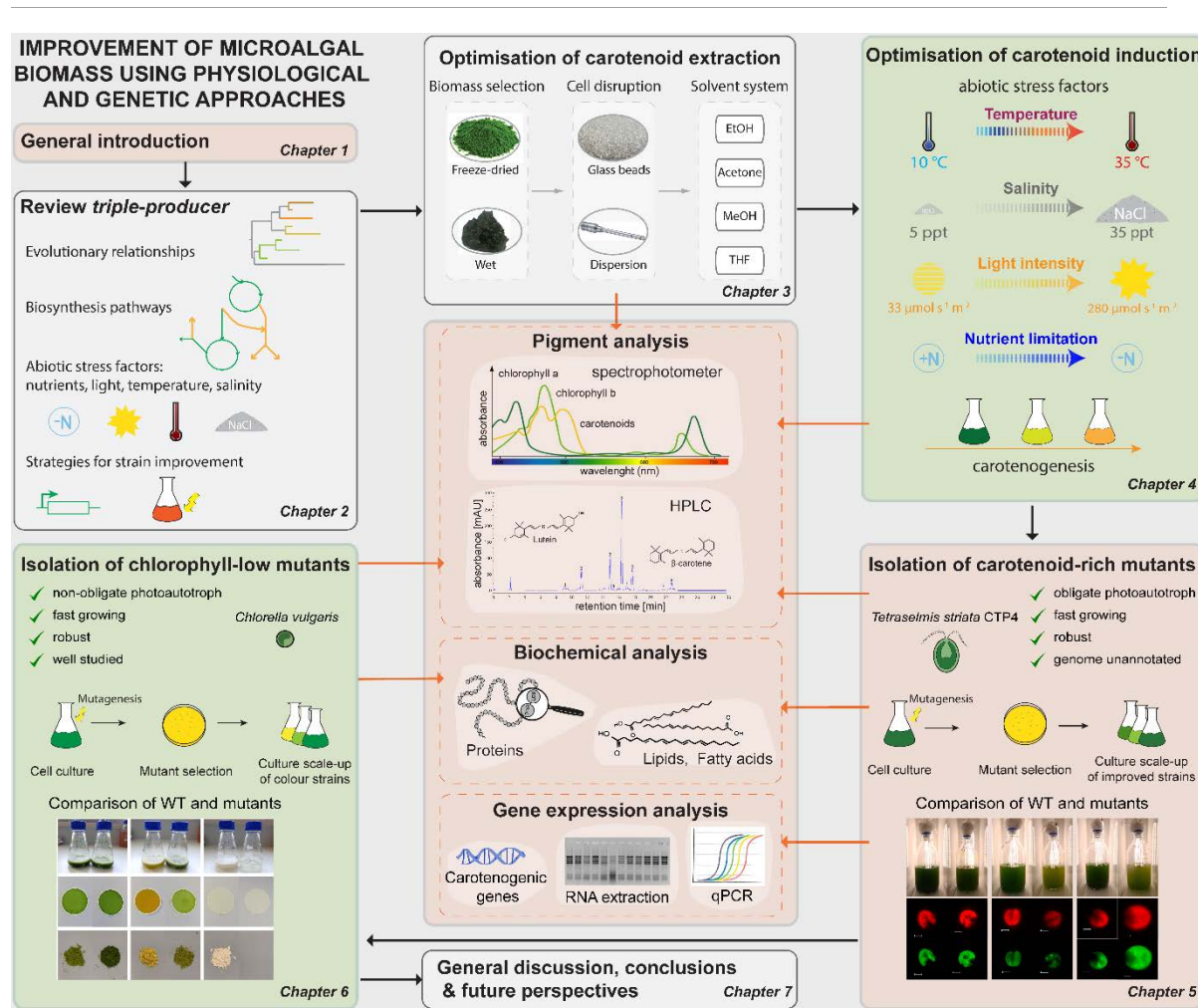
Figure 7.1. Diagram of the workflow performed in this thesis.

7.1.1 Extraction and analysis of carotenoids

A precise method of extraction and analysis of carotenoids is the first and most important step in the research and development of products rich in carotenoids. Although carotenoid extraction from microalgal biomass is a common effort, the short literature review in Chapter 3 revealed that a universal protocol is absent. The reason for this is that microalgae are a very diverse group of organisms producing different types of carotenoids and storing them within different cell coverings and intracellular compartments. Therefore, at the beginning of this thesis, the optimization of the extraction of carotenoids from *T. striata* CTP4 was crucial to later study the improvement of carotenoid content. Carotenoid extraction is challenging and a whole range of parameters need to be considered when selecting the type of biomass, cell disruption method and extracting solvent (Saini and Keum, 2018). Moreover, carotenoids outside of their natural environment are sensitive to heat, light and oxygen.

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Considering all these factors, the best extraction of carotenoids from *T. striata* CTP4 was achieved by using wet biomass, a glass bead-assisted cell disruption and acetone as extracting solvent. Although this method was mainly developed for laboratory use to analyse and quantify carotenoids throughout the thesis, a possible scale-up at industrial facilities took part in the decision of several parameters, namely solvent selection, cell disruption method and biomass pre-treatment. More specifically, since this microalga has already been produced industrially (Pereira et al., 2018a), the downstream processing of carotenoids is an important task for future industrialization of the process. Therefore, besides the existence of alternative extraction methods (e.g., supercritical fluids), the conventional solvent-based extraction was selected, which is already standardized to meet commercial applications (Ambati et al., 2019). Moreover, mechanical cell disruption was indispensable for this species because of its strong cell covering (Manton and Parke, 1965) and bead mills are available and applied at industrial scale (Schwenzfeier et al., 2011). At a later time during the implementation of this thesis, the aforementioned method was successfully applied to the biomass of *C. vulgaris*, a different microalga which is also known to possess a strong double-layered cell wall (Burczyk and Hesse, 1981). Even though a lower recovery of pigments from freeze-dried biomass of *T. striata* CTP4 as compared with wet biomass was obtained (Chapter 3), lyophilised biomass was used at a later time. The better handling, more precise quantification as well as easier transportation between the microalgal production facility and analysis laboratory were the reasons for this alteration. Moreover, as discussed in Chapter 3, the most important factors contributing to a lower carotenoid recovery from dry biomass were most probably the long storage time and high temperature. Hereafter, samples were kept at low temperatures and processed in a short time frame (<35 days) after lyophilisation. Additionally, the moisturizing of the biomass prior to extraction allowed for a better penetration of the solvent into the cells. Moreover, extraction efficiency was improved by using a bead beater acquired recently instead of using a vortex mixer. Another alteration was the use of methanol instead of acetone as both solvents proved to have similar extraction efficiencies (Chapter 3), and thus by using methanol a change of solvent prior to injection in the HPLC was avoided.

During the extraction, an alkaline saponification is often performed whereby xanthophyll esters are hydrolysed and chlorophyll as well as TAG are removed. Although this step aids to obtain a purer extract for better detection and quantification of carotenoids, it was not performed in this thesis. One reason for this is that saponification leads to structural alterations in carotenoids and to a decrease in proteins, lipids and carbohydrates, thus reducing the nutritional value of the extract (Kimura et al., 1990; T. Li et al., 2016). Moreover, all carotenoids in both species under study were present in the free form and an ester

hydrolysis was not necessary. When considering the application of extracts from these microalgal species, the presence of free carotenoids is preferred over esterified forms, as they are easily absorbed by the gastrointestinal tract (Saha et al., 2020). Moreover, instead of the application of pure extracts, oils rich in carotenoids can be used as, for example, nutraceuticals or cosmeceuticals, because they additionally contain other high-value compounds such as PUFAs or vitamins. Nevertheless, the presence of chlorophyll is often a disadvantage, not only because of its interference in quantification, as shown in Chapter 3, but also in the application of the extract or the whole microalgal biomass (Wold et al., 2005). Therefore, for both *T. striata* and *C. vulgaris*, novel mutants with low amounts of chlorophyll were obtained by strain improvement using a genetic approach (Chapters 5 and 6).

7.1.2   Strain improvement of *Tetraselmis striata* CTP4 by a physiological approach

Improvement of the production of high-value compounds can be achieved by optimisation of abiotic factors. Therefore, the primary goal of this thesis was to find out the best conditions to accumulate carotenoids in the euryhaline microalga *T. striata* CTP4. The fatty acid profile including LC-PUFA was analysed only at carotenoid-inducing growth conditions. A profound knowledge of the biosynthesis and accumulation of lipophilic compounds, such as TAGs, LC-PUFAs and carotenoids in microalgae is necessary to understand how different conditions affect compound production. During the extensive review carried out in Chapter 2, it became clear that not only each microalgal species produces different compounds but also senses, transduces and responds to environmental stress in a different way. Nevertheless, general trends showed that stressful conditions favoured TAG biosynthesis and the accumulation of photoprotective pigments, whereas the highest accumulation of LC-PUFAs and light harvesting pigments was usually obtained under growth-promoting conditions. Moreover, the term *triple-producer* was introduced, which is a species able to accumulate all three important compounds simultaneously. In such a strain, the accumulation of LC-PUFA-containing TAG together with the accumulation of carotenoids can be induced by a single abiotic stress factor e.g., nitrogen depletion.

Previous studies on *T. striata* CTP4 showed that this species is able to accumulate large amounts of lipids of up to 40% of DW mostly accumulated in lipid bodies under nutrient depletion and other stress conditions (Monteiro, 2014; Santos, 2014). Moreover, the analysis of the fatty acid profile revealed the presence of the LC-PUFA EPA, representing 2.8% of total fatty acids (Pereira et al., 2019). These results suggested a possible accumulation of

carotenoids in lipid bodies inside CTP4 cells under conditions favouring lipid induction; and together with the presence of EPA, this species could become a *triple-producer*.

With these concepts in mind, carotenoid production in *T. striata* CTP4 was investigated under a wide range of abiotic stress factors, namely light intensity (33, 170 and 280 µmols m^{-2} s^{-1}), salinity (5, 20 and 35 ppt), temperature (10, 20, 30 and 35 °C) and nutrient availability (nitrogen repletion and depletion; Chapter 4). The first important observation was that carotenoid contents decreased under nitrogen depletion in *T. striata* CTP4. Although the opposite was described for *T. marina* (Dahmen-Ben Moussa et al., 2017), this result is in agreement with other studies on species of the genus *Tetraselmis* (Sansone et al., 2017; Tsai et al., 2016). The observed decreased contents of carotenoids are most probably related with a reduced photosynthetic activity of the cells leading to a diversion of carbon towards lipid production instead of carotenoid biosynthesis. Indeed, lipid accumulation can be observed microscopically in the form of lipid bodies in this microalga. Moreover, a comparison of fluorescence micrographs of cells under nitrogen depletion and repletion conditions provided evidence that this species does not accumulate carotenoids in lipid bodies (Chapter 4). Carotenoids that are stored in lipid bodies need to be hydrophobic for a compact packing together with TAGs; therefore, they are either carotenes (e.g., *β*-carotene) or esterified xanthophylls. Although *T. striata* CTP4 synthesizes *β*-carotene and the xanthophyll lutein, they apparently do not accumulate in lipid bodies, most probably because they are not targeted into the lipid bodies and/or in the case of the latter are not esterified. Besides the known function of *β*-carotene in photoprotection (Mulders et al., 2014b), in this species the most important photoprotective pigment with a quenching and scavenging role seemed to be lutein. This is due to the observation that lutein contents increased under nitrogen limitation (after 10 days), conditions known to promote the accumulation of reactive oxygen species (ROS). Furthermore, carotenoid contents in *T. striata* CTP4 were studied under different temperatures (10, 20 and 30 °C) and salinities (5, 20 and 35 ppt). Previous studies showed that lipids can be induced under heat stress without nutrient depletion, although reaching lower contents of about 25% of DW, but promoting a faster induction already after 5 days of growth (Santos, 2014). As has already been seen for lipid induction, temperature had also a stronger influence on carotenoid contents than salinity. The highest content of neoxanthin, violaxanthin, lutein and *β*-carotene, were found under 30 °C and 35 ppt, accounting for 1.01 ± 0.06, 1.58 ± 0.16, 2.15 ± 0.25 and 3.37 ± 0.36 mg g^{-1} DW, respectively (Chapter 4). Heat stress affects membrane fluidity and produces ROS, conditions known to induce lipids and carotenoids in microalgae (Chapter 2). In the following experiments, these conditions were maintained constant, and the induction of carotenoids was studied under changing light intensities (33,

170 and 280 µmol m^{-2} s^{-1}). Interestingly, under changing light intensities, the contents of lutein and β-carotene showed an inverse response: higher contents of lutein were found under higher light intensity and growth-promoting conditions (170 and 280 µmol m^{-2} s^{-1}), while β-carotene contents increased under lower light intensities (33 µmol m^{-2} s^{-1}). Nevertheless, highest contents of carotenoids (8.48 ± 0.47 mg g^{-1} DW) were found upon a thermal upshift from 20 °C to 35 °C at a light intensity of 170 µmol m^{-2} s^{-1} after only two days of growth (Chapter 4). Under these conditions, high contents of both lutein and β-carotene were reached, accounting for 3.17 ± 0.18 and 3.21 ± 0.18 mg g^{-1} DW, respectively. These observations highlight that one pigment can have several functions. For example, β-carotene can act as a light harvesting pigment under low light, whereas under stress conditions its scavenging activity protects the cells from ROS. In a later experiment in Chapter 5, the inverse dynamics of lutein and β-carotene contents under different light intensities was confirmed. However, in this study, an even higher light intensity of 380 µmol m^{-2} s^{-1} led to the induction of lutein, though leading to lower contents (2.65 ± 0.13 mg g^{-1} DW) than in the previous experiment using a light intensity of 170 µmol m^{-2} s^{-1}. This is most probably related to the different reactors used in the study—the larger diameter of the 1-L reactor, as compared with that of 100-mL test tubes, corresponded to a longer light path and therefore the average light intensity inside the photobioreactor might have been lower. Nevertheless, when compared with the contents of the inoculum, both conditions (170 and 380 µmol m^{-2} s^{-1}) led to a 2-fold increase in lutein at high light intensities and heat stress. This showed once again the importance of this pigment in light harvesting and photoprotection of actively growing *T. striata* CTP4 cells. Moreover, in Chapter 5, the lipid contents and fatty acids profiles under changing growth conditions were addressed. As expected from previous studies on *T. striata* CTP4, the lipid contents increased 2-fold in cells under heat stress (30 °C) and high light intensities (380 µmol m^{-2} s^{-1}) as compared with mesophilic growth conditions. Nevertheless, a lower content as compared with previous studies (20% of DW, 400 µmol m^{-2} s^{-1}) was achieved, accounting for 17% of DW. When comparing fatty acid profiles, a higher fraction of EPA was found in microalgae under mesophilic growth conditions rather than under stress, accounting for 3.83 ± 0.21 and 2.92 ± 0.49% of TFA, respectively (Chapter 5). Most probably EPA is present, in this species, in the polar lipids as has been found in *T. suecica* (Bondioli et al., 2012). Polar lipids are part of thylakoid membranes which are probably increased under lower light conditions (100 µmol m^{-2} s^{-1}). Indeed, higher chlorophyll contents of 40.2 ± 2.6 mg g^{-1} DW have been found under these conditions as compared with stress (18.7 ± 1.6 mg g^{-1} DW), suggesting a proliferation of thylakoid membranes and their components in cells under light limitation. Taken together, the accumulation of high-value compounds in this microalga seem to follow the trends observed in the review in Chapter 2. Light harvesting pigments such as

neoxanthin, violaxanthin and *β*-carotene as well as EPA accumulate under lower light intensities and temperatures, while photoprotective pigments such as lutein, zeaxanthin and *β*-carotene as well as lipid bodies most probably composed of TAGs increase under stress conditions.

7.1.3   Strain improvement by a genetic approach

Besides the optimization of growth conditions for specific compound production, genetic approaches are a different way for improving key characteristics of microalgae. As suggested in Chapter 2, this can either be achieved by metabolic engineering or by strain selection upon spontaneous mutations or random mutagenesis. The drawbacks of metabolic engineering lie in the difficult and time-consuming development of transformation tools for different microalgal strains and the associated high costs (Hlavova et al., 2015). Furthermore, the genetics of the microalga under investigation need to be known as heterologous DNA is introduced in the cell to target specific sequences of the wildtype genome. The use of those generated genetically modified organisms (GMOs) in the food, feed and environmental sectors is restricted due to associated risks concerning human health and changes in biodiversity, among others (M. U. et al., 2019).

Therefore, in this thesis, random mutagenesis was used to improve the biomass value of two different microalgae, namely *T. striata* CTP4 and *C. vulgaris*. Both strains represent key features for large-scale production such as high growth rates, robustness and ease of harvest (Barros et al., 2019; Pereira et al., 2018a). Moreover, both species belong to the core chlorophytes and display similar pigment profiles presenting chlorophyll *a* and *b* as well as lutein and *β*-carotene as major carotenoids. Besides these similarities, the cultivation mode of both species is highly distinctive, which is most probably related to their evolutionary history. *T. striata* CTP4 belonging to the early diverging family of Chlorodendrophyceae is a euryhaline species and an obligate photoautotroph (Leliaert et al., 2012). Unlike earlier reports on the heterotrophic growth of *T. suecica* and *T. chui* and a strain closely related to *T. striata* (Azma et al., 2011; Day and Tsavalos, 1996; Lu et al., 2017; Mohamed et al., 2014), several attempts at growing *T. striata* CTP4 on different carbon sources in the dark have failed. *C. vulgaris*, however, is a freshwater species and can be grown photoauto-, mixo- or heterotrophically (Safi et al., 2014). Moreover, a typical feature of marine microalgae is the presence of LC-PUFAs such as EPA, which are absent in freshwater species. The comparison of mutant generation methodologies for these microalgae provided further evidence for their evolutionary differences.

During the process of random mutagenesis, hundreds of different mutants are obtained, making the selection of a good screening method for the desired mutant essential (Hlavova et al., 2015). As the main target of improvement for both microalgal species was the pigment production, a first evidence is the visual appearance of the new strains. Furthermore, upon mutagenesis different abiotic stress factors can be applied to make pressure towards mutant growth with desired compound accumulation. In the case of *C. vulgaris*, a key parameter of mutant selection was the heterotrophic growth in the dark resulting in the suppression of photosynthesis. In this way, upon chemical mutagenesis a yellow strain MT01 was obtained representing an 80% decrease in chlorophyll content as compared to the WT (Chapter 6). MT01 also contained a protein content 30% higher than that of the WT, reaching 39.5% of DW. This increase in protein content is most probably related with the shift of N-storage compounds, e.g., from chlorophyll to protein. However, carotenoid contents, mainly composed of lutein decreased significantly in the mutant as compared with the WT regardless of the growth conditions.

On the contrary, the obligate photoautotroph *T. striata* CTP4 was grown in the light and only different hues of green could provide an indication of changed pigment contents. Therefore, a more targeted approach was chosen by using the carotenoid biosynthesis inhibitor norflurazon. Besides phytoene desaturase, this herbicide has been shown to inhibit also fatty acid desaturation, in particular $\Delta 6$ desaturases of the LC-PUFA biosynthesis (Breitenbach et al., 2001; Cohen et al., 1993). Therefore, strains resistant to this herbicide are expected to present not only higher carotenoid but also higher LC-PUFA contents. Indeed, a selection of mutants from *T. striata* CTP4 by this method led to an improved strain ED5 with 1.5-fold increased carotenoid contents and a fatty acid profile enriched in EPA as compared to the WT (Chapter 5). Under heat stress and high light, the photoprotective pigments lutein and zeaxanthin were 1.5- and 1.3-fold increased as compared to the WT, reaching 3.81 and 0.09 mg g^{-1} DW, respectively. Moreover, this mutant displayed chlorophyll contents 60% lower than those of the WT when grown under high light conditions containing only 7.96 mg g^{-1} DW. However, these contents are still high as they are comparable to those of the *C. vulgaris* WT under dark conditions, representing 9.16 mg g^{-1} DW. Heat stress and high light conditions also led to a fatty acids profile containing 6.67% of EPA. The highest content of EPA in ED5 microalgae, however, was 8.67% of TFA. The enhanced production of photoprotective pigments and the antioxidant EPA accompanied by a decrease in chlorophyll is most probably related to a higher sensitivity of ED5 cells to stress conditions. Contrary to the mutants of *T. striata* CTP4, a selection in the dark using the same herbicide-inhibiting strategy on the yellow mutant MT01 of *C. vulgaris* led to the generation of a white mutant MT02 (Chapter 6).

In this case, the inhibition of the biosynthesis of photoprotective pigments by the herbicide norflurazon led most probably to an irreversible mutation in the phytoene desaturase (McCarthy et al., 2004). MT02 was only able to survive because of the absence of chlorophyll and light. As a result, MT02 presented a 99% decrease in chlorophyll content, whereas the phytoene contents rose 2-fold, as compared to the WT, respectively. Phytoene is a colourless carotenoid and has been already proven to be inefficient in photoprotection (León et al., 2005). Therefore, this mutant was not able to survive and thrive under light conditions. Moreover, the protein content of MT02 increased by 60% as compared to the WT, representing 48.7% of DW.

Another approach to strain improvement is the selection of desired traits by high throughput methods such as fluorescence activated cell sorting (FACS). This tool has been successfully applied for the isolation of lipid rich strains. For example, *T. striata* CTP4 was isolated by using a staining of lipid bodies with the fluorescent dye BODIPY 505/515 (Pereira et al., 2016). A correlation between the lipid staining dye Nile red and carotenoids was used to isolate carotenoid hyperproducing strains of *D. salina* upon random mutagenesis (Mendoza et al., 2008). However, previous experiments in Chapter 4 showed that lipid accumulation in *T. striata* CTP4 does not correlate with carotenoid induction thus requiring a different approach. A key characteristic of microalgae is the presence of autofluorescence of several pigments, not only the wine-red fluorescence of chlorophyll *a* but also of carotenoids in the green spectrum (J. Chen et al., 2017; Kleinegris et al., 2010). This green fluorescence was used to enrich carotenoid containing strains by FACS prior to the selection of *T. striata* CTP4 strains under lower light conditions (45 µmol m^{-2} s^{-1}). In combination with a confirmation of the resistance to the herbicide norflurazon another mutant B11 was developed from the previous mutant ED5. B11 showed a 1.4-fold increase in carotenoid contents as compared with the WT, in particular under lower light conditions, and *β*-carotene and lutein contents of 4.20 ± 0.27 and 3.09 ± 0.23 mg g^{-1} DW, respectively. The chlorophyll contents in B11 were up to 60% lower than those of the WT, suggesting an increased sensitivity of the cells to light. Furthermore, the fatty acid profile of this strain was enriched with EPA, reaching 5.81% of TFA.

Moreover, gene expression analysis of mutants obtained by random mutagenesis provide a better understanding of the microalgal metabolism regulation in cells under different stress conditions. Although this task contributes heavily to further improvements in the microalgal biotechnology, it is often not performed due to the lack of genomic sequences duly annotated. The recent publication of the genome of a related *T. striata* strain aided in the discovery and design of primers of carotenogenic genes used in this study (Steadman Tyler

et al., 2019). In this way, the expression profiles of six carotenogenic genes both in WT (*T. striata* CTP4) and respective mutants were determined in cells under mesophilic growth and heat stress combined with high light (Chapter 5). Because of the resistance of these mutants to the herbicide norflurazon, it was expected a visible effect on the expression of the gene encoding phytoene desaturase. Interestingly, the transcript levels of the other genes of the carotenoid biosynthetic pathway were significantly affected in both mutants, regardless of the growth conditions. More specifically, under stress conditions transcript levels of *PSY* and *PDS* were upregulated 1.9- and 2.0-fold in ED5 cells as compared with the WT, respectively. Under the same conditions, in B11 cells, the transcript levels of *PSY* and *PDS* were also found to be upregulated. PSY and PDS have been reported previously as rate limiting enzymes of the carotenoid biosynthetic pathway (Cordero et al., 2011a; Li et al., 2013). Moreover, their transcript upregulation has been reported under high light in *Chlamydomonas* sp. and *C. zofingiensis* (Cordero et al., 2012; Ma et al., 2019). However, genetic engineering attempts at enhancing the expression of enzymes involved in carotenoid biosynthetic pathways have often shown mixed and inconsistent results when *PSY* and/or *PDS* were overexpressed. This has led to the assumption that multiple rate-limiting steps exist in this complex pathway (Gimpel et al., 2015). Indeed, an up to 2.7-fold higher expression of *CYP97C3* was found in both mutants, as compared with the levels of the wildtype. This observation agreed well with the higher lutein levels found in the mutants as compared with those of the WT. Moreover, under mesophilic growth and in relation to WT microalgae, B11 cells showed a significant (3.4-fold) upregulation of *LCYB* gene expression. Taken together, the results obtained in *T. striata* CTP4 mutants provide, therefore, further evidence that the expression of genes encoding enzymes belonging to carotenogenic pathways is regulated coherently. Moreover, the changes observed in the fatty acid profiles and chlorophyll contents of the mutant may suggest that the mutations generated have affected the overall metabolism of the microalgal cell. A possible explanation could be that changes of gene expression of one enzyme might affect overall gene expression by feedback loops. However, a different possibility is that the mutation occurred in a gene coding for a regulatory factor (e.g., transcription factor), thus having a pleiotropic effect on overall gene expression.

7.1.4   Applications of improved microalgal biomass

7.1.4.1   *Tetraselmis striata* CTP4

Upon improvement by a physiological or genetic approach, *T. striata* showed higher carotenoid contents as compared with other microalgae of the genus of *Tetraselmis* (Table

7.1). Species of *Tetraselmis* are interesting carotenoid producers due to their large variety containing different carotenoids of both the *α*- and *β*-branch. Lutein is not only used as feed supplement to improve the yellow colour of eggs but has recently been gaining interest due to its health benefits related with improved vision and cognitive function (Johnson, 2014; Vishwanathan et al., 2011). Although marigold is the major natural source of lutein, microalgae have been considered as an alternative due to the advantages over crop production and the presence of lutein in the free form. The amounts of lutein produced by *T. striata* CTP4 are comparable to those of other microalgal species of lutein producers, for example, species of *Chlamydomonas* or *Chlorella* (Saha et al., 2020). Furthermore, violaxanthin is an interesting carotenoid due to its antiproliferative effects towards human mammary and colon cancer cells lines (Cha et al., 2008; Pasquet et al., 2011). Moreover, *β*-carotene is a provitamin A and an antioxidant which has been positively related with human health when included into the diet (Cicero and Colletti, 2017). In a recent study the carotenoid contents of *T. suecica* were compared with those of common food sources, which showed higher or comparable amounts (Di Lena et al., 2019). Taken together, the improved WT and mutants of *T. striata* in this thesis are interesting candidates for carotenoid production and alternative producers of lutein. Although microalgae of the genus *Tetraselmis* find their major application in the aquaculture sector, an application in the nutraceutical and cosmeceutical market might be considered. The direct human consumption, however, is hampered as *T. striata* CTP4 is not yet approved by the European Food Safety Agency (EFSA). Nevertheless, the nutritional value and absence of toxicity of its biomass revealed its suitability for food applications (Pereira et al., 2019).

Table 7.1. Carotenoid contents of different species of the genus *Tetraselmis*.

Species	Total carotenoids (μg g^{-1} DW)	Violaxanthin (μg g^{-1} DW)	Lutein (μg g^{-1} DW)	*β*-carotene (μg g^{-1} DW)	References
T. chui	2486	546	624	941	(Ahmed et al., 2014)
T. suecica	5806	1408	484	626	(Ahmed et al., 2014)
T. suecica	2972	819	854	433	(Di Lena et al., 2019)
T. chui	1887	33	653	845	(Lima et al., 2021)
T. striata CTP4	8475	1640	3166	3205	this study
T. striata ED5	9005	997	3811	3324	this study
T. striata B11	10196	1628	3086	4204	this study

The biomass of wildtype and mutants further displayed significant concentrations of EPA, ranging between 2.8 and 8.7 % of TFA. EPA is an essential fatty acid in human nutrition, which is important for growth and provides health promoting effects due to its antioxidant activity (Adarme-Vega et al., 2012). Microalgae have been considered to be a sustainable replacement to fish oils, the major source of EPA (Martins et al., 2013). Therefore, *T. striata* CTP4 could be a valuable and sustainable source of carotenoids and EPA in a biorefinery concept, where different fractions of the biomass are valorised. For the extraction of lipophilic compounds, a cost-efficient liquid-liquid triphasic system (LTPS) using the wet biomass of *T. striata* CTP4 and an ethanolic extraction has already been proposed, leading to three different fractions, namely a non-polar hexane phase, a colloidal phase and a water phase (Gangadhar, manuscript in prep). The non-polar hexane fraction contained TAG, free fatty acids, *β*-carotene and chlorophyll could find its application as edible oil in the feed or food market (Xue et al., 2018). However, the colloidal fraction was rich in phospholipids and xanthophylls (lutein and violaxanthin) with high antioxidant activity, suggesting an application to the nutraceutical and cosmeceutical industries. The water phase, rich in polysaccharides, can be used to produce bioethanol, however, other nutraceutical or feed applications are conceivable.

7.1.4.2 *Chlorella vulgaris*

Chlorella vulgaris is one of the few species approved for human consumption and can be used as food supplement or additive (Fradique et al., 2010). The heterotrophic cultivation of *C. vulgaris* has been shown to be easily scaled-up to industrial fermenters (Chapter 6), which are relatively cheap and offer a better control of process parameters. Furthermore, higher biomass concentrations and productivities can be achieved under heterotrophic growth than under photoautotrophic growth as the supply of light is omitted. When industrial or agricultural wastes are used as carbon source, heterotrophic cultivation is a promising mode for a sustainable and reliable production of microalgal biomass (Hu et al., 2018).

As already pointed out throughout this thesis the application of biomass containing high chlorophyll contents is hampered due to the unpleasant colour, taste and odour. Furthermore, the removal of chlorophyll is often accompanied by a loss of the nutritional value of the biomass. Therefore, the chlorophyll-deficient mutants isolated in this thesis can be used as whole biomass in food products, thus broadening the potential of algae-based food products. Recently, microalgae have been considered as potential ingredient in meat analogues, thus, contributing to a healthier and sustainable alternative for a growing

population (Fu et al., 2021). The higher protein contents found in the *C. vulgaris* mutants as compared to the WT make these strains suitable as meat analogues. Additionally, applications in nutraceutical or cosmeceutical products are conceivable due to the health benefits related to lutein and phytoene present in the yellow and white mutant, respectively.

7.2 Conclusions

The exploitation of novel strains for high-value compounds is an ongoing effort in microalgal biotechnology. A basic knowledge on cell physiology and reactions to changing environments is crucial to optimize the production of target metabolites. Moreover, in combination with genetic approaches the fields of microalgal biomass applications can be broadened. In the present thesis, a carotenoid extraction protocol was successfully optimized and applied to two robust industrial strains, namely *T. striata* CTP4 and *C. vulgaris*. Afterwards, different abiotic factors were studied to enhance carotenoid and EPA production in *T. striata* CTP4. However, as already expected from a literature review, the increase in one compound is often accompanied by the decrease in another compound. To further improve this euryhaline microalga, a genetic approach was used leading to the isolation of non-GMO strains with higher carotenoid contents and EPA-enriched fatty acid profiles as compared with the WT. Interestingly, the success of any random mutagenesis is highly dependent on the genetics of the species and the selection procedure. When the same genetic approach was applied to the freshwater species *C. vulgaris* under heterotrophic growth, mutants with chlorophyll deficiency and high protein contents were obtained. This thesis provides further evidence that the development of biotechnologically relevant microalgal strains by genetic means is a very powerful tool to gather knowledge and to improve strains; it is also a cost-effective way to facilitate microalgae-based products.

7.3 Future perspectives

Improvement of high-value compound production by optimization of abiotic stress factors is an important task to understand cellular processes. However, when it comes to large-scale production, not all environmental factors are easily controllable. Therefore, it would be interesting to study the performance concerning carotenoid and EPA production of *T. striata* CTP4 cultivated at large-scale. Most probably a nitrogen repletion strategy would be crucial to

achieve high contents of both high-value compounds. Moreover, high temperatures and high light intensities can be achieved during the summer in the South of Portugal; therefore, the seasonal production of these target metabolites could be interesting to investigate. The production can then be compared with the established mutants and their genetic stability could be tested at large-scale. Moreover, photoautotrophic cultivation depends heavily on a sufficient light supply and self-shading effects, in dense cultures, often limit cell growth at industrial scale. Because the mutants of *T. striata* represented lower chlorophyll contents as compared to the WT under all test conditions, the self-shading effect in these cells may be reduced leading to improved light penetrations. Therefore, it is possible that the mutants show higher biomass and carotenoid productivities than the WT at industrial scale.

In the case of *C. vulgaris*, the growth of the yellow mutant at industrial scale was proven in this study, while a trial for the white mutant at large scale was later performed successfully by the team of Allmicroalgae Natural Products S.A., Portugal. However, different growth conditions could be tested to improve the production of lutein and phytoene in the respective mutants due to the high market value of these compounds. Because of its photoprotective function, a common inducer for lutein production is light intensity. However, *C. vulgaris* mutants need to be grown under heterotrophic conditions and in the dark to maintain their phenotypes. Different strategies to induce carotenogenesis in microalgae under heterotrophic growth are other abiotic stress factors such as temperature, pH, salinity or nutrient availability (Hu et al., 2018). Furthermore, the induction of oxidative stress by the addition of ROS (e.g., hydrogen peroxide) led to an enhanced production of lutein in *C. protothecoides* (Wei et al., 2008). Moreover, high C/N ratios have been shown to stimulate the production of pigments in microalgae under heterotrophic growth. For example, an enhanced lutein production in *C. protothecoides* was found with increasing glucose concentrations (Shi et al., 1999).

Further studies will also be needed to evaluate the bioaccessibility, bioavailability and biological activity of the carotenoids produced by *T. striata* CTP4 and the novel mutants of *C. vulgaris*. For example, a recent study on *C. vulgaris* revealed that a food processing method is needed to increase the bioaccessibility of carotenoids (Gille et al., 2016).

Furthermore, rapidly emerging sequencing technologies will help to provide further insights into the developed strains in this study. In the case of *T. striata* CTP4, the genome sequencing using the current "gold standard" (PacBio) platform has been already performed. In collaboration with Dr. Cymon Cox's lab (CCMAR, CETA), the genome assembly and respective gene annotation are ongoing projects. Therefore, genomic and transcriptomic assessment of the novel mutants of this species would be important to find out the reasons

for the changes observed in the metabolism, namely high carotenoid and low chlorophyll contents as well as enrichment of the EPA fraction in the fatty acid profiles. Moreover, comparative genomics could be used to find out the mutations leading to the observed changes. Furthermore, a genetic analysis of the *C. vulgaris* mutants could provide a better understanding of what changed in the genome and transcriptome that led to lower chlorophyll and higher protein contents. The reduction in chlorophyll is most probably related to a mutation in the genes encoding elements of the photosynthetic machinery but only comparative genomics will tell whether this assumption is true (Tiwari et al., 2019). In this sense, the mutation of the white mutant of *C. vulgaris* could be revealed, which has been linked to the phytoene desaturase (McCarthy et al., 2004). However, since the study on *T. striata* showed not only changes in the *PDS* gene expression profiles but also in those of other genes encoding enzymes of the carotenogenic pathway, it is possible that the white *C. vulgaris* mutant may contain more than one mutation causing the absence of coloured carotenoids.

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