Gabriel da Cunha Bombo

Isolation of two novel *Chlorella vulgaris* and *Tetraselmis chui* mutants with improved protein contents and pigments for food applications

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologia

2021

Gabriel da Cunha Bombo

Isolation of two novel *Chlorella vulgaris* and *Tetraselmis chui* mutants with improved protein contents and pigments for food applications

Mestrado em Biologia Marinha
Supervisor:
João Carlos Serafim Varela

Co-supervisor

Hugo Galvão Pereira

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e

Tecnologia 2021

Isolation of two novel *Chlorella vulgaris* and *Tetraselmis chui* mutants with improved protein contents and pigments for food applications.

Declaração de autoria de trabalho

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

Copyright

ACKNOWLEDGEMENTS

Meu agradecimento ao Professor João Varela, que desde o início me apoiou e me disse sim, mesmo sem ainda me conhecer quando fui pedir uma oportunidade de estagiar no laboratório, e que desde então sempre esteve a me apoiar da melhor forma que pode, ao me aconselhar, tirar dúvidas e se esforçar para ajudar sempre que possível, e pela oportunidade de poder estar a trabalhar com o que sempre me fez brilhar os olhos.

Ao Hugo por me acolher e orientar durante esse período, assim como continua a fazer em todos os outros projetos e atividades que estão a se desenvolver nesta oportunidade incrível de trabalhar com a equipa do GreenColab.

Um muito obrigado à Lisa que sempre esteve no laboratório comigo a me ensinar e ajudar, a doar o precioso tempo e conhecimento que tinha e repartir comigo sempre, a me guiar e acalmar nos momentos mais difíceis.

À Tamara por ser sempre a força de todos no laboratório, por acalmar, brigar, motivar e estimular sempre que necessário. Por estar lá sempre que chamam, seja no laboratório ou não, sempre de coração aberto a escutar e ajudar de forma possível e impossível sem medir esforços. E sempre chamar para tomar um café nos momentos perfeitos.

Ao meus companheiros de laboratório e amigos Etiele, Nathana, Alexandre, Filipa, Zé Marques e todos os outros membros do Marbiotech.

Aos colegas de trabalho e amigos do GreenColab que estão sempre prontos a dar a ajuda que for necessária, Peter, Florinda, Cristina, Rita, Daniel Figueireido, Madalena, Laís, Mônia e todos os outros membros dessa equipa incrível que me orgulho de fazer parte.

A todos os amigos que fiz desde que cheguei a Portugal, seja de dentro da universidade ou de fora, que sempre estiveram a ouvir chorumelas desnecessárias e me acompanharam nessa jornada que está por acabar, aos amigos que deixei no Brasil mas não me esqueci de nenhum e ainda os carrego comigo e aos que por acaso nos encontramos por aqui vindos do mesmo lugar.

À Veronica, por acreditar em mim e me dar forças para seguir nos dias mais difíceis, por atravessar todas a dificuldades ao meu lado, sempre forte e pronta para o que der e vier, passamos por momentos difíceis, mas agora estamos em um melhor momento, onde podemos difrutar de risadas, alegrias e tudo que há de bom juntos e já ser parte indispensável da minha família.

Gratidão eterna à minha família, que apesar de estar longe nunca deixou de me apoiar e acreditar em mim, me motivando e me alegrando com amor incondicional que só a familia pode proporcionar, não passo um dia sem lembrar e ter saudades de estarmos juntos e que logo possamos matar essa saudade. Aos principalmente importantes minha mãe Nina Rosa, meu pai Celso Antonio, minha irmã Leticia, meu cunhado Felipe, e novamente a Veronica. Aos meus avós que já se foram, mas sempre se mostraram orgulhosos de mim e que sempre estarão junto a mim.

Muito obrigado a todos, sem as pessoas que me sercam nunca teria chego até aqui, esse trabalho é um esforço em conjunto de todas as pessoas que me apoiaram até então.

ABSTRACT

Microalgae are a worldwide food source, largely consumed by humans as food or, indirectly, as feed. Their biomass might contain high levels of protein, lipids, fatty acids, amino acids, and vitamins. They are unicellular organisms capable of living in the most diverse environments, being able to grow faster than other photoautotrophs and in non-conventional growth media that do not require either potable water or arable land. With such characteristics, microalgae can become an important alternative food source in the near future since the world population is estimated to reach 10 billion people before 2050. Microalgae are produced and consumed as high-quality nutritional food, but there is an urgent need to improve existing microalgae-based products since their organoleptic properties, such as their “grassy” taste, are not consensually accepted by the consumer. The present work aims to create new strains from microalgal species already registered as novel food, namely *Chlorella vulgaris* and *Tetraselmis chui*, to improve the quality of the produced biomass and the overall consumer acceptance. For this purpose, random chemical mutagenesis using the alkylating agent ethyl methane sulphonate (EMS) was carried out to generate mutants with higher protein contents and/or lower amounts of chlorophyll. Afterwards, the best performing strains were selected using visual scoring regarding pigmentation and flow cytometry techniques. Upon implementing this selection pipeline, two *C. vulgaris* strains were isolated, namely the C3 strain, a non-mutagenized isolate able to grow significantly faster on solid medium compared to the wildtype (WT) culture; and a second mutant, GL3, obtained from the C3 strain. Although the C3 strain presented higher protein contents, the GL3 displayed vestigial chlorophyll contents, lower carotenoid levels, and higher protein content than the original WT strain. The GL3 strain grown under heterotrophic conditions reached higher cell concentrations as compared to the WT, strongly suggesting that the mutant strain GL3 might become a relevant source of protein, being suitable to be produced on a larger scale to generate food products with enhanced organoleptic properties.

Keywords: Mutagenesis; Novel-food; Microalgae; protein; *Chlorella vulgaris*; *Tetraselmis chui*.

RESUMO

As microalgas são organismos aquáticos unicelulares, por vezes coloniais, que normalmente são eucariotas, à exceção das cianobactérias, que são procariotas. Uma parte significativa destes organismos são fototróficos obrigatórios. embora possam ser mixotróficos, ao consumirem fontes de carbono orgânico como a glucose e a frutose, para além de fixarem carbono na forma de CO_2. Podem ser ainda organismos heterotróficos facultativos, em que na falta de energia luminosa ou de carbono inorgânico, se especializaram no consumo de material orgânico. Esta forma de trofia pode ser encontrada em regiões de águas oligotróficas ou extremas, como ecossistemas cavernosos, debaixo de glaciares ou lagos subglaciais.

Em termos ecológicos, a principal função desses organismos no ambiente natural é a alta carga de produção primária que são responsáveis quando fazem fotossíntese, sendo os responsáveis pela maior parte do O_2 disponível no planeta, seja em ambientes aquáticos ou atmosfera.

As microalgas podem variar de tamanho, sendo consideradas como tal microrganismos cuja célula está na faixa entre os 200 a 20 µm. As história evolutiva das microalgas mostra que estes organismos foram capazes de colonizar praticamente qualquer ambiente com algum tipo de habitat aquático por menor que seja. Estes ambientes vão desde grandes lagos e mares até regiões escuras e enclausuradas, incluindo ambientes com corpos d’água periódicos, mesmo que assolados por longas épocas de seca como o deserto do Atacama.

A variabilidade genética e adaptabilidade das microalgas é uma questão chave para entender as caracteristicas biológicas, morfológicas e bioquímicas destes seres, que apresentam uma grande varíedade de lípidos, ácidos gordos, aminoácidos e proteínas. Essa rica composição torna os organismos deste grupo alvo de estudos para o desenvolvimento e pesquisa de produtos como ração para animais, alimento humano, produção de biodiesel, fármacos e cosméticos. Atualmente, as microalgas já são utilizadas na aquacultura como alimento vivo para criação de peixe, como biomassa para suplementação alimentar humana, fertilizantes e até mesmo como tratamento terciário de estações de tratamento de água residual de origem urbana ou industrial. A União Europeia já aceita cerca de 20 espécies diferentes de microalgas como suplemento alimentar ou alimento. Entre elas estão: *Chlorella vulgaris*, *Arthrospira platensis* (comummente designada por “Spirulina”), *Dunaliella salina* e *Tetraselmis chui*.

Uma grande variedade de espécies já são atualmente produzidas comercialmente por todo o mundo, especialmente para ração animal. Porém, a cada ano que passa, o mercado para a alimentação e suplementação humana vem crescendo exponencialmente. Isso se deve ao interesse do consumirdor em uma alimentação mais saudável e natural, que diminui a procura de alimento de origem animal e aumenta a apetência por sucedâneos de origem vegetal. Ainda

assim, estima-se que a produção alimentar mundial até o ano de 2050 será insuficiente, aumentando a necessidade do desenvolvimento de produtos que ocupem uma menor área para produção, sendo de alta qualidade e rendimento. A produção de microalgas se encaixa com as necessidades previstas, sendo que além de poder ser produziada em áreas inférteis e não aráveis, não necessita o uso de água potável. Tal é possível, pois há uma variada gama de espécies marinhas que podem ser domesticadas e produzidas em raceways, greenwalls e outros fotobiorreatores em que o meio de cultura pode ser feito com água do mar.

A produção industrial de microalgas depende de diversas etapas, que vai desde o isolamento e identificação da espécie até produção de biomassa em larga escala. E mesmo com a tecnologia atual a produção ainda se depara com algumas limitações que tornam o processo não tão barato quanto o esperado, seja pela presença de contaminações, como pela baixa produção de algumas espécies de microalgas. Para desenvolver melhores produtos, a prospecção por diferentes espécies ou estirpes de microalgas é uma atividade constante de empresas e laboratórios especializados nos estudos e produções de microalgas. Um dos métodos para aumentar a produção é o desenvolvimento de novas estirpes, seja por domesticação e aprimoração das condições fisicoquímicas de produção ou pela mutagénese aleatória em busca de novas caracteristicas favoráveis.

Algumas das propriedades mais procuradas é o aumneto da produção de proteina no conteúdo total da biomassa assim como o melhoramento das qualidades organolépticas da biomassa para aumentar ainda mais a aceitação do consumidor de produtos baseados em microalgas. Uma das causas principais para o cheiro e sabor da biomassa de microalgas ser tão acentuado é a clorofila, que, através da mutagenese, pode ser reduzida, melhorando as suas características organolépticas. Além disso, estas microalgas melhoradas tendem em apresentar uma maior percentagem de proteina total na sua biomassa, que é algo muito apreciado pela indústria alimentar.

Um dos métodos para promover a mutagenese aleatória de microalgas é a aplicação de um agente alquilante como o etilmetanosulfonato (EMS) que causa uma alteração em resíduos de guanina, alterando a composição genética do material genético (e.g., pares de bases GC são mutados para AT) de forma pontual e aleatória. O facto do EMS gerar mutações pontuais torna a mutagénese mais aceitável ao consumidor do que a introdução de genes heterólogos. Além disso, perante a União Européia, tal processo não é considerado como gerador de organismos genéticamente modificados, o que facilita a sua produção à escala industrial. Essa metodologia já foi testada em diversas espécies de microalgas, como *Dunaliella tertiolecta*, *Nanochloropsis* sp. e diversas espécies de *Chlorella* spp. Em todos os casos, foi possível desenvolver estirpes de alta qualidade, tanto na questão de pigmentos, como lípidos, ácidos gordos e proteínas.

Um dos pontos cruciais para garantir que a estirpe melhorada geneticamente continua a ser a mesma, após o processo, não sendo devido ao isolamento de algum contaminante originalmente presente na estirpe que sofreu o processo de mutagenese, é impotante realizar a identificação molecular, antes e depois de realizar a metodologia de mutagénese. A indentificação molecular se dá pela extração de material genético, seguido pela amplificação através de reação em cadeia da polimerase (PCR) de uma parte do gene 18S, que normalmente consiste em 1000 ou mais pares de base. Ainda assim, para algumas espécies de microalga a região 18S não é suficiente para se identificar diferenças interespecíficas, o que torna necessária a utilização de outros alvos como região 28S ou os espaçadores ITS1 e ITS2. Após a amplificação e sequenciação da região escolhida, é feita uma análise de similaridade com base de dados especializadas para identificar a espécie mais provável através do grau de semelhança de sequências.

Para este trabalho foram utilizadas duas estirpes de espécies diferentes, *Chlorella vulgaris* e *Tetraselmis* chui, tendo estas sido cedidas pela empresa Allmicroalgae, dentro do projeto conjunto ProFuture, para a produção de estirpes melhoradas por mutagénese química aleatória através do agente EMS. *Chlorella vulgaris* é uma espécia já amplamente estudada desde os anos 50, e foi pioneira para a produção de biomassa para o consumo humano, já sendo perspetivada como alimento para astronautas no começo da corrida espacial. Foi prontamente aceite como "novel food" pela União Europeia, por ter um longo histórico de consumo humano durante muitos anos. A mutagénese de microalgas começou por estudos realizados em *Chlorella vulgaris*, para o melhor entendimento e estudo do mecanismo fotossintético, sendo provada a possibilidade de produzir estirpes de diferentes cores desde então. *Tetrasselmis chui* é uma das espécies de microalgas mais recentes nos termos de "novel food", e tem sido uma espécie de grande interesse por apresentar uma alta produção de biomassa e ter uma boa qualidade bioquímica, por ter uma diversidade de carotenoides, lípidos e aminoácidos de interesse nutricional. O atual projeto tem como objetivo desenvolver mutantes de *Chorella vulgaris* e *Tetraselmis chui* como uma menor taxa de clorofila e maior taxa de proteína. No caso da *T. chui*, no período estipulado para o projeto, não foi possível desenvolver um mutante cuja a pigmentação fosse visivelmente diferente, o que já foi explicado em outros projetos pela probabilidade de a espécie ser um organismo fotoautotrófico obrigatório, dificultando a produção de mutantes, devido ao facto de mutações no aparelho fotossintético que impliquem alterações drásticas de pigmentos serem, em geral, letais. Já no caso da *Chlorella vulgaris*, foi

possível obter diferentes estirpes a partir da estirpe selvagem, nomeadamente a estirpe C3, que mostrou um melhor desenvolvimento em questão ao crescimento de unidades formadoras de colónias em heterotrofia e mixotrofia, mas sem revelar alterações visíveis em termos de pigmentos fotossintéticos. Assim sendo, foi selecionada a estirpe C3 para uma nova ronda de mutagénese aleatória, na qual foi possivel isolar e caracterizar a estirpe GL3 que apresentava visivelmente uma coloração amarelada. De facto, ao analisar a biomassa, foi detectado uma quantidade 20 vezes menor de clorofila e uma maior produção de proteína em gramas por 100 gramas de biomassa. A produção de uma estirpe como a GL3 foi considerada como um sucesso na aplicação da metodologia utilizada, sendo aquela enviada para o nosso parceiro empresarial para ser produzida em larga escala após uma prospecção mais detalhada da qualidade e aceitação do mercado desta biomassa inovadora.

Palavras-chave: Mutagénese; Novo alimento; Microalgas; proteina; *Chlorella vulgaris*; *Tetraselmis chui*.

Table of Contents

Abbreviations

AA	=	Amino acids
Acetyl-CoA	=	Acetyl Coenzyme A
CC	=	Cell concentration
CO_2	=	Carbon dioxide
Chl-a	=	Chlorophyll *a*
Chl-b	=	Chlorophyll *b*
CHN	=	Carbon Hydrogen Nitrogen (elemental analysis)
CRISPR	=	Clusters of Regularly Interspaced Short Palindromic Repeat
DNA	=	Deoxyribonucleic acid
DW	=	Dry weight
EMS	=	Ethyl methanesulfonate
FACS	=	Fluorescence activated cell sorting
gDNA	=	Genomic DNA
GMOs	=	Genetically Modified Organisms (with foreign DNA)
MAM	=	Modified Algal Medium
MNU	=	*N*-methyl-*N*-nitrosourea
PBR	=	Photobioreactor
PCA	=	Plate Counting Agar
PCB	=	Plate Counting Broth
Ti	=	Initial weight
Tf	=	Final weight
TCar	=	Total Carotenoids
WT	=	Wild type

1 Introduction

1.1 Microalgae

Microalgae are a diverse group of microscopic organisms that can be unicellular, colonial, or even pluricellular, comprising mainly photosynthetic eukaryotes and cyanobacteria [1]. These organisms consume CO_2, nutrients, and water and produce O_2 and biomass by using the energy of the sunlight. They are essential for the primary production in the world, having an excellent mechanism for carbon concentration [2].

These mechanisms include not only the capture of carbon as CO_2 but also as different types of dissolved inorganic carbon such as HCO^-_3. The latter is often accomplished by a reversible process catalyzed by carbonic anhydrase, which can convert HCO^-_3 into CO_2, releasing it into the pyrenoid, where the step of CO_2 fixation within the photosynthetic process takes place in many microalgae [3,4], including *Tetraselmis* spp. [5] and *Chlorella* spp. [6].

Microalgae vary in size and morphology, with several identified species from 2000 µm to less than 2 µm, with most species ranging from 200 to 20 µm [7]. During their evolutionary history, microalgae colonized almost all kinds of environments, from freshwater (e.g., *Chlorella vulgaris, Scenedesmus obliquus, Haematococcus pluvialis, Arthrospira platensis*) to brackish- and seawater (e.g., *Tetraselmis chui* and *Phaeodactylum tricornutum*) [8] as well as terrestrial habitats as dry as the desert of Atacama [9]. Moreover, these organisms can be found in extreme environments such as water with high salinity and exposed to high light intensity (*Dunaliella salina*) [10], underground lakes like Vostock [11] and alkaline/soda [12] lakes. They are also able to grow in the most oligotrophic to the most eutrophic habitats [7]. This adaptability is key for their high biological and biochemical diversity due to the biosynthesis of various metabolites, including lipids, proteins, carbohydrates, and pigments. Some of these metabolites are not only crucial to the survival of these microorganisms, but they are considered to be promising ingredients for food, feed [13], pharmaceutical and cosmetic applications [14] as well as feedstocks for biofuels. As such, they are often called third-generation (3G) biofuel feedstock [15]. Algae can be used as a fertilisation source and a plant protector to substitute products based on chemicals synthesized from fossil fuels that can degrade the local soil and impact near water sources [16]. Finally, they can be produced on non-arable land, using non-potable water, such as urban and agroindustrial effluents, whose water can be recycled for other usages [17].

1.2 Biotechnological and Industrial applications

Microalgal biomass is exploited due to its well-balanced biochemical profile containing high-value compounds for several applications. A broad range of species have been tested and used as a source of nutrients for crops, feed, and aquaculture [18]. The need to produce sustainable food sources is a common subject in the economic forecasts for 2050, and microalgae are, in many ways, products of high quality and interest when addressing sustainability and a steady food supply. The bioprospection for new species is essential to overcome one of the current limitations of using algae as food: the fact that only around 20 products from 10 different species are nowadays considered novel food and can be consumed in the EU (EU, 2017/2470). The improvement of techniques for the isolation of novel microalgae is necessary to make large scale cultivation economically feasible, considering its costly production [19]. There has been a strong effort from the research community to broaden the spectrum of useful microalgae for industrial algal producers as well as commercial users of microalgal biomass [20].

Known microalgae-based products include lipids extracted from microalgae such as *Desmodesmus armatus* and *Chlorella vulgaris*, which can be used as feedstock for biofuels [21]. However, because of the low value of biofuels, fatty acids, carbohydrates, and proteins (and their amino acids) of mainly marine microalgal species are often used for feed, a higher value commodity. Concerning non-marine microalgae such as *Arthrospira platensis* (often called “Spirulina”) and *Chlorella vulgaris*, they have often been used as ingredients for food supplementation worldwide for decades. Interestingly, these two latter species are not only robust and easily cultivated, but also, they have a stable, mature market that is able to pay for premium quality biomass. This has been key to the survival of current microalgal producers, as the production of microalgae is expensive, requiring improvement in terms of their growth rate and biomass quality [22]. Other species such as the euryhaline *Tetraselmis chui* and the diatom *Odontella aurita* have more recently been classified as “novel foods” (EU, 2017/2470). Another example is *Chaetoceros* sp., a large diatom containing biotechnologically relevant secondary metabolites such as polyunsaturated fatty acids with antioxidant properties for biomedical applications and hormone-like signalling molecules [23], which can be applied to the nutraceutical, cosmetic and personal care industries. Some other species have been used as, for example, *Dunaliella salina* and *Haematococcus pluvialis* as natural sources of carotenoids [1,24].

The industrial production of microalgae comprises several steps: cultivation of the initial inoculum, scale-up, harvesting, drying, and storing of the product. Each step of the production pipeline requires different technologies and know-how [25]. As some of them are labour- and energy intensive, microalgal biomass tends to be expensive, making the final product even costlier [26]. Microalgal production for food products can be carried out photoautotrophically in closed systems such as tubular photobioreactors (PBR) [27] or heterotrophically in fermenters [28]. Although the PBR uses a cheaper carbon source (CO_2), microalgae achieve lower growth rates and biomass concentrations as compared to heterotrophic growth. The reson for this is that the gorwth is dependent on light availability, which becomes a limiting factor in highly concentrated cultures and thus restricting growth. As a result, cultures grown heterotrophically can reach concentrations > 3-fold higher than those obtained under autotrophic production, as light availability is not required in fermenters [29]. Thus, heterotrophic cultivation is able to produce more biomass in less time, being also less prone to contamination due to a tighter control of the production system [30]. Despite their higher costs, microalgae have the advantage that they can be produced on nonarable land and in non-potable water, including wastewater [31]. However, large scale production of microalgae needs to be further optimized with respect to the enhancement of their biochemical composition, which impacts the organoleptic traits of the biomass and, therefore, its commercial value as food [19]. Improvement can come from production technology using innovative prototypes to increase light availability and enhance the composition of microalgal biomass in terms of (macro-)nutrients [32]. However, one cannot forget that microalgae can also be improved genetically to develop novel strains able to sustain better yields with higher quality biomass. The latter can be achieved by selecting improved microalgae with the desired traits using optimized growth media [33].

1.3 What and why to improve

From a global perspective, the human population should reach the milestone of 10 billion by 2050 (UN, 2019). The possibility that within a few decades, the capacity of food production will not be enough to feed so many people and that growing urbanization and wealth will bring changes in the consumption behaviour [34] requires new solutions. One strategy to solve protein scarcity is the growing market of meat substitutes, microalgal biomass being one of them [35]. Algae, unlike crops, are sources of protein that can be produced all year round, do not depend on arable land for most species [36], or potable water, as they can be grown in seawater. Moreover, they can be bioremediation agents (e.g., by removing nutrients that are viewed as pollutants) whose biomass can be used to produce commodities instead of being a waste that needs to be disposed of [37].

Two specific factors need to be taken into account for algal protein to be used by business-to-business and final consumers, which are: i) the quality of the product in terms of protein content and other health-linked compounds such as polyunsaturated fatty acids, lipids, and pigments [38]; and ii) the look, odour and taste of the biomass that tends to have a strong green colour, a characteristic "fishy" smell and a "grassy" flavour [35].

To solve these handicaps and further enhance the final product, strain improvement is needed. One way to achieve this goal is by performing random mutagenesis to enhance biomass production, pigment biosynthesis, compounds with biomedical applications [39], protein yield [28] and the profile of polyunsaturated fatty acids (PUFA) [40]. For the food industry, however, the abrogation of the green colour to get a more neutral appearance and enhance the appeal of the biomass while increasing protein yield is a more urgent and attractive strategy. The effect of this has already been seen on the food market with the launch of products such as the "honey chlorella", "white chlorella", and "blue spirulina" by companies such as Allmicroalgae.

Throughout the years, strain mutagenesis of microalgae was mostly for understanding the biochemical pathways linked to pigment production [41]. Since the 1930s, trials using different chemicals have been used in the generation and screening of mutants [28,42]. Microalgal strains with different pigmentation enabled the study of specific metabolic pathways in the early 1950s [43]. The species used in mutagenesis studies to elucidate biosynthetic pathways were mostly *Chlorella* sp. [44], *Scenedesmus* sp., *Chlorococcus* sp.[45], as well as some species of cyanobacteria such as *Anacystis nidulans* [46].

More recently, in the early years of the current century, the focus of mutagenesis has been research applied to industrial processes in order to increase yields of specific compounds such as the omega-3 PUFA eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid [47], among many other applications [48]. Other uses of mutagenesis have been enhanced production of carotenoids [40,49–51], biomass [52], or even protein content [28]. Moreover, there is an urgent need for screening and identifying mutant strains with improved traits such as higher robustness and the possibility of growing heterotrophically [53]. This leads to the consequent use of mutagenesis as a process that is able to accelerate the generation of mutants, which can be later selected as strains with improved properties [54].

1.4 Strain Improvement

Several methods can be used to generate strains capable of producing compounds in larger concentrations and purity [55]. The methods vary from genetic modification by cutting-edge technologies such as CRISPR-Cas9 gene editing tools or more traditional approaches such as breeding and random mutagenesis [56]. The former type of mutagenesis involves targeted mutations to engineer specific enzymes as well as other gene products [57]. However, this technology often leads to the insertion of non-homologous sequences, generating genetically modified organisms (GMOs). Because of their potential environmental impact and unknown health issues, the EU legislation, comprising the directives 2001/18/EC, 2009/41/EC, 2015/412, and the regulations (EC) 1829/2003 and (EC) 1830/2003, can be quite strict with companies that want to produce them, which increases costs. Conversely, random mutagenesis usually requires a non-specific chemical or radiation to randomly target DNA. Examples of mutagens used in this technique are UV irradiation [58] and chemical mutagenic agents such as ethyl methanesulfonate (EMS) [28] and *N*-methyl-*N*-nitrosourea (MNU) [59]. As this type of methodologies does not require the insertion of non-homologous sequences, it is generally accepted as an acceleration of a natural process of mutation and selection, which can be used in microalgae to be used as food or food supplements, according to the regulation EU, 2017/2470 and the decision 111/18 of the Court of Justice of the EU.

1.5 The mutagenic agent ethyl methanesulfonate (EMS)

Ethyl methanesulfonate (EMS) is a chemical agent capable of ethylating several sites of the DNA molecule, thus, promoting the introduction of nucleotide substitutions, insertions, or deletions [60]. The history of using ethyl methanesulfonate to promote mutations in the genetic material of a biological entity started with its application to bacteriophage mutagenesis [61], when the ability of a chemical agent to increase the number of mutants in an *in vivo* experiment was demonstrated for the first time. Since then, it has been broadly studied and used as a mutagenic agent throughout the years [60] and continues to be used. EMS can alkylate DNA, leading to the generation of 6-ethyl-guanine, which is able to pair with thiamine. This mispairing yields a conversion from a CG to a TA pair. Alternatively, EMS can also form 6-

ethyl-thiamine, which upon a round of replication, promotes the conversion of TA to CG pairs [62]. As these random mutations occur at different sites along the genome of the target organism, loss-of-function alterations can disrupt essential structures in the cell, leading to cell impairment or even death [48]. Gain-of-function phenotypes can also be observed, but they are much less frequent because these require that mutated DNA sequence acquires a novel molecular role instead of losing its original function.

The EMS mutagenesis methodology has been used in different studies, including mutant generation in microalgae. Species such as *Dunaliella tertiolecta* were mutagenized for a better yield of pigments like zeaxanthin production, getting a yield of up to 8 mg/L [39]. Other research groups increased protein production in *Nannochloropsis* sp. by 20% using using 0.1M and 0.5M EMS solutions [63]. Conversely, another study described a 69 to 75% faster growth of the lipid overproducing mutant as compared to the wild type (WT) *Chlorella* strain [54]. In addition, the produced biomass and lipids increased by 100 and 50%, respectively, in the mutant strain. The same authors also described the development of a viable strong mutant using the suppression of acetyl-CoA by the herbicide quizalofop. In fact, herbicides such as norflurazon and nicotine can be used to inhibit specific metabolic pathways to affect genes coding for proteins involved in the biosynthesis of target metabolites as, for example, carotenoids [28]. Besides the use of herbicides, different abiotic factors can be used as a screening tool for mutants with higher resistance to harsh environments [50]. In addition, mutants can be selected by simple visual colourimetric analysis or by fluorescence activated cell sorting (FACS) if the target compounds have specific colours or are autofluorescent, respectively. Other selection procedures might use microscopy techniques such as confocal Raman [55] or the use of specific dyes (e.g., BODIPY and Nile red) to identify cells with the desired traits[64].

1.6 Identification

When understanding and developing products utilizing microalgal biomass and considering the European Union standards and regulations for its use in biotechnological applications, the first step is to characterize and know the taxonomical classification of the alga in use.
Microalgae can be divided into eight different phylogenetic clades, namely Cyanophyta (cyanobacteria), Chlorophyta, Bacillariophyta, Eustigmatophyta, Chrysophyta, Dinophyta, and Xanthophyta, Cryptophyta and Haptophyta [65]. The taxonomical classifications of these organisms have long been performed by morphological characters and behavioural traits [66].

However, with the development of molecular identification techniques, microalgae have also been identified via sequencing of specific genomic regions, in particular the 18S or 28S ribosomal DNA (rDNA) genes. These genomic loci are universally distributed among eukaryotic taxa, having similar sizes and often reflecting common evolutionary histories [67]. However, the latter genes are often not enough to resolve all taxa. Thus, the sequencing of the internal transcribed spacers (ITS) [68] have become increasingly necessary as means to identify isolates down to the species level. Therefore, for_the analysis of DNA sequences, multiple pipelines and models have been developed [69].

1.7 *Chlorella vulgaris*

The species *Chlorella vulgaris* has the following classification: Domain: Eukaryota; Division: Archaeplastida; Sub-division: Chlorophyta, Class: Trebouxiophyceae, Order: Chlorellales, Family: Chlorellaceae, Genus: Chlorella, Species: *Chlorella vulgaris* [70,71]. The genus has been present on Earth for millions of years. This microalga has been commercialized as food for more than 40 years and has been studied for its high protein and lipid production since the 1950s [72].

C. vulgaris has been reported as a microalga capable of growing in freshwater, although it can be used in wastewater treatment with simultaneous CO_2 capture. Moreover, it has been proposed that its biomass can be used in biomedical, cosmetic, feed and food supplement applications [70]. It is often able to outcompete contaminants and grow in harsh environments [73] from wastewater [74] to heavy metal contaminated waters [75]. More recently, several studies reported on the use of *Chlorella* microalgae as potential live feed using wastewater for their production [76]. This strategy could allow nutrient recovery from treated water [77] with a concomitant removal of ammonia and CO_2 fixation [74]. The produced biomass could later be used as a high-value source of lipids [25] and proteins [78]. The biomass harvesting of this alga has also been studied to decrease the biomass production costs [79].

This alga can be photoautotrophic because of its ability to use light as an energy source and fixate CO_2 [80]. However, it is also able to grow heterotrophically using organic carbon sources for its growth and energy production [29]. In addition, it can be cultivated mixotrophically due to its capacity to adapt itself to each growth condition, such as the addition of acetate to its growth medium [81].

1.8 *Tetraselmis chui*

Tetralsemis chui is also a green alga but diverges from *C. vulgaris* on its Class: Chlorodendrophyceae; Order: Chlorodendrales; Family: Chlorodendraceae; Genus: *Tetraselmis*, Species: *Tetraselmis chui*. The genus *Tetraselmis* was first registered in 1986 and separated from the subgenus *Parviselmis*, originating the species *T. striata; T. levis, T. suecica, T. alacris* and *T. chui*. These species have been frequently studied for their high contents of lipids, proteins and fatty acids in their cell [82] as well as for their high resistance to environmental stress [64,83].

T. chui [84] is a species that has been studied for commercialization of its biomass as feed for molluscs, shrimps, rotifers and *Artemia nauplii* because of its balanced nutritional value in terms of protein, lipids, carbohydrates, essential fatty acids and sterols as well as their positive impact on the immune system of the feeders [82]. It has been approved as a commercial product for the European Union since 2017 (Reg. EU, 2017/2470) and has been commercialized in the EU since 2014 [85,86].

The latest studies about *T. chui* have emphasized the quality of its biomass and its health-promoting properties as a novel and powerful food source. In addition, a very recent study described the generation of the first tools to study the molecular biology of this organism [87]. Being a feedstock for production and commercialization, like *C. vulgaris, T. chui* is also a valuable target for strain improvement.

2 Aim of Thesis

In light of the strict policy on food products containing organisms with modifications with foreign, non-homologous DNA, the ProFuture consortium decided that an adequate methodology to obtain the strains with improved traits was by means of random mutagenesis. Thus, this thesis results from the partnership between the Marbiotech laboratory from the University of Algarve - FCT and collaborative laboratory GreenCoLab for the development and production of novel, non-GMO mutant strains with higher productivity in terms of protein content and lower chlorophyll levels. For this purpose, industrially relevant *Chlorella vulgaris* and *Tetraselmis chui* strains were selected to be further improved by chemically induced random mutagenesis. The most promising strain(s) will be chosen to be scaled up by Allmicroalgae in order to develop novel food products with higher commercial value due to improved nutritional contents and organoleptic properties of the biomass.

References

1. Morocho-Jácome, A.L.; Ruscinc, N.; Martinez, R.M.; de Carvalho, J.C.M.; Santos de Almeida, T.; Rosado, C.; Costa, J.G.; Velasco, M.V.R.; Baby, A.R. (Bio)Technological Aspects of Microalgae Pigments for Cosmetics. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 9513–9522, doi:10.1007/s00253-020-10936-x.
2. Pereira, H.; Barreira, L.; Custódio, L.; Alrokayan, S.; Mouffouk, F.; Varela, J.; Abu-Salah, K.; Ben-Hamadou, R. Isolation and Fatty Acid Profile of Selected Microalgae Strains from the Red Sea for Biofuel Production. *Energies* **2013**, *6*, 2773–2783, doi:10.3390/en6062773.
3. Aizawa, K.; Miyachi, S. Carbonic Anhydrase and CO_2 Concentrating Mechanisms in Microalgae and Cyanobacteria. *FEMS Microbiol. Lett.* **1986**, *39*, 215–233, doi:10.1111/j.1574-6968.1986.tb01860.x.
4. Giordano, M.; Beardall, J.; Raven, J.A. CO_2 CONCENTRATING MECHANISMS IN ALGAE: Mechanisms, Environmental Modulation, and Evolution. *Annu. Rev. Plant Biol.* **2005**, *56*, 99–131, doi:10.1146/annurev.arplant.56.032604.144052.
5. Rigobello-Masini, M.; Aidar, E.; Masini, J.C. Extra and Intracelular Activities of Carbonic Anhydrase of the Marine Microalga *Tetraselmis Gracilis* (Chlorophyta). *Braz. J. Microbiol.* **2003**, *34*, doi:10.1590/S1517-83822003000300017.
6. Fan, J.; Xu, H.; Luo, Y.; Wan, M.; Huang, J.; Wang, W.; Li, Y. Impacts of CO2 Concentration on Growth, Lipid Accumulation, and Carbon-Concentrating-Mechanism-Related Gene Expression in Oleaginous Chlorella. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2451–2462, doi:10.1007/s00253-015-6397-4.
7. Castell, J.P.; Krug, L.C. *Introdução às ciências do mar*; 2015; ISBN 978-85-68539-00-2.
8. Santos, F.M.; Gonçalves, A.L.; Pires, J.C.M. Negative emission technologies. In *Bioenergy with Carbon Capture and Storage*; Elsevier, 2019; pp. 1–13 ISBN 978-0-12-816229-3.
9. Wierzchos, J.; DiRuggiero, J.; Vítek, P.; Artieda, O.; Souza-Egipsy, V.; Škaloud, P.; Tisza, M.; Davila, A.F.; Vílchez, C.; Garbayo, I.; et al. Adaptation Strategies of Endolithic Chlorophototrophs to Survive the Hyperarid and Extreme Solar Radiation Environment of the Atacama Desert. *Front. Microbiol.* **2015**, *6*, doi:10.3389/fmicb.2015.00934.
10. Varela, J.C.; Pereira, H.; Vila, M.; León, R. Production of Carotenoids by Microalgae: Achievements and Challenges. *Photosynth. Res.* **2015**, *125*, 423–436, doi:10.1007/s11120-015-0149-2.
11. Vishnivetskaya Viable Phototrophs: Cyanobacteria and Green Algae from the Permafrost Darkness. In *Life in Ancient Ice*; Princeton University Press, 2016; p. 328 ISBN 978-1-4008-8018-8.
12. Rogers, S.; Shtarkman, Y.; Koçer, Z.; Edgar, R.; Veerapaneni, R.; D’Elia, T. Ecology of Subglacial Lake Vostok (Antarctica), Based on Metagenomic/Metatranscriptomic Analyses of Accretion Ice. *Biology* **2013**, *2*, 629–650, doi:10.3390/biology2020629.
13. Vigani, M.; Parisi, C.; Rodríguez-Cerezo, E.; Barbosa, M.J.; Sijtsma, L.; Ploeg, M.; Enzing, C. Food and Feed Products from Micro-Algae: Market Opportunities and Challenges for the EU. *Trends Food Sci. Technol.* **2015**, *42*, 81–92, doi:10.1016/j.tifs.2014.12.004.
14. Pereira, H.; Custódio, L.; Rodrigues, M.; de Sousa, C.; Oliveira, M.; Barreira, L.; Neng, N.; Nogueira, J.; Alrokayan, S.; Mouffouk, F.; et al. Biological Activities and Chemical Composition of Methanolic Extracts of Selected Autochthonous Microalgae Strains from the Red Sea. *Mar. Drugs* **2015**, *13*, 3531–3549, doi:10.3390/md13063531.

15. Satari, B.; Jaiswal, A.K. Green Fractionation of 2G and 3G Feedstocks for Ethanol Production: Advances, Incentives and Barriers. *Curr. Opin. Food Sci.* **2021**, *37*, 1–9, doi:10.1016/j.cofs.2020.07.004.
16. Gonçalves, A.L. The Use of Microalgae and Cyanobacteria in the Improvement of Agricultural Practices: A Review on Their Biofertilising, Biostimulating and Biopesticide Roles. *Appl. Sci.* **2021**, *11*, 871, doi:10.3390/app11020871.
17. Kumar, A.; Singh, J.S. Microalgal bio-fertilizers. In *Handbook of Microalgae-Based Processes and Products*; Elsevier, 2020; pp. 445–463 ISBN 978-0-12-818536-0.
18. Herrera, A.; D'Imporzano, G.; Acién Fernandez, F.G.; Adani, F. Sustainable Production of Microalgae in Raceways: Nutrients and Water Management as Key Factors Influencing Environmental Impacts. *J. Clean. Prod.* **2020**, 125005, doi:10.1016/j.jclepro.2020.125005.
19. Torres-Tiji, Y.; Fields, F.J.; Mayfield, S.P. Microalgae as a Future Food Source. *Biotechnol. Adv.* **2020**, *41*, 107536, doi:10.1016/j.biotechadv.2020.107536.
20. Lafarga, T.; Acien, G. *Cultured Microalgae for the Food Industry Current and Potential Applications*; 2021; ISBN 978-0-12-821081-9.
21. Smalley, T.; Fields, F.J.; Berndt, A.J.E.; Ostrand, J.T.; Heredia, V.; Mayfield, S.P. Improving Biomass and Lipid Yields of Desmodesmus Armatus and *Chlorella Vulgaris* through Mutagenesis and High-Throughput Screening. *Biomass Bioenergy* **2020**, *142*, 105755, doi:10.1016/j.biombioe.2020.105755.
22. Pierobon, S.C.; Cheng, X.; Graham, P.J.; Nguyen, B.; Karakolis, E.G.; Sinton, D. Emerging Microalgae Technology: A Review. *Sustain. Energy Fuels* **2018**, *2*, 13–38, doi:10.1039/C7SE00236J.
23. Pereira, H.; Barreira, L.; Figueiredo, F.; Custódio, L.; Vizetto-Duarte, C.; Polo, C.; Rešek, E.; Engelen, A.; Varela, J. Polyunsaturated Fatty Acids of Marine Macroalgae: Potential for Nutritional and Pharmaceutical Applications. *Mar. Drugs* **2012**, *10*, 1920–1935, doi:10.3390/md10091920.
24. Yarkent, Ç.; Gürlek, C.; Oncel, S.S. Potential of Microalgal Compounds in Trending Natural Cosmetics: A Review. *Sustain. Chem. Pharm.* **2020**, *17*, 100304, doi:10.1016/j.scp.2020.100304.
25. Koutra, E.; Mastropetros, S.G.; Ali, S.S.; Tsigkou, K.; Kornaros, M. Assessing the Potential of *Chlorella Vulgaris* for Valorization of Liquid Digestates from Agro-Industrial and Municipal Organic Wastes in a Biorefinery Approach. *J. Clean. Prod.* **2021**, *280*, 124352, doi:10.1016/j.jclepro.2020.124352.
26. Muhammad, G.; Alam, M.A.; Mofijur, M.; Jahirul, M.I.; Lv, Y.; Xiong, W.; Ong, H.C.; Xu, J. Modern Developmental Aspects in the Field of Economical Harvesting and Biodiesel Production from Microalgae Biomass. *Renew. Sustain. Energy Rev.* **2021**, *135*, 110209, doi:10.1016/j.rser.2020.110209.
27. Schade, S.; Meier, T. Techno-Economic Assessment of Microalgae Cultivation in a Tubular Photobioreactor for Food in a Humid Continental Climate. *Clean Technol. Environ. Policy* **2021**, *23*, 1475–1492, doi:10.1007/s10098-021-02042-x.
28. Schüler, L.; Greque de Morais, E.; Trovão, M.; Machado, A.; Carvalho, B.; Carneiro, M.; Maia, I.; Soares, M.; Duarte, P.; Barros, A.; et al. Isolation and Characterization of Novel *Chlorella Vulgaris* Mutants With Low Chlorophyll and Improved Protein Contents for Food Applications. *Front. Bioeng. Biotechnol.* **2020**, *8*, 469, doi:10.3389/fbioe.2020.00469.
29. Cai, Y.; Liu, Y.; Liu, T.; Gao, K.; Zhang, Q.; Cao, L.; Wang, Y.; Wu, X.; Zheng, H.; Peng, H.; et al. Heterotrophic Cultivation of *Chlorella Vulgaris* Using Broken Rice Hydrolysate as Carbon Source for Biomass and Pigment Production. *Bioresour. Technol.* **2021**, *323*, 124607, doi:10.1016/j.biortech.2020.124607.

30. Vuppaladadiyam, A.K.; Prinsen, P.; Raheem, A.; Luque, R.; Zhao, M. Microalgae Cultivation and Metabolites Production: A Comprehensive Review. *Biofuels Bioprod. Biorefining* **2018**, *12*, 304–324, doi:10.1002/bbb.1864.
31. Geada, P.; Moreira, C.; Silva, M.; Nunes, R.; Madureira, L.; Rocha, C.M.R.; Pereira, R.N.; Vicente, A.A.; Teixeira, J.A. Algal Proteins: Production Strategies and Nutritional and Functional Properties. *Bioresour. Technol.* **2021**, *332*, 125125, doi:10.1016/j.biortech.2021.125125.
32. Borella, L.; Ortolan, D.; Barbera, E.; Trivellin, N.; Sforza, E. A Multiwavelength Model to Improve Microalgal Productivity and Energetic Conversion in a Red-Blue Light Emitting Diodes (LEDs) Continuous Photobioreactor. *Energy Convers. Manag.* **2021**, *243*, 114330, doi:10.1016/j.enconman.2021.114330.
33. Zimny, T.; Sowa, S.; Tyczewska, A.; Twardowski, T. Certain New Plant Breeding Techniques and Their Marketability in the Context of EU GMO Legislation – Recent Developments. *New Biotechnol.* **2019**, *51*, 49–56, doi:10.1016/j.nbt.2019.02.003.
34. Fu, Y. The Potentials and Challenges of Using Microalgae as an Ingredient to Produce Meat Analogues. **2021**, 13.
35. Michel, F.; Knaapila, A.; Hartmann, C.; Siegrist, M. A Multi-National Comparison of Meat Eaters' Attitudes and Expectations for Burgers Containing Beef, Pea or Algae Protein. *Food Qual. Prefer.* **2021**, *91*, 104195, doi:10.1016/j.foodqual.2021.104195.
36. Sharif, M.; Zafar, M.H.; Aqib, A.I.; Saeed, M.; Farag, M.R.; Alagawany, M. Single Cell Protein: Sources, Mechanism of Production, Nutritional Value and Its Uses in Aquaculture Nutrition. *Aquaculture* **2021**, *531*, 735885, doi:10.1016/j.aquaculture.2020.735885.
37. Yadav, G.; Shanmugam, S.; Sivaramakrishnan, R.; Kumar, D.; Mathimani, T.; Brindhadevi, K.; Pugazhendhi, A.; Rajendran, K. Mechanism and Challenges behind Algae as a Wastewater Treatment Choice for Bioenergy Production and Beyond. *Fuel* **2021**, *285*, 119093, doi:10.1016/j.fuel.2020.119093.
38. Ullmann, J.; Grimm, D. Algae and Their Potential for a Future Bioeconomy, Landless Food Production, and the Socio-Economic Impact of an Algae Industry. *Org. Agric.* **2021**, *11*, 261–267, doi:10.1007/s13165-020-00337-9.
39. Kim, M.; Ahn, J.; Jeon, H.; Jin, E. Development of a *Dunaliella Tertiolecta* Strain with Increased Zeaxanthin Content Using Random Mutagenesis. *Mar. Drugs* **2017**, *15*, 189, doi:10.3390/md15060189.
40. Schüler, L.M.; Bombo, G.; Duarte, P.; Santos, T.F.; Maia, I.B.; Pinheiro, F.; Marques, J.; Jacinto, R.; Schulze, P.S.C.; Pereira, H.; et al. 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. *Bioresour. Technol.* **2021**, *337*, 125385, doi:10.1016/j.biortech.2021.125385.
41. Burczyk, J.; Hesse, M. The Ultrastructure of the Outer Cell Wall-Layer Of *Chlorella Mutants* with and without Sporopollenin. *Plant Syst. Evol.* **1981**, *138*, 121–137, doi:10.1007/BF00984613.
42. Irmer, U.; Heuer, K.; Weber, A. Effects of Various Organic Chemicals on the Regreening of Red Colored *Chlorella Zofingiensis*. *Ecotoxicol. Environ. Saf.* **1985**, *9*, 121–133, doi:10.1016/0147-6513(85)90043-0.
43. Claes, H. Analyse Der Biochemischen Synthesekette Für Carotinoide Mit Hilfe von Chlorella-Mutanten. *Z. Für Naturforschung B* **1954**, *9*, 461–469, doi:10.1515/znb-1954-0705.
44. Czygan, F.-C. Effect of Chloramphenicol on Mutants of *Chlorella Pyrenoidosa*. *Nature* **1966**, *212*, 960–960, doi:10.1038/212960a0.

45. Burczyk, J.; Czygan, F.-C. Ocurrence of Carotenoids and Sporopollenin in the Cell Wall of *Chlorella Fusca* and of Its Mutants. *Z. Für Pflanzenphysiol.* **1983**, *111*, 169–174, doi:10.1016/S0044-328X(83)80042-7.
46. Gavrilă, L.; Mihăescu, G. Mutagenesis in Blue-Green Algae (Cyano-Bacteria) and Some Evolutionary Considerations. *Caryologia* **1982**, *35*, 11–22, doi:10.1080/00087114.1982.10796918.
47. Meireles, L.A.; Guedes, A.C.; Malcata, F.X. Increase of the Yields of Eicosapentaenoic and Docosahexaenoic Acids by the MicroalgaPavlova Lutheri Following Random Mutagenesis. *Biotechnol. Bioeng.* **2003**, *81*, 50–55, doi:10.1002/bit.10451.
48. Fu, W.; Chaiboonchoe, A.; Khraiwesh, B.; Nelson, D.; Al-Khairy, D.; Mystikou, A.; Alzahmi, A.; Salehi-Ashtiani, K. Algal Cell Factories: Approaches, Applications, and Potentials. *Mar. Drugs* **2016**, *14*, 225, doi:10.3390/md14120225.
49. Sandesh Kamath, B.; Vidhyavathi, R.; Sarada, R.; Ravishankar, G.A. Enhancement of Carotenoids by Mutation and Stress Induced Carotenogenic Genes in *Haematococcus Pluvialis* Mutants. *Bioresour. Technol.* **2008**, *99*, 8667–8673, doi:10.1016/j.biortech.2008.04.013.
50. Huang, W.; Lin, Y.; He, M.; Gong, Y.; Huang, J. Induced High-Yield Production of Zeaxanthin, Lutein, and β-Carotene by a Mutant of *Chlorella Zofingiensis*. *J. Agric. Food Chem.* **2018**, *66*, 891–897, doi:10.1021/acs.jafc.7b05400.
51. Teco-Bravo, J.I.; Barahona-Pérez, L.F.; Peraza-Echeverria, S.; Baas-Espínola, F.M.; Reyes-Sosa, C.F.; Herrera-Valencia, V.A. Lipid Profiles of Acid-Tolerant Mutants of the Green Microalga *Chlorella Saccharophila* Reveal Hydrocarbons and High-Value Lipids with Potential Industrial Applications. *Bioresour. Technol. Rep.* **2021**, *13*, 100636, doi:10.1016/j.biteb.2021.100636.
52. Guardini, Z.; Dall'Osto, L.; Barera, S.; Jaberi, M.; Cazzaniga, S.; Vitulo, N.; Bassi, R. High Carotenoid Mutants of *Chlorella Vulgaris* Show Enhanced Biomass Yield under High Irradiance. *Plants* **2021**, *10*, 911, doi:10.3390/plants10050911.
53. Liang, Y.; Sarkany, N.; Cui, Y. Biomass and Lipid Productivities of *Chlorella Vulgaris* under Autotrophic, Heterotrophic and Mixotrophic Growth Conditions. *Biotechnol. Lett.* **2009**, *31*, 1043–1049, doi:10.1007/s10529-009-9975-7.
54. Tanadul, O.; Noochanong, W.; Jirakranwong, P.; Chanprame, S. EMS-Induced Mutation Followed by Quizalofop-Screening Increased Lipid Productivity in *Chlorella* Sp. *Bioprocess Biosyst. Eng.* **2018**, *41*, 613–619, doi:10.1007/s00449-018-1896-1.
55. Kumar, G.; Shekh, A.; Jakhu, S.; Sharma, Y.; Kapoor, R.; Sharma, T.R. Bioengineering of Microalgae: Recent Advances, Perspectives, and Regulatory Challenges for Industrial Application. *Front. Bioeng. Biotechnol.* **2020**, *8*, 914, doi:10.3389/fbioe.2020.00914.
56. Xie, F.; Zhang, F.; Zhou, K.; Zhao, Q.; Sun, H.; Wang, S.; Zhao, Y.; Fu, J. Breeding of High Protein *Chlorella Sorokiniana* Using Protoplast Fusion. *Bioresour. Technol.* **2020**, *313*, 123624, doi:10.1016/j.biortech.2020.123624.
57. Dubey, K.K.; Pramanik, A.; Ankush; Khushboo; Yadav, J. Enzyme Engineering. In *Advances in Enzyme Technology*; Elsevier, 2019; pp. 325–347 ISBN 978-0-444-64114-4.
58. Rachmayati, R.; Agustriana, E.; Rahman, D.Y. UV Mutagenesis as a Strategy to Enhance Growth and Lipid Productivity of *Chlorella* Sp. 042. *J. Trop. Biodivers. Biotechnol.* **2020**, *5*, 218, doi:10.22146/jtbb.56862.
59. Chaturvedi, R.; Uppalapati, S.R.; Alamsjah, M.A.; Fujita, Y. Isolation of Quizalofop-Resistant Mutants of *Nannochloropsis Oculata* (Eustigmatophyceae) with High Eicosapentaenoic Acid Following N-Methyl-N-Nitrosourea-Induced Random Mutagenesis. *J. Appl. Phycol.* **2004**, *16*, 135–144, doi:10.1023/B:JAPH.0000044826.70360.8e.

60. Sega, G.A. A Review of the Genetic Effects of Ethyl Methanesulfonate. *Mutat. Res. Genet. Toxicol.* **1984**, *134*, 113–142, doi:10.1016/0165-1110(84)90007-1.
61. Loveless, A. Increased Rate of Plaque-Type and Host-Range Mutation Following Treatment of Bacteriophage in Vitro with Ethyl Methane Sulphonate. *Nature* **1958**, *181*, 1212–1213, doi:10.1038/1811212a0.
62. Amini, M. Ethyl Methanesulfonate. In *Encyclopedia of Toxicology*; Elsevier, 2014; pp. 522–524 ISBN 978-0-12-386455-0.
63. Kawaroe, M.; Sudrajat, A.; Hwangbo, J.; Augustine, D. Chemical Mutagenesis of Microalgae *Nannochloropsis* Sp. Using EMS (Ethyl Methanesulfonate). *Br. J. Appl. Sci. Technol.* **2015**, *8*, 494–505, doi:10.9734/BJAST/2015/16862.
64. Pereira, H.; Gangadhar, K.N.; Schulze, P.S.C.; Santos, T.; de Sousa, C.B.; Schueler, L.M.; Custódio, L.; Malcata, F.X.; Gouveia, L.; Varela, J.C.S.; et al. Isolation of a Euryhaline Microalgal Strain, *Tetraselmis* Sp. CTP4, as a Robust Feedstock for Biodiesel Production. *Sci. Rep.* **2016**, *6*, doi:10.1038/srep35663.
65. Fawley, M.W.; Fawley, K.P. Identification of Eukaryotic Microalgal Strains. *J. Appl. Phycol.* **2020**, *32*, 2699–2709, doi:10.1007/s10811-020-02190-5.
66. Moestrup, Ø. Algal Taxonomy: Historical Overview. In *Encyclopedia of Life Sciences*; John Wiley & Sons, Ltd, Ed.; John Wiley & Sons, Ltd: Chichester, UK, 2006; p. a0000328.pub2 ISBN 978-0-470-01617-6.
67. Khaw, Y.S.; Khong, N.M.H.; Shaharuddin, N.A.; Yusoff, F.Md. A Simple 18S RDNA Approach for the Identification of Cultured Eukaryotic Microalgae with an Emphasis on Primers. *J. Microbiol. Methods* **2020**, *172*, 105890, doi:10.1016/j.mimet.2020.105890.
68. Tran, D.; Louime, C.; Vo ,T.; Giordano, M.; Portilla, S. Identification of Dunaliella ViridisUsing ITS Markers. *Int. J. Appl. Sci. Technol.* **2013**, *3*.
69. Dereeper, A.; Guignon, V.; Blanc, G.; Audic, S.; Buffet, S.; Chevenet, F.; Dufayard, J.-F.; Guindon, S.; Lefort, V.; Lescot, M.; et al. Phylogeny.Fr: Robust Phylogenetic Analysis for the Non-Specialist. *Nucleic Acids Res.* **2008**, *36*, W465–W469, doi:10.1093/nar/gkn180.
70. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Morphology, Composition, Production, Processing and Applications of *Chlorella Vulgaris*: A Review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278, doi:10.1016/j.rser.2014.04.007.
71. Keeling, P.J. The Number, Speed, and Impact of Plastid Endosymbioses in Eukaryotic Evolution. *Annu. Rev. Plant Biol.* **2013**, *64*, 583–607, doi:10.1146/annurev-arplant-050312-120144.
72. Burlew, J.S.; Carnegie Institution of Washington *Algal Culture: From Laboratory to Pilot Plant / Edited by John S. Burlew.*; Carnegie Institution of Washington: Washington, D. C., 1976; ISBN 978-0-87279-611-9.
73. Přibyl, P.; Cepák, V.; Zachleder, V. Production of Lipids and Formation and Mobilization of Lipid Bodies in *Chlorella Vulgaris*. *J. Appl. Phycol.* **2013**, *25*, 545–553, doi:10.1007/s10811-012-9889-y.
74. Kong, W.; Kong, J.; Ma, J.; Lyu, H.; Feng, S.; Wang, Z.; Yuan, P.; Shen, B. *Chlorella Vulgaris* Cultivation in Simulated Wastewater for the Biomass Production, Nutrients Removal and CO2 Fixation Simultaneously. *J. Environ. Manage.* **2021**, *284*, 112070, doi:10.1016/j.jenvman.2021.112070.
75. Bajguz, A. An Enhancing Effect of Exogenous Brassinolide on the Growth and Antioxidant Activity in *Chlorella Vulgaris* Cultures under Heavy Metals Stress. *Environ. Exp. Bot.* **2010**, *68*, 175–179, doi:10.1016/j.envexpbot.2009.11.003.

76. Mtaki, K.; Kyewalyanga, M.S.; Mtolera, M.S.P. Supplementing Wastewater with NPK Fertilizer as a Cheap Source of Nutrients in Cultivating Live Food (*Chlorella Vulgaris*). *Ann. Microbiol.* **2021**, *71*, 7, doi:10.1186/s13213-020-01618-0.
77. Huo, S.; Necas, D.; Zhu, F.; Chen, D.; An, J.; Zhou, N.; Liu, W.; Wang, L.; Cheng, Y.; Liu, Y.; et al. Anaerobic Digestion Wastewater Decolorization by H2O2-Enhanced Electro-Fenton Coagulation Following Nutrients Recovery via Acid Tolerant and Protein-Rich Chlorella Production. *Chem. Eng. J.* **2021**, *406*, 127160, doi:10.1016/j.cej.2020.127160.
78. Soto-Sierra, L.; Kulkarni, S.; Woodard, S.L.; Nikolov, Z.L. Processing of Permeabilized *Chlorella Vulgaris* Biomass into Lutein and Protein-Rich Products. *J. Appl. Phycol.* **2020**, *32*, 1697–1707, doi:10.1007/s10811-020-02055-x.
79. Japar, A.S.; Takriff, M.S.; Mohd Yasin, N.H.; Mahmod, S.S. Optimization of Chlorella Biomass Harvesting by Flocculation and Its Potential for Biofuel Production. *J. Appl. Phycol.* **2021**, doi:10.1007/s10811-021-02393-4.
80. Battistuzzi, F.U.; Feijao, A.; Hedges, S.B. A Genomic Timescale of Prokaryote Evolution: Insights into the Origin of Methanogenesis, Phototrophy, and the Colonization of Land. *BMC Evol. Biol.* **2004**, *4*, 44, doi:10.1186/1471-2148-4-44.
81. Morais, K.C.C.; Conceição, D.; Vargas, J.V.C.; Mitchell, D.A.; Mariano, A.B.; Ordonez, J.C.; Galli-Terasawa, L.V.; Kava, V.M. Enhanced Microalgae Biomass and Lipid Output for Increased Biodiesel Productivity. *Renew. Energy* **2021**, *163*, 138–145, doi:10.1016/j.renene.2020.08.082.
82. Meseck, S.L.; Alix, J.H.; Wikfors, G.H. Photoperiod and Light Intensity Effects on Growth and Utilization of Nutrients by the Aquaculture Feed Microalga, *Tetraselmis Chui* (PLY429). *Aquaculture* **2005**, *246*, 393–404, doi:10.1016/j.aquaculture.2005.02.034.
83. Fabregas, J.; Abalde, J.; Herrero, C.; Cabezas, B.; Veiga, M. Growth of the Marine Microalga *Tetraselmis Suecica* in Batch Cultures with Different Salinities and Nutrient Concentrations. *Aquaculture* **1984**, *42*, 207–215, doi:10.1016/0044-8486(84)90101-7.
84. Butcher, R.W. *An Introductory Account of the Smaller Algae of British Coastal Waters. PART I: Introduction and Chlorophyceae*; IV; Fisheries Investigation.; London, 1959; Vol. I;
85. Mantecón, L.; Moyano, R.; Cameán, A.M.; Jos, A. Safety Assessment of a Lyophilized Biomass of *Tetraselmis Chuii* (TetraSOD®) in a 90 Day Feeding Study. *Food Chem. Toxicol.* **2019**, *133*, 110810, doi:10.1016/j.fct.2019.110810.
86. EFSA Panel on Biological Hazards (BIOHAZ); Koutsoumanis, K.; Allende, A.; Alvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Hilbert, F.; et al. Update of the List of QPS-recommended Biological Agents Intentionally Added to Food or Feed as Notified to EFSA 11: Suitability of Taxonomic Units Notified to EFSA until September 2019. *EFSA J.* **2020**, *18*, doi:10.2903/j.efsa.2020.5965.
87. Torres, S.; Lama, C.; Mantecón, L.; Flemetakis, E.; Infante, C. Selection and Validation of Reference Genes for Quantitative Real-Time PCR in the Green Microalgae *Tetraselmis Chui*. *PLOS ONE* **2021**, *16*, e0245495, doi:10.1371/journal.pone.0245495.

[1]Master thesis

Isolation of two novel *Chlorella vulgaris* and *Tetraselmis chui* mutants with improved protein contents and pigments for food applications

Gabriel Bombo [1,2]

[1] Marine Biotechnology Group, Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal;
[2] GreenColab - Associação Oceano Verde, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal; e-mail@e-mail.com
* Correspondence: gabrielbombo@greencolab.com; Tel.: +351910705203

Abstract: Microalgae are unicellular organisms capable of living in the most diverse environments, being able to grow faster than other photoautotrophs in non-conventional growth media that do not require either potable water or arable land. Organisms with such characteristics can become an important alternative food source for the future since the world population is estimated to reach 10 billion people before 2050. Microalgae are already produced and consumed as high-quality nutritional food, but there is an urgent need to improve existing microalgae-based products since their organoleptic properties, such as their “grassy” taste, are not consensually accepted by the consumer. The present work aims to create new strains from microalgal species already registered as novel food, namely *Chlorella vulgaris* and *Tetraselmis chui,* to improve the quality of the produced biomass and the overall consumer acceptance. For this purpose, random chemical mutagenesis using the alkylating agent ethyl methane sulphonate (EMS) was carried out to generate mutants with higher protein contents and/or lower amounts of chlorophyll. Afterwards, the best performing strains were selected using visual scoring regarding pigmentation and flow cytometry techniques. Upon the implementation of this selection pipeline, two *C. vulgaris* strains were isolated, namely the C3 strain, a non-mutagenized isolate able to grow significantly faster on solid medium as compared to the wildtype (WT) culture and a second a mutant (GL3) obtained from the C3 strain. Interestingly, although the C3 strain presented higher protein contents, the GL3 displayed vestigial chlorophyll contents, lower carotenoid levels, and higher protein content than the WT. The GL3 strain grown under heterotrophic conditions reached higher cell concentrations as compared to the WT, strongly suggesting that the mutant strain GL3 might become a relevant source of protein, being suitable to be produced on a larger scale to generate food products with enhanced organoleptic properties.

Keywords: Mutagenesis; Novel-food; Microalgae; protein; *Chlorella vulgaris*; *Tetraselmis chui.*

1. Introduction

Microalgae are a diverse group of microscopic organisms that can be unicellular, colonial, or even multicellular, comprising mainly photosynthetic eukaryotes and cyanobacteria [1]. These organisms consume CO_2, nutrients, and water, producing O_2 and biomass by using sunlight energy. They are essential for the primary production in the biosphere, having an excellent mechanism for carbon capture [2]. These organisms vary in morphology and size, the latter ranging between 2000 µm and less than 2 µm, with most species displaying a cell size between 200 and 20 µm [7]. During evolution, microalgae colonized almost all kinds of environments, from fresh-, brackish- and seawater to terrestrial habitats as dry as the Atacama desert [8,9].

This diversity of environments where this group of organisms can thrive is related to its high biological and biochemical diversity that includes the biosynthesis of a wide array of metabolites, including lipids, proteins, carbohydrates, and pigments. As these metabolites are often used as nutrients by other organisms, including humans, microalgae are considered to be a promising source of food and feed [13].

The need for producing sustainable food stocks is a common subject in the economic forecasts for 2050 (EU,2019), and microalgae are, in many ways, products of great quality and interest when addressing sustainability and food. Microalgae display a higher growth rate than crop plants and can be cultivated on ponds and bioreactors placed non-arable land, using sea- or wastewater as sources of nutrients. However, microalgal production needs to be further developed [55], because currently only 20 products from 10 different species are considered as novel food (EU, 2017/2470), despite the enormous microalgal biodiversity present in the oceans and other water bodies. As a result, the scientific community has made a strong effort to broaden the spectrum of useful microalgae for the commercial industry through bioprospection campaigns and strain improvement [20].

Microalgal production for food products can be carried out photoautotrophically in closed systems such as tubular photobioreactors (PBR) [27] or heterotrophically in fermenters [28]. Although growth in PBRs is achieved by using a cheaper carbon source (CO_2), microalgae attain lower growth rates and biomass concentrations than cultures grown in fermenters. In the latter case, maximum biomass concentrations can be enhanced 3-fold as compared to those of PBR-grown autotrophic cultures [29]. However, currently used microalgal strains need to be further optimized, as their biochemical/nutritional content and their organoleptic traits are not optimal regarding consumer acceptance [19]. The improvement can come from novel photobioreactor engineering to augment light availability and nutrient consumption [32] or, in alternative, selection of novel microalgal strains and/or growth conditions able to sustain better yields with a higher quality biomass in terms of human nutrition (e.g., higher protein content) and/or organoleptic properties (e.g., taste) [33]. Taking into count that the current capacity for producing food will certainly not be enough for so many people and consumers in western countries have changed their consumption behaviour towards a more vegetable-rich diet without animal protein [34], there is a need to identify new protein sources and meat substitutes. Thus, protein-rich microalgae might qualify as high-quality feedstocks with enhanced environmental sustainability [35].

Two specific factors will enable the use of algal protein by the final consumer, which are: i) the quality of the product in terms of protein content and other health-linked compounds such as polyunsaturated fatty acids, lipids and pigments [38]; and ii) organoleptic properties such as the odour and taste of the biomass. In general, microalgae tend to have relatively low to average protein contents, a strong green colour, a characteristic “fishy” smell and a “grassy” taste [35]. For food consumption, biomass with a more neutral appearance has, in general, a stronger appeal. Moreover, increased protein contents in the biomass are properties highly sought after by the food industry in order to create better, more nutritious products for consumption. Some of these trends are already seen in the algae-based food market with the introduction of “yellow chlorella”, “white chlorella”, and “blue spirulina” product lines produced by companies such as Allmicroalgae. A few of these new products were obtained by obtaining mutants using random mutagenesis, a well-known method that usually requires a non-specific chemical (e.g., ethyl methanesulfonate [40) or radiation (e.g., ultraviolet radiation [58]) to randomly target the DNA. The advantage of using this type of method to generate mutants is that it does not require non-homologous DNA to be inserted in the target genome. Thus, according to the decision 111/18 of the Court of Justice of the EU, organisms mutagenized conventionally do not fall under the EU GMO directive.

The EMS mutagenesis methodology has been used in different studies, including mutant generation in microalgae. Species such as *Dunaliella tertiolecta* have been mutagenized for a better yield of pigments like zeaxanthin, leading to concentrations of up to 1.2 mg/L [39]. The same methodology was used to increase growth rates by 69 to 75% in the lipid overproducing mutant as compared to the wild type (WT) *Chlorella* strain. This led to a 100 and 50% enhancement of the biomass and lipids produced, respectively, in the mutant strain [54]. To further increase the likelihood of finding mutants with the desired traits, herbicides such as norflurazon and nicotine can be used to inhibit specific metabolic pathways by affecting enzymes or genes coding for proteins involved in the biosynthesis of target metabolites as, for example, carotenoids [28]. Another important factor for mutant selection, is the ease of detection of phenotypic changes. With pigments, a simple visual colourimetric analysis is often enough. However, as many of them produce fluorescence upon excitation, fluorescence activated cell sorting (FACS) can also be used to target cells rich in compounds that have specific colours or are autofluorescent. Other selection procedures might use microscopy techniques such as confocal Raman [55] or the use of specific dyes (e.g., BODIPY and Nile red) to identify cells with the desired traits [64] when the target compounds cannot be scored visually or by autofluorescence.

From the biodiversity of algae that have been recognized as novel food sources by the EU, two species were targeted for random mutagenesis in this work, namely *Chlorella vulgaris* and *Tetraselmis chui*. *C. vulgaris* is a chlorophyte that has been on the market for more than 40 years, and is highly regarded for its protein and lipid contents [72]. The second targeted species was *Tetraselmis chui* [84], which is a species that has been commercialized as feed for molluscs, shrimps, rotifers and *Artemia nauplii* due to its protein, lipid, carbohydrate and essential fatty acids profile [82]. More recently, this microalgal species was approved as “novel food” by the European Union in 2017 (Reg. EU, 2017/2470) even though it has been commercialized in the EU since 2014 [85,86].

Considering the strict policy on food products containing organisms with modifications with foreign, non-homologous DNA, the ProFuture consortium decided that an adequate methodology to achieve the desired strains was by means of random mutagenesis. Therefore, here is described the development and production of novel mutant strains with higher protein contents and/or lower chlorophyll contents to improve the colour and decrease the “grassy” taste of the original WT strains.

2. Materials and Methods

The microalgal strains used for this project were kindly provided by the Allmicroalgae’s production unit, Pataias, Portugal. The axenic cultures of *Chlorella vulgaris* 440 and *Tetraselmis chui* 439 were then added to the GreenCoLab algal collection. The experimental work was carried out in the MarBiotech group located at the Centre of Marine Sciences, University of Algarve, Portugal. The work was divided into five steps: 1) growth performance; 2) ethyl methanesulfonate (EMS) mutagenesis; 3) colony screening; 4) identification; and 5) mutant biochemical characterization.

2.1 Growth performance

2.1.1 Culture conditions

To establish a pre-culture, the microalgal strains were transferred from PCA plates to 250 mL Erlenmeyer flasks containing PCB media enriched with 1 mL/L modified Algal medium MAM [64] as described in Table 1. For all *T. chui* cultures, filtered, sterile seawater with a salinity of 20 ppt was used to dilute the concentrated growth medium, whereas cultures of *C. vulgaris* were diluted with distilled water. Lab-scale growth was performed in triplicate under continuous illumination (60 µmol/s^1.m^2) in 50 mL Erlenmeyer flasks, with sample collection every 24 h until the senescence phase. WT and mutant cells were grown at 30 °C in the dark, using an orbital shaker set to 140 rpm.

2.1.2 Culture measurement

Culture growth was followed by correlating cell concentration, optical density and dry weight. Upon calibration, optical density was used to estimate cell concentration daily until the stationary phase was reached [88].

2.1.3 Cell concentration (CC)

CC was obtained by cell counting using a Motic BA310 LED Digital (Motic, Kowloon Bay, Kowloon) microscope in a Neubauer standard chamber utilizing a 10 µL algal suspension. The counts were performed in the 1 x 1 mm side squares, and the number of cells per mL was calculated using the following formula:

$$\text{CC} = \text{mean number of counted cells} \times 10^4 \times \text{dilution factor}. \quad (1)$$

2.1.4 Optical density (OD)

OD was measured using 96-wells plates filled with 200 µL of algal suspension in triplicate and read in a BioTek Synergy 4 spectrophotometer (BioTek Instruments, Inc., headquartered in Winooski, VT, USA) at a wavelength of 750 nm. OD for measuring growth of cells under heterotrophy was carried out at a wavelength of 600 nm.

2.1.5 Dry weight (DW)

DW was determined in triplicates using 1-mL samples filtered by vacuum pump to pre-weighed (Ti) 0.5-µm pore glass fibre filters, followed by washing with 1 mL of 31.5 g/L ammonium sulfate to remove salts when needed. The filters were then dried in an oven for 24 hours at 60 °C until constant weight (Tf) the dry biomass together with Ti. The value of the Tf minus the Ti divided by the volume of filtered sample is equal to the dry weight biomass per litre of algae culture (DW). The DW was calculated as follows:

$$DW(g/L) = \frac{\text{Tf(g)}-\text{Ti(g)}}{\text{Volume (L)}}. \quad (2)$$

For heterotrophy experiments, dry weight was estimated by a correlation of OD and DW obtained previously.

Table 1. Media composition used throughout the workflow. PCA media was used for solid heterotrophic and mixotrophic cultures, the PCB media was used for liquid heterotrophic and mixotrophic cultures and the MAM media was used for the photoautotrophic and mixotrophic solid and liquid cultures.

Media	**Reagent**	**Concentration**
PCA	Water	Solvent
	Tryptone	5 g/L
	Yeast Extract	2.5 g/L
	Glucose	1 g/L
	Agar	15 g/L
	Obs.:	pH regulated to 7 ± 0.02
PCB	Water	Solvent
	Tryptone	5 g/L
	Yeast Extract	2.5 g/L
	Glucose	1 g/L
	Obs.:	pH regulated to 7 ± 0.02
Modified Algal media (MAM)	$ZnCl_2$ ()	0.1 mM
	$ZnSO_4H_2O$ (0,1mM)	0.10 mM

based on the original Algal medium by Fabregas et al. (1984)	$MnCl_2.4H_2O$ (0,1mM)	0.10 mM
	$Na_2MoO_4.2\ H_2O$ (0,01mM)	0.01 mM
	$CoCl_2.6H_2O$ (0,01mM)	0.01 mM
	$CuSO_4.5H_2O$ (0,01mM)	0.01 mM
	EDTA-Na (2,64mM)	2.64 mM
	$MgSO_4.7H_2O$ (0,2mM)	0.20 mM
	$FeCl_3$ (2mM)	2.00 mM
	$NaNO_3$ (2M)	2.00 M
	KH_2PO_4 (100mM)	100.00 mM

2.2 EMS mutagenesis

2.2.1 Culture concentration

For the killing curve and mutagenesis protocols, the algae culture was used at a high cellular concentration ($1x10^7$ – $1x10^8$) to increase the chances of exposure to the mutagen and thus obtain the desired response to the mutagenic events. Therefore, cultures with a lower cellular concentration were concentrated 10 times by centrifugation at 3000 × *g* for 5 min. Furthermore, the culture used was always at the exponential phase for a faster fixation of the generated mutation upon replication.

2.2.2 EMS killing curve

The killing curve for the EMS was to identify the mutagenizing agent concentration, which would yield a 90 – 95% killing of the wildtype cells. Cultures of *C. vulgaris* and *T. chui* were aliquoted to six tubes containing 1 mL of MAM, and to each EMS was added (0, 100, 150, 200, 250, 300 mM) [28]. The cultures were incubated in the dark for 1 h under mild agitation (140 rpm) to avoid photo-induced DNA reparation. Afterwards, EMS was inactivated by the addition of 1 mL 0.4 M sodium thiosulfate; the cells were pelleted at 300 × *g* for 3 minutes, and fresh PCB medium was added. This washing procedure was repeated twice, followed by incubation in the dark for 16 to 24 hours under mild agitation (140 rpm). For each concentration of the killing curve, serial culture dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were performed and 100 µL were spread onto PCA or MAM-Agar plates for *C. vulgaris* and *T. chui*, respectively. Cell viability was estimated by counting the number of colony forming units (CFU).

2.2.3 Herbicide growth tests

To find out the minimum lethal concentration of the two herbicides norflurazon (Figure 1) and nicotine (Figure 2) for *C. vulgaris* and *T. chui* wildtype strains (WT), herbicide growth tests were performed in 24-well plates containing solid media, PCA, in the dark, or MAM, in the light (60 µmol/s^1.m^2). Different concentrations of herbicides, namely norflurazon (0, 5, 10, 15, 20, 30 and 35 µM) and nicotine (0, 2,4, 6, 7, 8, 10 and 12 mM) were used.

The plates were inoculated with 10 µL of concentrated algal suspensions and incubated at a light intensity of 60 µmol/m^2 s^1 until growth became apparent in the control wells.

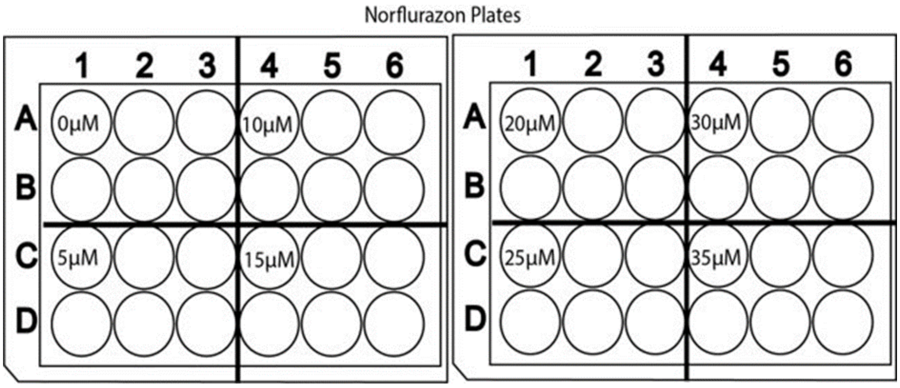

Figure 1. Representation of norflurazon growth test on 24-well plates with the respective concentration of each group of six wells.

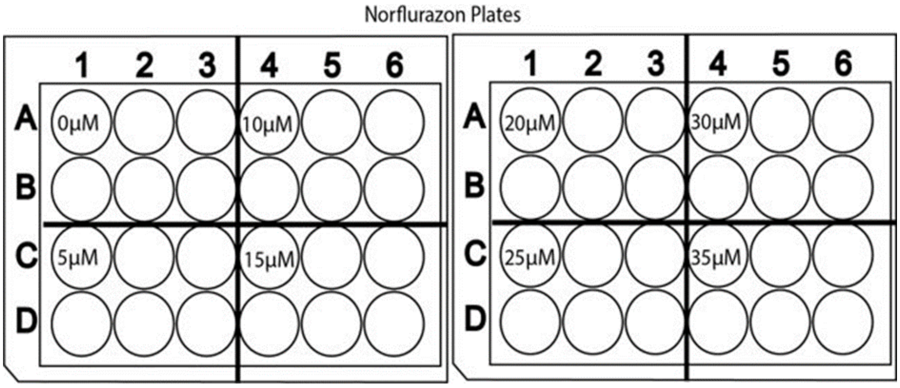

Figure 2. Representation of the nicotine growth test on 24-well plates with the respective concentration of each group of six wells.

2.3 Colony Screening

2.3.1 Mutant generation

For the generation of mutants, exponentially growing *C. vulgaris* and *T. chui* cells were treated with 250 mM of EMS and, upon the overnight culture recovery, different screening procedures were applied. Cells were plated onto: 1) PCA followed by incubation in the dark (heterotrophic); 2) PCA + MAM followed by incubation at a light intensity of 60 µmol/s^1.m^2 (mixotrophic), 3) onto MAM + Agar followed by incubation at a light intensity of 60 µmol/s^1.m^2 (autotrophic) and 4) MAM + Agar containing nicotine (2 mM for *T. chui* and 6.5 mM for *C. vulgaris*) or norflurazon (2.5 µM for *T. chui* and 25 µM for *C. vulgaris*) and incubated at a light intensity of 60 µmol/s^1.m^2. Mutants were then selected by fluorescence activated cell sorting (FACS).

2.3.2 FACS selection

The selection of mutants with desired properties was carried in a FACS Becton Dickinson FACS Aria II (BD Biosciences, Erembodegem, Belgium) equipped with a blue and red laser (488 and 633 nm, respectively) and FACSDiva (version 6.1.3) software. Three channels to record the fluorescence signal, P1, P2, and P3 centred at 670/30, 530/30 and 610/20 nm, respectively, were used. Cells emitting higher levels of fluorescence in P1 (chlorophyll *a*), higher levels of fluorescence in P2 (carotenoids) and lower levels of fluorescence in P3 (phycoerythrin-like fluorescence) were sorted onto 96-well plates with solid media containing herbicides with different concentrations for *C. vulgaris* (6.5 mM nicotine and 15 µM norflurazon) and *T. chui* (2 mM nicotine and 2.5 µM norflurazon) (Figure 3).

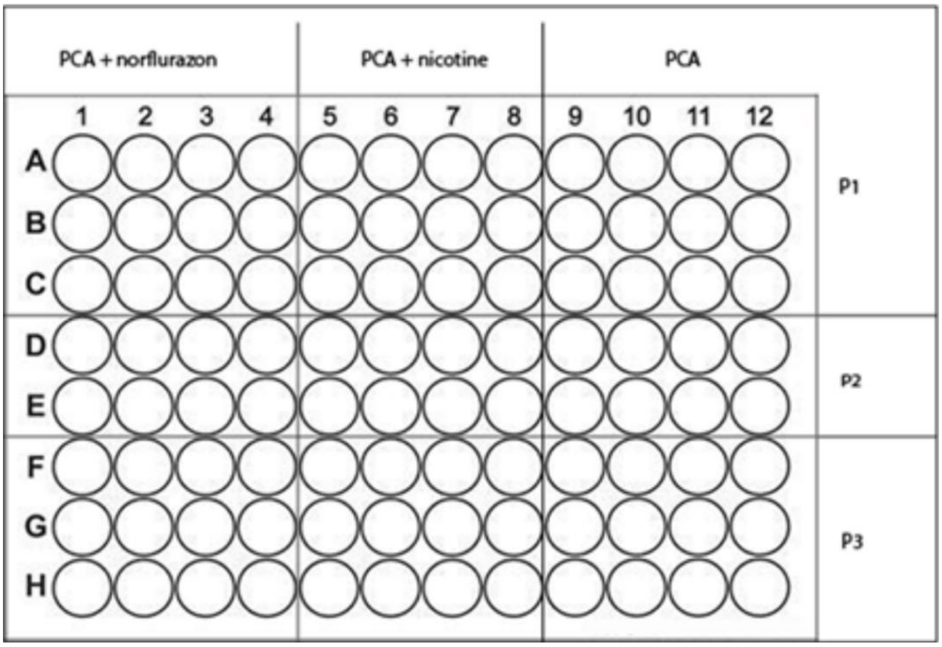

Figure 3. Representation of the 96 well plates used to sort cells in the FACS.

2.3.3 Colour Screening

The mutant colonies displaying less greenish or yellow hues were picked up and streaked on PCA plates several times before starting the liquid batch culture to ensure the stability of the mutant. Upon at least 5 serial streaking events, the mutant was cultivated in PCB (culture in the dark) or MAM (culture in the light). A diagram representing typical colonies growing on a plate upon EMS mutagenesis is shown in Fig. 4.

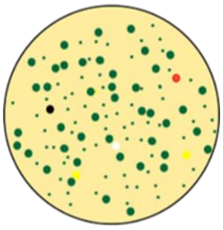

Figure 4. Representation of the expected results of the random mutagenesis protocol using EMS.

2.4 Molecular Identification

DNA was extracted with the EZNA DNA plant extraction kit (Omega Bio-Tek, Norcross, GA). To this end, biomass was harvested by centrifugation at 21,500 × *g*, 10 °C for 5 minutes, and then the extraction was performed in accordance with the guidelines from the manufacturer's extended protocol. The 18S rDNA was amplified from the extracted genomic DNA using the Promega GoTaq G2 flexi DNA Polymerase master mix on Table 2 together with the primers 18SUnivFor (5'–ACCTGGTTGATCCTGCCAGT–3') and 18SUnivRev (5'–TCAGCCTTGCGACCATAC–3') [2,64,89]. The PCR amplification was done using a Thermo Cycler (Applied Biosystems 2720 Thermo Cycler, California, USA) with the initial conditions of 5 min 94 °C followed by 35 cycles of 0.5 min 95 °C, 0.5 min at 55 °C and 0.75 min at 72 °C, and final extension of 10 min at 72 °C. PCR products were stored at 10 °C until further analysis (Groff and Moon, 1993). Sequencing of the products was carried out by Sanger DNA sequencing at an in-house facility equipped with an Applied Biosystems 3130XL DNA Sequencer (Life Technologies BV, Porto, Portugal).

The bioinformatics was carried out using the software packages Finch TV, CLC Genomics Workbench, NCBI BLAST tool, with the bioinformatic pipeline as follows: Sequence alignment by CLC Genomics Workbench, followed by Gblocks alignment curation [90] and maximum likelihood PhyML phylogenetic inference [91] using the approximate likelihood-ratio test to estimate branch support values [92]. Finally, phylogenetic trees were rendered by TreeDyn [93]. The proposed pipeline was accessed via the website located at phylogeny.fr [69]. The distance-based neighbour joining inference software, BioNJ [94], was used to test the congruence of the PhyML-generated trees.

Table 2. List of reagents used for the master mixture and PCR amplification. The volumes of the reagents are given for one sample.

Ingredients	**Concentration**
5x colorless GoTaq Flexi buffer	5 X
MgCl2 solution	25 mM
Primer 18SUnivF	10 µM
Primer 18SUnivR	10 µM
dNTPs (10mM)	10 mM
GoTaq G2 Flexi DNA polymerase	0.2x

2.5 Mutant characterization

2.5.1 Cultivation

Growth performance was done according to section 2.1. The strains used for this experiment were the respective wildtype and stable mutants of each species. Every 24 h, OD read at a wavelength of 750 nm, cell counting, and dry weight of the biomass were determined. By the end of the growth curve experiment, biomass was harvested by centrifugation at 3000 × *g* for 5 min and stored at -20 °C until further analysis.

2.5.2 Carotenoids profile

Carotenoid extraction of 10 mg of lyophilised biomass was performed by mechanical means using glass beads (425 – 600 nm) and methanol using the Mixer Mill MM400 (Retsch, Germany) at 30 Hz for 2 min. The supernatant was collected by centrifugation at 10 °C 21,500 × *g* for 3 min. The extraction procedure was repeated until the pellet and supernatant became completely colourless [95]. The extracts were dried under a gentle flow of nitrogen and resuspended in 1 mL of methanol.

The extracts were used for spectrophotometric analysis of total chlorophyll and carotenoids. To this end, the absorbance was read at three different wavelengths 662, 645 and 470 nm, from the specific absorbance ratios were used (fix numbers on the equations) based on the solvent used. For the estimation of the concentration of chlorophyll *a* (Chl a) and *b* (Chl b) and total carotenoids (TCar) in µg.mL^{-1}, the following equations were used:

$$\text{Chla} = 11.75\ \text{A662} - 2.35\ \text{A645}; \tag{3}$$

$$\text{Chlb} = 18.61\ \text{A645} - 3.96\ \text{A662}; \tag{4}$$

$$\text{TCar} = 1000\ \text{A470} - 2.27\ \text{Chla} - 81.4\ \text{Chlb}/227. \tag{5}$$

where A*n* is the absorbance at the wavelength *n* in nm [96].

2.5.3 Protein content

The determination of the protein content was done by means of CHN Elemental Analysis through total nitrogen measurement in a Vario EL III (Elementar Analysensysteme GmbH, Germany). One milligram of lyophilized biomass was encapsulated in aluminium caps for reading. It was calculated the crude protein content by the macro-Kjedal method, multiplying the nitrogen content percentage on the biomass by the coefficient 6.25 [97].

3. Results

3.1 Chlorella vulgaris

Wild type (WT) *C. vulgari*s was grown in solid and liquid media in the dark to allow the microalgal cells to adapt to the growth conditions required for random mutagenesis. This strain displayed a deep green colour. Molecular identification confirmed that the strain used was a bona fide *C. vulgaris* strain (data not shown).

After cultures were submitted to serial dilutions, different populations were observed that must have evolved from spontaneous mutations, as seen in Figure 5. Therefore, larger colony forming units or colonies with a lighter green colour were found growing on the plates (Figure 6). These larger colonies, which apparently corresponded to isolates with higher growth rates, and possibly higher protein productivities, were isolated. Growth of the mutant C3 on plate was compared with that of the WT (Figure 7). Interestingly, as can be seen by the streaking experiment, strain C3 showed faster growth than the WT after 7 days of incubation under heterotrophic conditions.

The *C. vulgaris* C3 strain was subjected to random mutagenesis for further improvement. A killing curve was not possible to establish due to the fact that the WT strain, when cells were highly diluted, failed to grow on plate growth media, except for a few colonies that were able to spontaneously grow under such conditions (Figure 5). In contrast, fast growth was observed in concentrated cultures, suggesting that a significant crowding effect is needed for the WT strain to grow effectively on solid media. However, as the C3 mutant was recovered as a fast-growing isolate from plate, it was possible to achieve a killing curve of the tested concentrations, even though it generated a large standard error at the lowest concentrations of EMS (Figure 8).

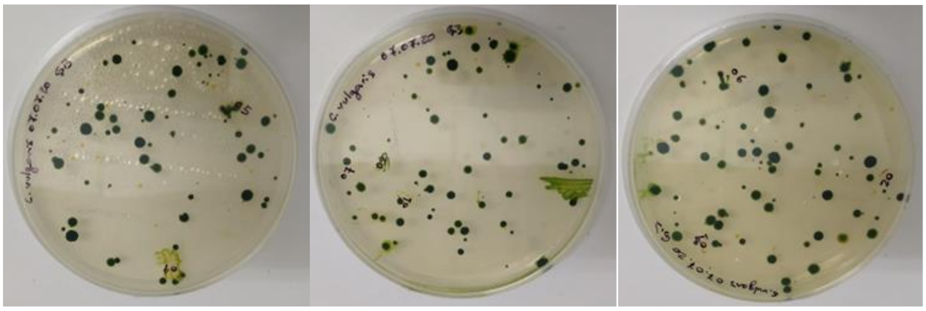

Figure 5. Plating of a concentrated wildtype (WT) *C. vulgaris* culture, which resulted in colonies of different sizes (07 of July of 2020 to day 27 of July of 2020) and various shades of green.

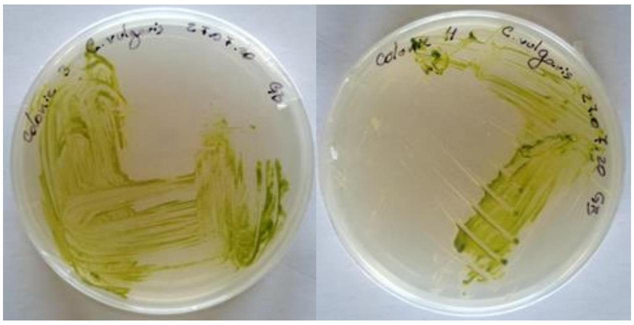

Figure 6. Isolated colonies from the WT strain of *C. vulgaris,* cultivated on PCA and isolated for their yellowish-green colour.

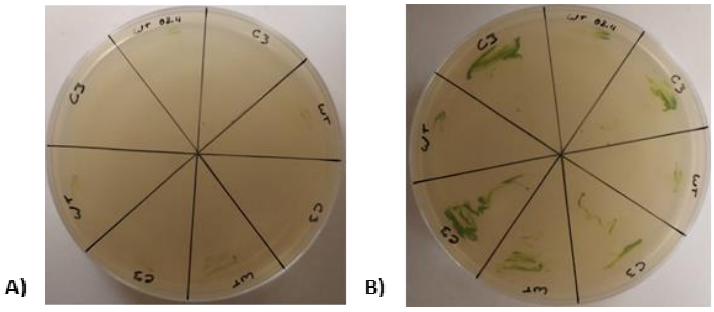

Figure 7. Growth comparison between the WT and C3 strains; the incubation period was 7 days at 25°C in the dark. A) Plate on day 1 of the incubation period with little to no apparent streaks with growing cells. B) The same plate on day 7 showing a significant difference in growth between the WT and C3 cells, where the latter strain appears to grow faster.

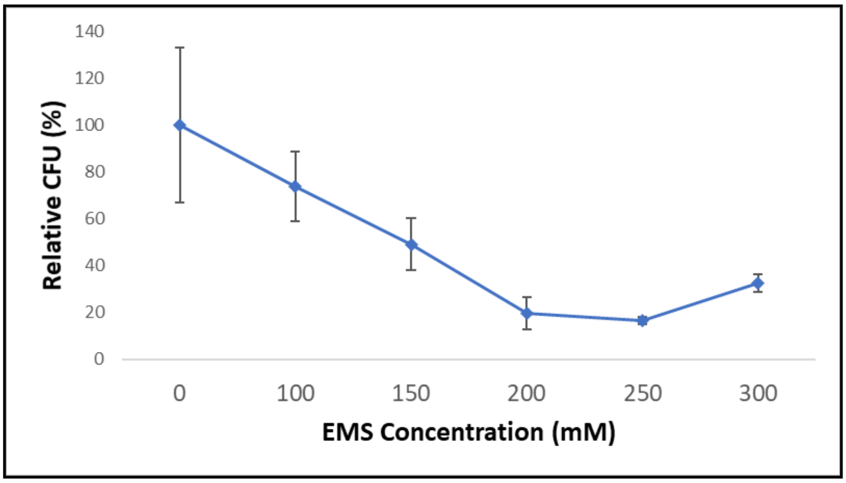

Figure 8. Killing curve for *Chlorella vulgaris* C3 growing in dark and 25ºC on PCA solid media.

Despite these aforementioned technical constraints, the minimal lethal concentration for norflurazon and nicotine of 15µM and 6mM, respectively, was determined to optimize the isolation of WT and C3 mutants in terms of chlorophyll and/or protein contents, as the former parameter seems to influence the latter [28]. Moreover, upon mutagenesis using 250 mM of EMS, several yellow colonies appeared on the plates. These colonies were isolated and then streaked onto agar plates, resulting in the isolation of one yellow mutant (GL3) of *C. vulgaris*. The yellow GL3 strain, together with the WT and C3 strains, were scaled up and grown in the dark for further analysis (Figure 9). FACS sorting of the C3 strain did not result in new isolates; however, it is possible to observe in the FACS event plots that different cell subpopulations occur, suggesting that cultures of this mutant were heterogeneous (Figure 10). Upon phylogenetic analysis, the 18S ribosomal DNA sequences of the two mutants clustered together with that of the WT, ensuring that the isolates were bona fide *C. vulgaris* strains rather than a contaminant microalga.

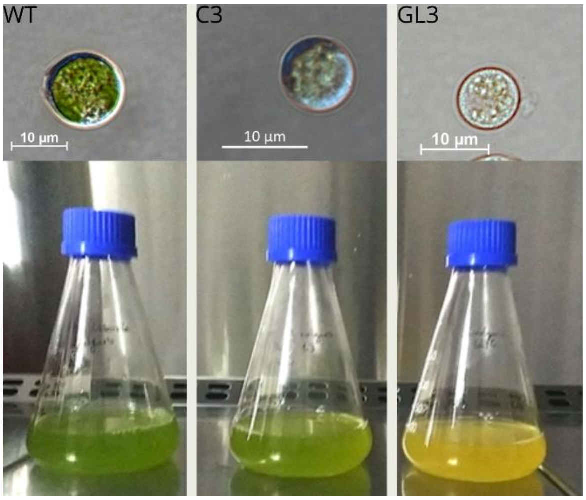

Figure 9. Liquid cultures of *Chlorella vulgaris* strains WT, C3 and GL3, where it is possible to perceive the different colours of the respective cultures resulting from the mutations (bottom panel). This phenotypic change is also visible upon microscopic observation, where the decrease in chlorophyll content between the WT and GL3 strains is striking.

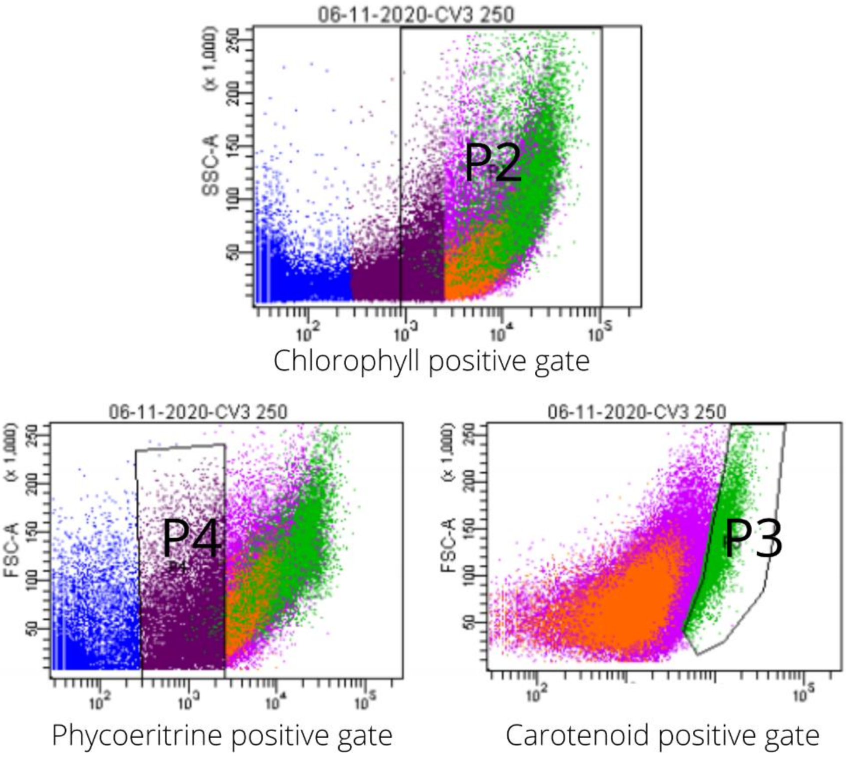

Figure 10. Fluorescence gates for screening *Chlorella vulgaris* C3 mutant strains. The events in the gates P2 refer to a 670/30nm passband where higher fluorescence levels on the *x*-axis are linked to the presence of chlorophyll *a*; P3, a 530/30nm passband, where the measured carotenoid autofluorescence is shown on the *x*-axis; and P4, a 610/20 passband, where phycoerythrin autofluorescence levels are indicated on the *x*-axis. FSC-A and SSC-A are, in general and

respectively, linked to the size and complexity of the cells passing through the flow cytometer interrogation point.

When growth between the *C. vulgaris* WT and the GL3 strains was compared, at the peak of growth, i.e., on day 5, the GL3 cells showed an average cell concentration (CC) of $4.37 \times 10^8 \pm 1.10 \times 10^7$ cells/mL of culture. Conversely, the WT only showed an average CC of $3.57 \times 10^8 \pm 5.70 \times 10^7$ cells/mL, suggesting an apparent 22% enhancement of this parameter in the mutant strain ($p < 0.05$).

Mutants were biochemically analysed concerning protein and pigment contents (Table 3). These parameters were compared with those of the WT strain. As expected by their differential phenotypes, the concentration of pigments in the mutants were lower than those of the WT cells. Overall, chlorophyll levels in the GL3 mutant decreased 20-fold as compared by WT levels, whereas they remained virtually unchanged in the C3 strain. Conversely, total carotenoid contents of C3 and GL3 decreased by half as compared to those of the WT cells, although the specific carotenoid affected by the mutant genotype was strain-specific. Between the WT and C3, it is possible to observe a 2-fold decrease in neoxanthin and violaxanthin levels in the mutant. Although lutein and *β*-carotene levels also decreased in C3 cells, the change appears to have been less significant. However, the same cannot be said when comparing C3 and GL3 cells, where a very significant suppression of *β*-carotene contents is evident in the GL3 strain. This suppression was accompanied with a further drop in the neoxanthin and lutein levels when comparing the contents of these carotenoids in GL3, WT, and C3 cells. Taken together, these results suggest that the phenotypic changes observed in the GL3 cells were mainly due to a dramatic decrease in the chlorophyll levels and that the observed yellow colour is mainly due to lutein rather than a combination of this xanthophyll with *β*-carotene. Interestingly, the mutant strains, either C3 or GL3, showed also a slight but significant increase ($p<0.05$) in protein concentration as compared to the WT.

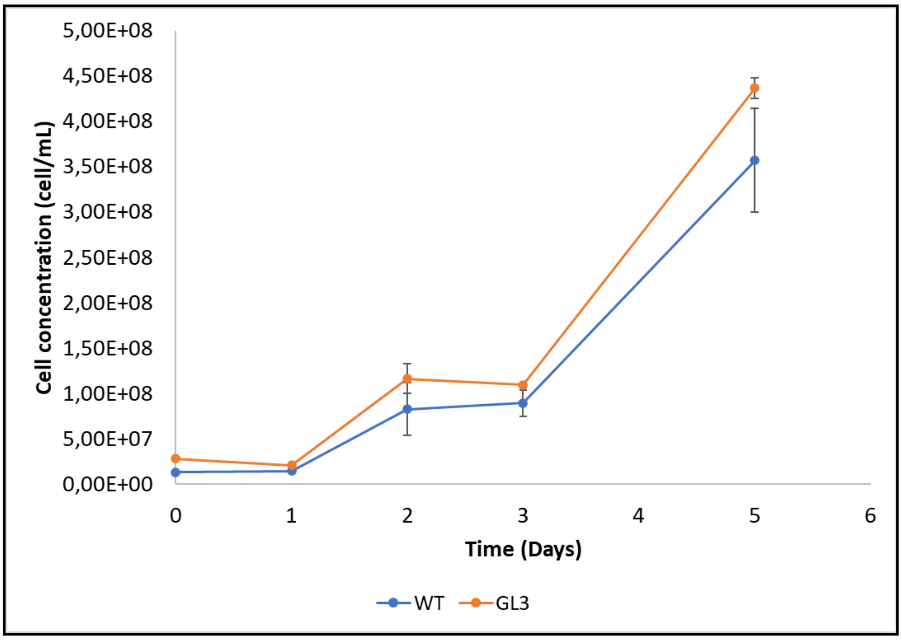

Figure 11. Growth of *C. vulgaris* WT and the *C. vulgaris* GL3 strain, which was derived from the C3 strain. The growth is presented in cell counts of a culture under heterotrophic conditions, which was essential for the maintaining and growing the latter strain (*n* = 9).

Table 3. Protein and pigment composition in the WT, C3 and GL3 strains. The results were analysed upon growth in heterotrophic conditions utilizing 200 mL PCB media on 500-mL baffled Erlenmeyers for 5 days ($n = 9$).

Composition	WT	C3	GL3
Protein (%)	35.99 ± 0.76	43.15 ± 2.23	40.47 ± 0.05
Chlorophyll *a* (g/100g)	0.578 ± 0.07	0.568 ± 0.17	0.028 ± 0.00
Chlorophyll *b* (g/100g)	0.272 ± 0.06	0.351 ± 0.11	0.021 ± 0.00
Chlorophyll *a,b* (g/100g)	0.850 ± 0.13	0.919 ± 0.28	0.049 ± 0.01
Total carotenoids (g/100g)	0.167 ± 0.00	0.083 ± 0.00	0.081 ± 0.00
Neoxanthin (g/100g)	0.034 ± 0.007	0.017 ± 0.002	0.000
Violaxanthin	0.012 ± 0.003	0.006 ± 0.006	0.004 ± 0.001
Lutein (g/100g)	0.171 ± 0.029	0.145 ± 0.008	0.096 ± 0.038
Zeaxanthin (g/100g)	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
***β*-carotene (g/100g)**	0.097 ± 0.033	0.089 ± 0.011	0.009 ± 0.003

3.2 *Tetraselmis chui*

To determine the conditions needed for mutagenizing *T. chui*, a killing curve was established using different concentrations of EMS. At a concentration of 250 mM, a survival of only 0.9% of WT cells was observed (Figure 12). In growth assays on plates containing different herbicides, a minimal lethal concentration for norflurazon and nicotine could not be found, as the concentrations to inoculate the control, i.e., *T. chui* WT cells, showed no consistent response (data not shown).

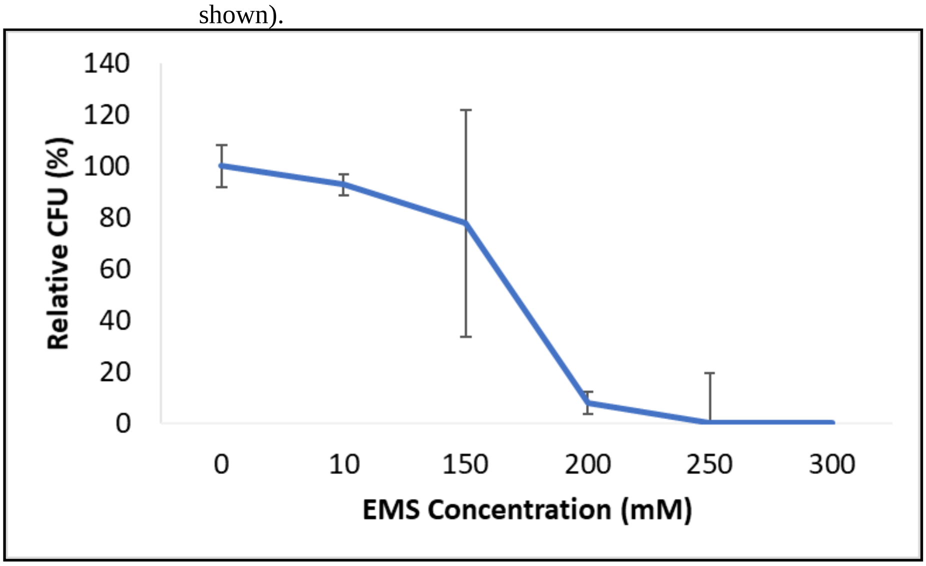

Figure 12. Killing curve for WT *Tetraselmis chui* growing in dark and 25ºC on PCA solid media.

Mutagenesis of this strain was performed several times and at different light intensities, using a diverse array of carbon sources during the selection procedures. However, it was not possible to make this strain grow in the dark nor generate yellow or other mutants with differential pigmentation. Moreover, the FACS selection only resulted in one colony, which was kept for further analysis, even though the screening by FACS showed the existence of at least two different populations (Figure 13). However, even this colony failed to grow in the dark.

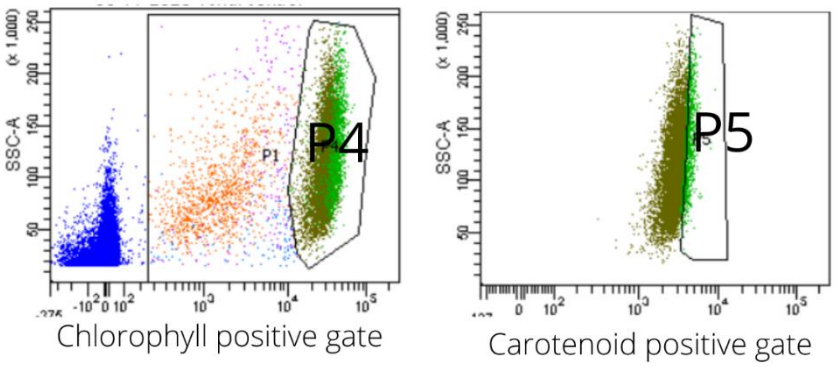

Figure 13. Fluorescence gates to screen for *Tetraselmis chui* chlorophyll and carotenoid mutant strains. The events in the gate P4 referred to a 670/30nm passband, where fluorescence levels on the *x*-axis are linked to chlorophyll contents; the gate P3, using a 530/30nm passband, was used to isolate cells with higher carotenoid levels as measured by the fluorescence on the *x*-axis. FSC-A and SSC-A are, in general and respectively, linked to the size and complexity of the cells passing through the flow cytometer interrogation point.

4. Discussion

One of the earliest observations during the generation of mutants for *C. vulgaris* is that we distinguished different morphological and physiological characteristics within the WT cultures, which resulted in the isolation of the fast-growing C3 strain. For example, a significant growth difference scored visually was observed when the WT and the C3 strains were grown side-by-side on agar plates. As the C3 strain derived from a non-mutagenized batch culture, this suggests that the WT strain was composed of different subpopulations with different abilities to grow on solid media. The C3 strain showed better growth performance than the WT and other strains that were isolated at the same time. Comparing to other *C. vulgaris* strains described in the literature, growth seems to be highly variable, with some mutant and wild type strains being able to achieve the peak of the exponential phase within 50 h [28]. Other strains required 160 hours to reach the same growth phase [98], whereas, in some other cases, cells reached a maximum biomass concentration within 120 hours (≈5 days) [54], as was the case for the industrial WT strain used in this work and its yellow derivative, GL3. The growth differences observed might be caused by the environmental adaptability of each strain, which can influence growth and ease of domestication for industrial use, even if among strains from the same species [99]. Moreover, abiotic conditions such as temperature, nutrients, light source (or its absence) or even the use of an organic carbon source can be leading factors to yield different growth patterns [52]. This seems to be the case for the industrial WT strain used as the starting point for strain improvement in this work, as these *C. vulgaris* cultures were very recalcitrant to growing on solid media if diluted to low cell concentrations. Such results might be expected, as industrial strains are seldom grown under such conditions, unlike strains normally used in fundamental research.

Even though several strains underwent random chemical mutagenesis, including the WT, only the C3 strain yielded a promising mutant not only in terms of growth but also regarding its pigmentation. The main reason for this lower number of mutants obtained was linked to the fact that growth on plates after mutagenesis either yielded full plates on which it was impossible to differentiate one colony from the other or plates with no colonies at all if the cells were diluted in order to have isolated colonies. This odd behaviour from this particular *C. vulgaris* strain strongly suggests that this microalgal WT strain requires a kind of "crowding effect" in order to form colonies on plate. Conversely, the C3 strain, which was isolated as a fast-growing colony from plate, upon dilution down to concentrations of 10^2 to 10^3 cells per mL, was able to form individual colonies that could be counted and differentiated from other colonies. Because of this key improvement, it finally became possible to obtain mutants from the C3 strain, which yielded the yellow strain, *Chlorella vulgaris* GL3. Its yellow phenotype was mainly due to the fact that it contained 20 times less chlorophyll and the presence of lutein, which is known for its yellow colour. Interestingly, this mutant also showed increased protein content, which is also a desired trait for microalgae used for food production. The protein content from microalgae is directly linked to the chloroplast biogenesis and metabolization of CO_2 [4]. In general, the production of protein and chloroplast formation are directly related to the nitrogen content of the media [100] and a diversity of studies have been carried out to better understand the relationship between protein production and chlorophyll contents in microalgal cells [101,102]. A better yield of protein has been reported when there is less chlorophyll in the biomass [64,103], suggesting that the relative protein content may be higher on chlorophyll-deficient mutants or simply in cells with lower amounts of this pigment.

To confirm the stability of the GL3 mutant, these cells were repeatedly streaked on PCA for ten generations, thus confirming that its yellow phenotype was stable. This process is an essential step to ensure that the mutation(s) causing this phenotype did not revert, as there is a strong selective pressure to isolate cells that regain the ability to biosynthesize chlorophyll and re-acquire their typical green pigmentation [104]. Most probably, a mutation in the photosynthetic machinery is the cause for the observed decrease in the contents of pigments, either chlorophyll or carotenoids, in this mutant [105]. Indeed, it has been put forward that this factor might be responsible for the permanent alteration of the colour of microalgal biomass over many generations [28]. This hypothesis seems to be supported by the fact that in the GL3 mutant β-carotene levels were drastically reduced; interestingly, this particular carotenoid is an intrinsic component of the photosystems [40]. However, colour changes in photosynthetic cells can be due to a myriad of reasons—from a block in chloroplast biogenesis to mutations in the biosynthetic pathways that produce the precursors needed for chlorophyll and carotenoid biosynthesis [106]. As GL3 is a relatively new strain, further research is needed to identify which molecular and cellular changes led to generated phenotype.

Concerning *T. chui,* the isolation of mutants displaying a differential pigmentation, such as chlorophyll deficiency, was not observed in this work. A likely explanation for this result is that, unlike *C. vulgaris*, *T. chui* apparently lacks an organic carbon transporter, or some other gene product, which forces this microalga to be fully dependent on photosynthesis to acquire energy as well as the building blocks for its macromolecules [82]. These types of organisms are often called "obligate photoautotrophs". However, a *T. chui* strain has recently been described [107] that apparently is able to support heterotrophic growth. Nevertheless, these authors did not demonstrate unequivocal growth in the dark, as growth was only shown in the presence of light, which suggests that the observed growth was mixotrophic rather than truly heterotrophic, as indicated by the same authors elsewhere [108]. Furthermore, the cells in the micrographs of the heterotrophic culture do not appear to have any similarity with other *T. chui* cells, raising the possibility that the authors were growing a contaminant or at least a misidentified microalgal strain. On the other hand, other *Tetraselmis* species have been described as "facultative heterotrophic organisms", i.e., as being able to survive without photosynthesis, if need be. One example of these is *T. suecica*, where an increase in the omega-3 PUFA EPA was reported when cultivated heterotrophically as compared to cells grown photoautotrophically [109].

The capability of growing heterotrophically as displayed by *T. suecica* and *C. vulgaris* is a necessary factor for the development of chlorophyll-deficient mutants, as that minimizes the selective pressure to correct the mutation in the photosynthetic machinery, including the biosynthetic pathways that produce the necessary molecular components for photosynthesis to take place [104]. In evolutionary terms, heterotrophy is a common trend in microalgae that are often found in environments with lower amounts of light or none at all, as underneath ice sheets [12] or in sediment [110]. Either way, the common factor of these places is the lack of an appropriate condition for photosynthesis to take place constantly, light being absent at some point of the algal life cycle [111], which might explain why *T. suecica* can grow heterotrophically in absence of light while *T. chui* cannot. Moreover, heterotrophy in photosynthetic organisms normally derive from a gain-of-function mutation (e.g., acquirement of a sugar transporter via horizontal gene transfer), which is a significantly more complex, rarer event than a functional loss, such as lower amounts of photosynthetic pigments caused by a mutation in a gene encoding an enzyme involved in a metabolic pathway [112]. *Chlorella vulgaris* GL3 is precisely an example of a loss-of-function mutant, which only became possible as it is able to grow heterotrophically on an organic carbon source such as glucose. Conversely, as *T. chui* seems to be an obligate photoautotroph, any drastic mutations in the photosynthetic apparatus or in the pathways leading to the biosynthesis of pigments might be lethal.

Another interesting observation is that heterotrophy and mixotrophy in microalgae are mostly observed in freshwater organisms. Aside from adaptation to low luminosity environments as discussed above, habitats with higher competition among organisms for scarcer nutrients seem to promote the appearance of microalgae able to find food using mechanisms other than photoautotrophy [113,114]. Even in situations when the availability of organic carbon sources is high, some microalgae species have a tendency to consume the organic carbon source first rather than carry out photosynthesis, since the metabolization is faster, thus providing a competitive advantage [115]. For example, the pathway linked to glycerol catabolism can drastically increase biomass production in *C. vulgaris* [116].

However, as the occurrence of marine microalgae that can be grown heterotrophically is scarce, new strategies to enable these microalgae to be produced in fermenters and increase the overall yield might be necessary in the near future [98,106]. One possibility is the introduction of genes encoding proteins that promote organic carbon intake as is the case of *Phaeodactylum tricornutum* [112]. However, this can be problematic as the generated strains will fall under the EU GMO directive. On the other hand, random mutagenesis of *Chlorella vulgaris* has been considered as a promising strategy for generating novel commercial strains for the production of “White Chlorella” and “Yellow Chlorella”, which already hit the market [117].

Finally, it is worth mentioning that the current interest in non-animal sources of nutrients, including protein, has been rising. This trend is not only due to the perceived notion that additional food resources will be needed to feed the whole human population, but mostly because consumers prefer to have healthier, more sustainable life styles, shifting to vegan diets or at least diets containing “natural products” rather than processed food [118]. Another trend is that the food industry is looking for non-animal sources of protein. As the C3 and GL3 strains showed higher protein contents than the WT, being able to grow heterotrophically in fermenters, it might be possible to produce high quality biomass at lower costs using these novel mutants [29]. There is also the expectation that the GL3 strain will lose the “grassy taste” of the WT biomass, because this organoleptic property is usually linked to chlorophyll [34]. In addition, there is the possibility of using the biomass of these microalgae as meat analogues as suggested previously [35]. Indeed, alternative protein sources of high quality have been used to substitute meat with flavours that meet consumer expectations, as is the case of non-animal protein being included in burgers and extruded meat products [34,119].

5. Conclusions

Even though not all goals of this thesis were achieved, as it was not possible to obtain a mutant strain of *Tetraselmis chui*, one *Chlorella vulgaris* mutant, GL3, with a yellow colour and a low concentration of chlorophyll was isolated. This mutant also displayed higher protein contents than the WT, a trait highly valuable for the food market. Because of its lower chlorophyll contents, it is highly likely that phenotypic changes displayed by GL3 cells will lead to improved organoleptic properties, such a less obvious grassy taste. Moreover, their yellow colour is also preferred by the food industry, as bulk biomass with this hue is often used for certain types of food products, such as soups.

Another conclusion from this work is that the mutagenesis of obligate phototrophic species such as *Tetraselmis chui* is not as simple as for non-obligate photoautotrophic microalgae. However, the task of pursuing mutants of this species with better commercial qualities should not be halted, but novel strategies need to be devised to overcome their inability to grow in the dark and, therefore, in fermenters. Together with the already promising results from *Chlorella vulgaris*, the strains can be further improved with new trials for mutant generation, testing novel stress factors upon EMS treatment to test the inhibition of other metabolic functions for the generation of higher quality strains.

To summarise, the idea of mutagenizing already domesticated species for a better product is valid and has been tested several times over the years, for, sometimes, this process can be long and not so profitable in the early stages but can yield very interesting results with time, effort, and perseverance. Because of the current understanding of what falls under the EU GMO directive, EMS-based mutagenesis is by far the best tool to obtain novel products, benefiting not only producers but also consumers with the possibility of purchasing better supplements for a healthier life and, in the near future, finding a solution for the forthcoming global lack of food and food production limitations.

References

1. Morocho-Jácome, A.L.; Ruscinc, N.; Martinez, R.M.; de Carvalho, J.C.M.; Santos de Almeida, T.; Rosado, C.; Costa, J.G.; Velasco, M.V.R.; Baby, A.R. (Bio)Technological Aspects of Microalgae Pigments for Cosmetics. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 9513–9522, doi:10.1007/s00253-020-10936-x.
2. Pereira, H.; Barreira, L.; Custódio, L.; Alrokayan, S.; Mouffouk, F.; Varela, J.; Abu-Salah, K.; Ben-Hamadou, R. Isolation and Fatty Acid Profile of Selected Microalgae Strains from the Red Sea for Biofuel Production. *Energies* **2013**, *6*, 2773–2783, doi:10.3390/en6062773.
3. Aizawa, K.; Miyachi, S. Carbonic Anhydrase and CO_2 Concentrating Mechanisms in Microalgae and Cyanobacteria. *FEMS Microbiol. Lett.* **1986**, *39*, 215–233, doi:10.1111/j.1574-6968.1986.tb01860.x.
4. Giordano, M.; Beardall, J.; Raven, J.A. CO_2 CONCENTRATING MECHANISMS IN ALGAE: Mechanisms, Environmental Modulation, and Evolution. *Annu. Rev. Plant Biol.* **2005**, *56*, 99–131, doi:10.1146/annurev.arplant.56.032604.144052.
5. Rigobello-Masini, M.; Aidar, E.; Masini, J.C. Extra and Intracelular Activities of Carbonic Anhydrase of the Marine Microalga *Tetraselmis Gracilis* (Chlorophyta). *Braz. J. Microbiol.* **2003**, *34*, doi:10.1590/S1517-83822003000300017.
6. Fan, J.; Xu, H.; Luo, Y.; Wan, M.; Huang, J.; Wang, W.; Li, Y. Impacts of CO2 Concentration on Growth, Lipid Accumulation, and Carbon-Concentrating-Mechanism-Related Gene Expression in Oleaginous Chlorella. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2451–2462, doi:10.1007/s00253-015-6397-4.
7. Castell, J.P.; Krug, L.C. *Introdução às ciências do mar*; 2015; ISBN 978-85-68539-00-2.
8. Santos, F.M.; Gonçalves, A.L.; Pires, J.C.M. Negative emission technologies. In *Bioenergy with Carbon Capture and Storage*; Elsevier, 2019; pp. 1–13 ISBN 978-0-12-816229-3.
9. Wierzchos, J.; DiRuggiero, J.; Vítek, P.; Artieda, O.; Souza-Egipsy, V.; Škaloud, P.; Tisza, M.; Davila, A.F.; Vílchez, C.; Garbayo, I.; et al. Adaptation Strategies of Endolithic Chlorophototrophs to Survive the Hyperarid and Extreme Solar Radiation Environment of the Atacama Desert. *Front. Microbiol.* **2015**, *6*, doi:10.3389/fmicb.2015.00934.
10. Varela, J.C.; Pereira, H.; Vila, M.; León, R. Production of Carotenoids by Microalgae: Achievements and Challenges. *Photosynth. Res.* **2015**, *125*, 423–436, doi:10.1007/s11120-015-0149-2.
11. Vishnivetskaya Viable Phototrophs: Cyanobacteria and Green Algae from the Permafrost Darkness. In *Life in Ancient Ice*; Princeton University Press, 2016; p. 328 ISBN 978-1-4008-8018-8.
12. Rogers, S.; Shtarkman, Y.; Koçer, Z.; Edgar, R.; Veerapaneni, R.; D'Elia, T. Ecology of Subglacial Lake Vostok (Antarctica), Based on Metagenomic/Metatranscriptomic Analyses of Accretion Ice. *Biology* **2013**, *2*, 629–650, doi:10.3390/biology2020629.
13. Vigani, M.; Parisi, C.; Rodríguez-Cerezo, E.; Barbosa, M.J.; Sijtsma, L.; Ploeg, M.; Enzing, C. Food and Feed Products from Micro-Algae: Market Opportunities and Challenges for the EU. *Trends Food Sci. Technol.* **2015**, *42*, 81–92, doi:10.1016/j.tifs.2014.12.004.

14. Pereira, H.; Custódio, L.; Rodrigues, M.; de Sousa, C.; Oliveira, M.; Barreira, L.; Neng, N.; Nogueira, J.; Alrokayan, S.; Mouffouk, F.; et al. Biological Activities and Chemical Composition of Methanolic Extracts of Selected Autochthonous Microalgae Strains from the Red Sea. *Mar. Drugs* **2015**, *13*, 3531–3549, doi:10.3390/md13063531.
15. Satari, B.; Jaiswal, A.K. Green Fractionation of 2G and 3G Feedstocks for Ethanol Production: Advances, Incentives and Barriers. *Curr. Opin. Food Sci.* **2021**, *37*, 1–9, doi:10.1016/j.cofs.2020.07.004.
16. Gonçalves, A.L. The Use of Microalgae and Cyanobacteria in the Improvement of Agricultural Practices: A Review on Their Biofertilising, Biostimulating and Biopesticide Roles. *Appl. Sci.* **2021**, *11*, 871, doi:10.3390/app11020871.
17. Kumar, A.; Singh, J.S. Microalgal bio-fertilizers. In *Handbook of Microalgae-Based Processes and Products*; Elsevier, 2020; pp. 445–463 ISBN 978-0-12-818536-0.
18. Herrera, A.; D'Imporzano, G.; Acién Fernandez, F.G.; Adani, F. Sustainable Production of Microalgae in Raceways: Nutrients and Water Management as Key Factors Influencing Environmental Impacts. *J. Clean. Prod.* **2020**, 125005, doi:10.1016/j.jclepro.2020.125005.
19. Torres-Tiji, Y.; Fields, F.J.; Mayfield, S.P. Microalgae as a Future Food Source. *Biotechnol. Adv.* **2020**, *41*, 107536, doi:10.1016/j.biotechadv.2020.107536.
20. Lafarga, T.; Acien, G. *Cultured Microalgae for the Food Industry Current and Potential Applications*; 2021; ISBN 978-0-12-821081-9.
21. Smalley, T.; Fields, F.J.; Berndt, A.J.E.; Ostrand, J.T.; Heredia, V.; Mayfield, S.P. Improving Biomass and Lipid Yields of Desmodesmus Armatus and *Chlorella Vulgaris* through Mutagenesis and High-Throughput Screening. *Biomass Bioenergy* **2020**, *142*, 105755, doi:10.1016/j.biombioe.2020.105755.
22. Pierobon, S.C.; Cheng, X.; Graham, P.J.; Nguyen, B.; Karakolis, E.G.; Sinton, D. Emerging Microalgae Technology: A Review. *Sustain. Energy Fuels* **2018**, *2*, 13–38, doi:10.1039/C7SE00236J.
23. Pereira, H.; Barreira, L.; Figueiredo, F.; Custódio, L.; Vizetto-Duarte, C.; Polo, C.; Rešek, E.; Engelen, A.; Varela, J. Polyunsaturated Fatty Acids of Marine Macroalgae: Potential for Nutritional and Pharmaceutical Applications. *Mar. Drugs* **2012**, *10*, 1920–1935, doi:10.3390/md10091920.
24. Yarkent, Ç.; Gürlek, C.; Oncel, S.S. Potential of Microalgal Compounds in Trending Natural Cosmetics: A Review. *Sustain. Chem. Pharm.* **2020**, *17*, 100304, doi:10.1016/j.scp.2020.100304.
25. Koutra, E.; Mastropetros, S.G.; Ali, S.S.; Tsigkou, K.; Kornaros, M. Assessing the Potential of *Chlorella Vulgaris* for Valorization of Liquid Digestates from Agro-Industrial and Municipal Organic Wastes in a Biorefinery Approach. *J. Clean. Prod.* **2021**, *280*, 124352, doi:10.1016/j.jclepro.2020.124352.
26. Muhammad, G.; Alam, M.A.; Mofijur, M.; Jahirul, M.I.; Lv, Y.; Xiong, W.; Ong, H.C.; Xu, J. Modern Developmental Aspects in the Field of Economical Harvesting and Biodiesel Production from Microalgae Biomass. *Renew. Sustain. Energy Rev.* **2021**, *135*, 110209, doi:10.1016/j.rser.2020.110209.
27. Schade, S.; Meier, T. Techno-Economic Assessment of Microalgae Cultivation in a Tubular Photobioreactor for Food in a Humid Continental Climate. *Clean Technol. Environ. Policy* **2021**, *23*, 1475–1492, doi:10.1007/s10098-021-02042-x.
28. Schüler, L.; Greque de Morais, E.; Trovão, M.; Machado, A.; Carvalho, B.; Carneiro, M.; Maia, I.; Soares, M.; Duarte, P.; Barros, A.; et al. Isolation and Characterization of Novel *Chlorella Vulgaris* Mutants With Low Chlorophyll and Improved Protein Contents for Food Applications. *Front. Bioeng. Biotechnol.* **2020**, *8*, 469, doi:10.3389/fbioe.2020.00469.
29. Cai, Y.; Liu, Y.; Liu, T.; Gao, K.; Zhang, Q.; Cao, L.; Wang, Y.; Wu, X.; Zheng, H.; Peng, H.; et al. Heterotrophic Cultivation of *Chlorella Vulgaris* Using Broken Rice Hydrolysate as Carbon Source for Biomass and Pigment Production. *Bioresour. Technol.* **2021**, *323*, 124607, doi:10.1016/j.biortech.2020.124607.
30. Vuppaladadiyam, A.K.; Prinsen, P.; Raheem, A.; Luque, R.; Zhao, M. Microalgae Cultivation and Metabolites Production: A Comprehensive Review. *Biofuels Bioprod. Biorefining* **2018**, *12*, 304–324, doi:10.1002/bbb.1864.

31. Geada, P.; Moreira, C.; Silva, M.; Nunes, R.; Madureira, L.; Rocha, C.M.R.; Pereira, R.N.; Vicente, A.A.; Teixeira, J.A. Algal Proteins: Production Strategies and Nutritional and Functional Properties. *Bioresour. Technol.* **2021**, *332*, 125125, doi:10.1016/j.biortech.2021.125125.
32. Borella, L.; Ortolan, D.; Barbera, E.; Trivellin, N.; Sforza, E. A Multiwavelength Model to Improve Microalgal Productivity and Energetic Conversion in a Red-Blue Light Emitting Diodes (LEDs) Continuous Photobioreactor. *Energy Convers. Manag.* **2021**, *243*, 114330, doi:10.1016/j.enconman.2021.114330.
33. Zimny, T.; Sowa, S.; Tyczewska, A.; Twardowski, T. Certain New Plant Breeding Techniques and Their Marketability in the Context of EU GMO Legislation – Recent Developments. *New Biotechnol.* **2019**, *51*, 49–56, doi:10.1016/j.nbt.2019.02.003.
34. Fu, Y. The Potentials and Challenges of Using Microalgae as an Ingredient to Produce Meat Analogues. **2021**, 13.
35. Michel, F.; Knaapila, A.; Hartmann, C.; Siegrist, M. A Multi-National Comparison of Meat Eaters' Attitudes and Expectations for Burgers Containing Beef, Pea or Algae Protein. *Food Qual. Prefer.* **2021**, *91*, 104195, doi:10.1016/j.foodqual.2021.104195.
36. Sharif, M.; Zafar, M.H.; Aqib, A.I.; Saeed, M.; Farag, M.R.; Alagawany, M. Single Cell Protein: Sources, Mechanism of Production, Nutritional Value and Its Uses in Aquaculture Nutrition. *Aquaculture* **2021**, *531*, 735885, doi:10.1016/j.aquaculture.2020.735885.
37. Yadav, G.; Shanmugam, S.; Sivaramakrishnan, R.; Kumar, D.; Mathimani, T.; Brindhadevi, K.; Pugazhendhi, A.; Rajendran, K. Mechanism and Challenges behind Algae as a Wastewater Treatment Choice for Bioenergy Production and Beyond. *Fuel* **2021**, *285*, 119093, doi:10.1016/j.fuel.2020.119093.
38. Ullmann, J.; Grimm, D. Algae and Their Potential for a Future Bioeconomy, Landless Food Production, and the Socio-Economic Impact of an Algae Industry. *Org. Agric.* **2021**, *11*, 261–267, doi:10.1007/s13165-020-00337-9.
39. Kim, M.; Ahn, J.; Jeon, H.; Jin, E. Development of a *Dunaliella Tertiolecta* Strain with Increased Zeaxanthin Content Using Random Mutagenesis. *Mar. Drugs* **2017**, *15*, 189, doi:10.3390/md15060189.
40. Schüler, L.M.; Bombo, G.; Duarte, P.; Santos, T.F.; Maia, I.B.; Pinheiro, F.; Marques, J.; Jacinto, R.; Schulze, P.S.C.; Pereira, H.; et al. 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. *Bioresour. Technol.* **2021**, *337*, 125385, doi:10.1016/j.biortech.2021.125385.
41. Burczyk, J.; Hesse, M. The Ultrastructure of the Outer Cell Wall-Layer Of *Chlorella Mutants* with and without Sporopollenin. *Plant Syst. Evol.* **1981**, *138*, 121–137, doi:10.1007/BF00984613.
42. Irmer, U.; Heuer, K.; Weber, A. Effects of Various Organic Chemicals on the Regreening of Red Colored *Chlorella Zofingiensis*. *Ecotoxicol. Environ. Saf.* **1985**, *9*, 121–133, doi:10.1016/0147-6513(85)90043-0.
43. Claes, H. Analyse Der Biochemischen Synthesekette Für Carotinoide Mit Hilfe von Chlorella-Mutanten. *Z. Für Naturforschung B* **1954**, *9*, 461–469, doi:10.1515/znb-1954-0705.
44. Czygan, F.-C. Effect of Chloramphenicol on Mutants of *Chlorella Pyrenoidosa*. *Nature* **1966**, *212*, 960–960, doi:10.1038/212960a0.
45. Burczyk, J.; Czygan, F.-C. Ocurrence of Carotenoids and Sporopollenin in the Cell Wall of *Chlorella Fusca* and of Its Mutants. *Z. Für Pflanzenphysiol.* **1983**, *111*, 169–174, doi:10.1016/S0044-328X(83)80042-7.
46. Gavrilă, L.; Mihăescu, G. Mutagenesis in Blue-Green Algae (Cyano-Bacteria) and Some Evolutionary Considerations. *Caryologia* **1982**, *35*, 11–22, doi:10.1080/00087114.1982.10796918.
47. Meireles, L.A.; Guedes, A.C.; Malcata, F.X. Increase of the Yields of Eicosapentaenoic and Docosahexaenoic Acids by the MicroalgaPavlova Lutheri Following Random Mutagenesis. *Biotechnol. Bioeng.* **2003**, *81*, 50–55, doi:10.1002/bit.10451.

48. Fu, W.; Chaiboonchoe, A.; Khraiwesh, B.; Nelson, D.; Al-Khairy, D.; Mystikou, A.; Alzahmi, A.; Salehi-Ashtiani, K. Algal Cell Factories: Approaches, Applications, and Potentials. *Mar. Drugs* **2016**, *14*, 225, doi:10.3390/md14120225.
49. Sandesh Kamath, B.; Vidhyavathi, R.; Sarada, R.; Ravishankar, G.A. Enhancement of Carotenoids by Mutation and Stress Induced Carotenogenic Genes in *Haematococcus Pluvialis* Mutants. *Bioresour. Technol.* **2008**, *99*, 8667–8673, doi:10.1016/j.biortech.2008.04.013.
50. Huang, W.; Lin, Y.; He, M.; Gong, Y.; Huang, J. Induced High-Yield Production of Zeaxanthin, Lutein, and β-Carotene by a Mutant of *Chlorella Zofingiensis*. *J. Agric. Food Chem.* **2018**, *66*, 891–897, doi:10.1021/acs.jafc.7b05400.
51. Teco-Bravo, J.I.; Barahona-Pérez, L.F.; Peraza-Echeverria, S.; Baas-Espínola, F.M.; Reyes-Sosa, C.F.; Herrera-Valencia, V.A. Lipid Profiles of Acid-Tolerant Mutants of the Green Microalga *Chlorella Saccharophila* Reveal Hydrocarbons and High-Value Lipids with Potential Industrial Applications. *Bioresour. Technol. Rep.* **2021**, *13*, 100636, doi:10.1016/j.biteb.2021.100636.
52. Guardini, Z.; Dall'Osto, L.; Barera, S.; Jaberi, M.; Cazzaniga, S.; Vitulo, N.; Bassi, R. High Carotenoid Mutants of *Chlorella Vulgaris* Show Enhanced Biomass Yield under High Irradiance. *Plants* **2021**, *10*, 911, doi:10.3390/plants10050911.
53. Liang, Y.; Sarkany, N.; Cui, Y. Biomass and Lipid Productivities of *Chlorella Vulgaris* under Autotrophic, Heterotrophic and Mixotrophic Growth Conditions. *Biotechnol. Lett.* **2009**, *31*, 1043–1049, doi:10.1007/s10529-009-9975-7.
54. Tanadul, O.; Noochanong, W.; Jirakranwong, P.; Chanprame, S. EMS-Induced Mutation Followed by Quizalofop-Screening Increased Lipid Productivity in *Chlorella* Sp. *Bioprocess Biosyst. Eng.* **2018**, *41*, 613–619, doi:10.1007/s00449-018-1896-1.
55. Kumar, G.; Shekh, A.; Jakhu, S.; Sharma, Y.; Kapoor, R.; Sharma, T.R. Bioengineering of Microalgae: Recent Advances, Perspectives, and Regulatory Challenges for Industrial Application. *Front. Bioeng. Biotechnol.* **2020**, *8*, 914, doi:10.3389/fbioe.2020.00914.
56. Xie, F.; Zhang, F.; Zhou, K.; Zhao, Q.; Sun, H.; Wang, S.; Zhao, Y.; Fu, J. Breeding of High Protein *Chlorella Sorokiniana* Using Protoplast Fusion. *Bioresour. Technol.* **2020**, *313*, 123624, doi:10.1016/j.biortech.2020.123624.
57. Dubey, K.K.; Pramanik, A.; Ankush; Khushboo; Yadav, J. Enzyme Engineering. In *Advances in Enzyme Technology*; Elsevier, 2019; pp. 325–347 ISBN 978-0-444-64114-4.
58. Rachmayati, R.; Agustriana, E.; Rahman, D.Y. UV Mutagenesis as a Strategy to Enhance Growth and Lipid Productivity of *Chlorella* Sp. 042. *J. Trop. Biodivers. Biotechnol.* **2020**, *5*, 218, doi:10.22146/jtbb.56862.
59. Chaturvedi, R.; Uppalapati, S.R.; Alamsjah, M.A.; Fujita, Y. Isolation of Quizalofop-Resistant Mutants of *Nannochloropsis Oculata* (Eustigmatophyceae) with High Eicosapentaenoic Acid Following N-Methyl-N-Nitrosourea-Induced Random Mutagenesis. *J. Appl. Phycol.* **2004**, *16*, 135–144, doi:10.1023/B:JAPH.0000044826.70360.8e.
60. Sega, G.A. A Review of the Genetic Effects of Ethyl Methanesulfonate. *Mutat. Res. Genet. Toxicol.* **1984**, *134*, 113–142, doi:10.1016/0165-1110(84)90007-1.
61. Loveless, A. Increased Rate of Plaque-Type and Host-Range Mutation Following Treatment of Bacteriophage in Vitro with Ethyl Methane Sulphonate. *Nature* **1958**, *181*, 1212–1213, doi:10.1038/1811212a0.
62. Amini, M. Ethyl Methanesulfonate. In *Encyclopedia of Toxicology*; Elsevier, 2014; pp. 522–524 ISBN 978-0-12-386455-0.
63. Kawaroe, M.; Sudrajat, A.; Hwangbo, J.; Augustine, D. Chemical Mutagenesis of Microalgae *Nannochloropsis* Sp. Using EMS (Ethyl Methanesulfonate). *Br. J. Appl. Sci. Technol.* **2015**, *8*, 494–505, doi:10.9734/BJAST/2015/16862.
64. Pereira, H.; Gangadhar, K.N.; Schulze, P.S.C.; Santos, T.; de Sousa, C.B.; Schueler, L.M.; Custódio, L.; Malcata, F.X.; Gouveia, L.; Varela, J.C.S.; et al. Isolation of a Euryhaline Microalgal Strain,

Tetraselmis Sp. CTP4, as a Robust Feedstock for Biodiesel Production. *Sci. Rep.* **2016**, *6*, doi:10.1038/srep35663.

65. Fawley, M.W.; Fawley, K.P. Identification of Eukaryotic Microalgal Strains. *J. Appl. Phycol.* **2020**, *32*, 2699–2709, doi:10.1007/s10811-020-02190-5.
66. Moestrup, Ø. Algal Taxonomy: Historical Overview. In *Encyclopedia of Life Sciences*; John Wiley & Sons, Ltd, Ed.; John Wiley & Sons, Ltd: Chichester, UK, 2006; p. a0000328.pub2 ISBN 978-0-470-01617-6.
67. Khaw, Y.S.; Khong, N.M.H.; Shaharuddin, N.A.; Yusoff, F.Md. A Simple 18S RDNA Approach for the Identification of Cultured Eukaryotic Microalgae with an Emphasis on Primers. *J. Microbiol. Methods* **2020**, *172*, 105890, doi:10.1016/j.mimet.2020.105890.
68. Tran, D.; Louime, C.; Vo ,T.; Giordano, M.; Portilla, S. Identification of Dunaliella ViridisUsing ITS Markers. *Int. J. Appl. Sci. Technol.* **2013**, *3*.
69. Dereeper, A.; Guignon, V.; Blanc, G.; Audic, S.; Buffet, S.; Chevenet, F.; Dufayard, J.-F.; Guindon, S.; Lefort, V.; Lescot, M.; et al. Phylogeny.Fr: Robust Phylogenetic Analysis for the Non-Specialist. *Nucleic Acids Res.* **2008**, *36*, W465–W469, doi:10.1093/nar/gkn180.
70. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Morphology, Composition, Production, Processing and Applications of *Chlorella Vulgaris*: A Review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278, doi:10.1016/j.rser.2014.04.007.
71. Keeling, P.J. The Number, Speed, and Impact of Plastid Endosymbioses in Eukaryotic Evolution. *Annu. Rev. Plant Biol.* **2013**, *64*, 583–607, doi:10.1146/annurev-arplant-050312-120144.
72. Burlew, J.S.; Carnegie Institution of Washington *Algal Culture: From Laboratory to Pilot Plant / Edited by John S. Burlew.*; Carnegie Institution of Washington: Washington, D. C., 1976; ISBN 978-0-87279-611-9.
73. Přibyl, P.; Cepák, V.; Zachleder, V. Production of Lipids and Formation and Mobilization of Lipid Bodies in *Chlorella Vulgaris*. *J. Appl. Phycol.* **2013**, *25*, 545–553, doi:10.1007/s10811-012-9889-y.
74. Kong, W.; Kong, J.; Ma, J.; Lyu, H.; Feng, S.; Wang, Z.; Yuan, P.; Shen, B. *Chlorella Vulgaris* Cultivation in Simulated Wastewater for the Biomass Production, Nutrients Removal and CO2 Fixation Simultaneously. *J. Environ. Manage.* **2021**, *284*, 112070, doi:10.1016/j.jenvman.2021.112070.
75. Bajguz, A. An Enhancing Effect of Exogenous Brassinolide on the Growth and Antioxidant Activity in *Chlorella Vulgaris* Cultures under Heavy Metals Stress. *Environ. Exp. Bot.* **2010**, *68*, 175–179, doi:10.1016/j.envexpbot.2009.11.003.
76. Mtaki, K.; Kyewalyanga, M.S.; Mtolera, M.S.P. Supplementing Wastewater with NPK Fertilizer as a Cheap Source of Nutrients in Cultivating Live Food (*Chlorella Vulgaris*). *Ann. Microbiol.* **2021**, *71*, 7, doi:10.1186/s13213-020-01618-0.
77. Huo, S.; Necas, D.; Zhu, F.; Chen, D.; An, J.; Zhou, N.; Liu, W.; Wang, L.; Cheng, Y.; Liu, Y.; et al. Anaerobic Digestion Wastewater Decolorization by H2O2-Enhanced Electro-Fenton Coagulation Following Nutrients Recovery via Acid Tolerant and Protein-Rich Chlorella Production. *Chem. Eng. J.* **2021**, *406*, 127160, doi:10.1016/j.cej.2020.127160.
78. Soto-Sierra, L.; Kulkarni, S.; Woodard, S.L.; Nikolov, Z.L. Processing of Permeabilized *Chlorella Vulgaris* Biomass into Lutein and Protein-Rich Products. *J. Appl. Phycol.* **2020**, *32*, 1697–1707, doi:10.1007/s10811-020-02055-x.
79. Japar, A.S.; Takriff, M.S.; Mohd Yasin, N.H.; Mahmod, S.S. Optimization of Chlorella Biomass Harvesting by Flocculation and Its Potential for Biofuel Production. *J. Appl. Phycol.* **2021**, doi:10.1007/s10811-021-02393-4.
80. Battistuzzi, F.U.; Feijao, A.; Hedges, S.B. A Genomic Timescale of Prokaryote Evolution: Insights into the Origin of Methanogenesis, Phototrophy, and the Colonization of Land. *BMC Evol. Biol.* **2004**, *4*, 44, doi:10.1186/1471-2148-4-44.
81. Morais, K.C.C.; Conceição, D.; Vargas, J.V.C.; Mitchell, D.A.; Mariano, A.B.; Ordonez, J.C.; Galli-Terasawa, L.V.; Kava, V.M. Enhanced Microalgae Biomass and Lipid Output for Increased Biodiesel Productivity. *Renew. Energy* **2021**, *163*, 138–145, doi:10.1016/j.renene.2020.08.082.

82. Meseck, S.L.; Alix, J.H.; Wikfors, G.H. Photoperiod and Light Intensity Effects on Growth and Utilization of Nutrients by the Aquaculture Feed Microalga, *Tetraselmis Chui* (PLY429). *Aquaculture* **2005**, *246*, 393–404, doi:10.1016/j.aquaculture.2005.02.034.
83. Fabregas, J.; Abalde, J.; Herrero, C.; Cabezas, B.; Veiga, M. Growth of the Marine Microalga *Tetraselmis Suecica* in Batch Cultures with Different Salinities and Nutrient Concentrations. *Aquaculture* **1984**, *42*, 207–215, doi:10.1016/0044-8486(84)90101-7.
84. Butcher, R.W. *An Introductory Account of the Smaller Algae of British Coastal Waters. PART I: Introduction and Chlorophyceae*; IV; Fisheries Investigation.; London, 1959; Vol. I;
85. Mantecón, L.; Moyano, R.; Cameán, A.M.; Jos, A. Safety Assessment of a Lyophilized Biomass of *Tetraselmis Chuii* (TetraSOD®) in a 90 Day Feeding Study. *Food Chem. Toxicol.* **2019**, *133*, 110810, doi:10.1016/j.fct.2019.110810.
86. EFSA Panel on Biological Hazards (BIOHAZ); Koutsoumanis, K.; Allende, A.; Alvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Hilbert, F.; et al. Update of the List of QPS-recommended Biological Agents Intentionally Added to Food or Feed as Notified to EFSA 11: Suitability of Taxonomic Units Notified to EFSA until September 2019. *EFSA J.* **2020**, *18*, doi:10.2903/j.efsa.2020.5965.
87. Torres, S.; Lama, C.; Mantecón, L.; Flemetakis, E.; Infante, C. Selection and Validation of Reference Genes for Quantitative Real-Time PCR in the Green Microalgae *Tetraselmis Chui*. *PLOS ONE* **2021**, *16*, e0245495, doi:10.1371/journal.pone.0245495.
88. Singh, P.; Gupta, S.K.; Guldhe, A.; Rawat, I.; Bux, F. Microalgae Isolation and Basic Culturing Techniques. In *Handbook of Marine Microalgae*; Elsevier, 2015; pp. 43–54 ISBN 978-0-12-800776-1.
89. Pereira, H.; Barreira, L.; Mozes, A.; Florindo, C.; Polo, C.; Duarte, C.V.; Custódio, L.; Varela, J. Microplate-Based High Throughput Screening Procedure for the Isolation of Lipid-Rich Marine Microalgae. *Biotechnol. Biofuels* **2011**, *4*, 61, doi:10.1186/1754-6834-4-61.
90. Castresana, J. Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. *Mol. Biol. Evol.* **2000**, *17*, 540–552, doi:10.1093/oxfordjournals.molbev.a026334.
91. Guindon, S.; Gascuel, O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.* **2003**, *52*, 696–704, doi:10.1080/10635150390235520.
92. Anisimova, M.; Gascuel, O. Approximate Likelihood-Ratio Test for Branches: A Fast, Accurate, and Powerful Alternative. *Syst. Biol.* **2006**, *55*, 539–552, doi:10.1080/10635150600755453.
93. Chevenet, F.; Brun, C.; Bañuls, A.-L.; Jacq, B.; Christen, R. TreeDyn: Towards Dynamic Graphics and Annotations for Analyses of Trees. *BMC Bioinformatics* **2006**, *7*, doi:10.1186/1471-2105-7-439.
94. Elias, I.; Lagergren, J. Fast Computation of Distance Estimators. *BMC Bioinformatics* **2007**, *8*, 89, doi:10.1186/1471-2105-8-89.
95. Schüler, L.M.; Gangadhar, K.N.; Duarte, P.; Placines, C.; Molina-Márquez, A.M.; Léon-Bañares, R.; Sousa, V.S.; Varela, J.; Barreira, L. Improvement of Carotenoid Extraction from a Recently Isolated, Robust Microalga, *Tetraselmis* Sp. CTP4 (Chlorophyta). *Bioprocess Biosyst. Eng.* **2020**, doi:10.1007/s00449-019-02273-9.
96. LICHTENTHALER, H.K.; WELLBURN, A.R. Determinations of Total Carotenoids and Chlorophylls a and b of Leaf Extracts in Different Solvents. *Biochem. Soc. Trans.* **1983**, *11*, 591–592, doi:10.1042/bst0110591.
97. Barreira, L.; Resek, E.; Rodrigues, M.J.; Rocha, M.I.; Pereira, H.; Bandarra, N.; da Silva, M.M.; Varela, J.; Custódio, L. Halophytes: Gourmet Food with Nutritional Health Benefits? *J. Food Compos. Anal.* **2017**, *59*, 35–42, doi:10.1016/j.jfca.2017.02.003.
98. Vidotti, A.D.S.; Riaño-Pachón, D.M.; Mattiello, L.; Giraldi, L.A.; Winck, F.V.; Franco, T.T. Analysis of Autotrophic, Mixotrophic and Heterotrophic Phenotypes in the Microalgae *Chlorella Vulgaris* Using Time-Resolved Proteomics and Transcriptomics Approaches. *Algal Res.* **2020**, *51*, 102060, doi:10.1016/j.algal.2020.102060.

99. Aigner, S.; Glaser, K.; Arc, E.; Holzinger, A.; Schletter, M.; Karsten, U.; Kranner, I. Adaptation to Aquatic and Terrestrial Environments in *Chlorella Vulgaris* (Chlorophyta). *Front. Microbiol.* **2020**, *11*, 585836, doi:10.3389/fmicb.2020.585836.
100. Shigeji, A.; Eiji, H. De- And Re-Generation Of Chloroplasts In The Cells Of *Chlorella Protothecoidesi*. Syntheses Of Nucleic Acids And Protein In Relation To The Process Of Regeneration Of Chloroplast. *Plant Cell Physiol.* **1964**, doi:10.1093/oxfordjournals.pcp.a079067.
101. Porra, R.J.; Grimme, L.H. Chlorophyll Synthesis and Intracellular Fluctuations of 5-Aminolaevulinate Formation during the Regreening of Nitrogen-Deficient *Chlorella Fusca*. *Arch. Biochem. Biophys.* **1974**, *164*, 312–321, doi:10.1016/0003-9861(74)90036-8.
102. Tan, C.H.; Show, P.L.; Lam, M.K.; Fu, X.; Ling, T.C.; Chen, C.-Y.; Chang, J.-S. Examination of Indigenous Microalgal Species for Maximal Protein Synthesis. *Biochem. Eng. J.* **2020**, *154*, 107425, doi:10.1016/j.bej.2019.107425.
103. Seyfabadi, J.; Ramezanpour, Z.; Amini Khoeyi, Z. Protein, Fatty Acid, and Pigment Content of *Chlorella Vulgaris* under Different Light Regimes. *J. Appl. Phycol.* **2011**, *23*, 721–726, doi:10.1007/s10811-010-9569-8.
104. Fu, M.; Cheng, S.; Xu, F.; Chen, Z.; Liu, Z.; Zhang, W.; Zheng, J.; Wang, L. Advance in Mechanism of Plant Leaf Colour Mutation. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2021**, *49*, 12071, doi:10.15835/nbha49212071.
105. Kaur, I.; Goyal, D. Cartap Hydrochloride Induced Stress Response in Anabaena Variabilis ARM 441. *Pestic. Biochem. Physiol.* **2021**, 104904, doi:10.1016/j.pestbp.2021.104904.
106. Perez-Garcia, O.; Escalante, F.M.E.; de-Bashan, L.E.; Bashan, Y. Heterotrophic Cultures of Microalgae: Metabolism and Potential Products. *Water Res.* **2011**, *45*, 11–36, doi:10.1016/j.watres.2010.08.037.
107. Lu, L.; Wang, J.; Yang, G.; Zhu, B.; Pan, K. Heterotrophic Growth and Nutrient Productivities of *Tetraselmis Chuii* Using Glucose as a Carbon Source under Different C/N Ratios. *J. Appl. Phycol.* **2017**, *29*, 15–21, doi:10.1007/s10811-016-0919-z.
108. Lu, L.; Wang, J.; Yang, G.; Zhu, B.; Pan, K. Biomass and Nutrient Productivities of *Tetraselmis Chuii* under Mixotrophic Culture Conditions with Various C:N Ratios. *Chin. J. Oceanol. Limnol.* **2017**, *35*, 303–312, doi:10.1007/s00343-016-5299-3.
109. Penhaul Smith, J.K.; Hughes, A.D.; McEvoy, L.; Thornton, B.; Day, J.G. The Carbon Partitioning of Glucose and DIC in Mixotrophic, Heterotrophic and Photoautotrophic Cultures of *Tetraselmis Suecica*. *Biotechnol. Lett.* **2021**, *43*, 729–743, doi:10.1007/s10529-020-03073-y.
110. *Advances in Algal Biology: A Commemoration of the Work of Rex Lowe*; Stevenson, R.J., Pan, Y., Kociolek, J.P., Kingston, J.C., Eds.; Springer Netherlands: Dordrecht, 2006; ISBN 978-1-4020-4782-4.
111. Neilson, A.H.; Lewin, R.A. The Uptake and Utilization of Organic Carbon by Algae: An Essay in Comparative Biochemistry. *Phycologia* **1974**, *13*, 227–264, doi:10.2216/i0031-8884-13-3-227.1.
112. Zaslavskaia, L.A. Trophic Conversion of an Obligate Photoautotrophic Organism Through Metabolic Engineering. *Science* **2001**, *292*, 2073–2075, doi:10.1126/science.160015.
113. Legendre, L.; Rassoulzadegan, F. Plankton and Nutrient Dynamics in Marine Waters. *Ophelia* **1995**, *41*, 153–172, doi:10.1080/00785236.1995.10422042.
114. Morales-Sánchez, D.; Martinez-Rodriguez, O.A.; Kyndt, J.; Martinez, A. Heterotrophic Growth of Microalgae: Metabolic Aspects. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1–9, doi:10.1007/s11274-014-1773-2.
115. Morais, E.G. de; Druzian, J.I.; Nunes, I.L.; Morais, M.G. de; Costa, J.A.V. Glycerol Increases Growth, Protein Production and Alters the Fatty Acids Profile of Spirulina (Arthrospira) Sp LEB 18. *Process Biochem.* **2019**, *76*, 40–45, doi:10.1016/j.procbio.2018.09.024.
116. Ma, X.; Zheng, H.; Addy, M.; Anderson, E.; Liu, Y.; Chen, P.; Ruan, R. Cultivation of *Chlorella Vulgaris* in Wastewater with Waste Glycerol: Strategies for Improving Nutrients Removal and

Enhancing Lipid Production. *Bioresour. Technol.* **2016**, *207*, 252–261, doi:10.1016/j.biortech.2016.02.013.

117. Allmicroalgae Natural Products: New-Look Microalgae: Brigt Future for Light-Coloured Chlorella. Available online: Allmicroalgae - New-look microalgae: Bright future for light-coloured (Accessed on 07/2021).
118. Gheihman, N. Veganism as a Lifestyle Movement. *Sociol. Compass* **2021**, *15*, doi:10.1111/soc4.12877.
119. Caporgno, M.P.; Böcker, L.; Müssner, C.; Stirnemann, E.; Haberkorn, I.; Adelmann, H.; Handschin, S.; Windhab, E.J.; Mathys, A. Extruded Meat Analogues Based on Yellow, Heterotrophically Cultivated *Auxenochlorella Protothecoides* Microalgae. *Innov. Food Sci. Emerg. Technol.* **2020**, *59*, 102275, doi:10.1016/j.ifset.2019.102275.