Development of novel *Scenedesmus rubescens* mutants with improved pigmentation and protein contents

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Preface

The work presented in this thesis was performed at the R&D Department of Allmicroalgae Natural Products S.A. (Pataias, Portugal), during the period March-September 2022, under the supervision of Specialis Mafalda Trovão and Dr Helena Cardoso. The thesis was co-supervised at Instituto Superior Técnico by Prof. Marília Mateus.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Abstract

Common protein sources, such as animal-based food, have a high environmental impact, which will be worsened by the rise in world population and food demand. Microalgae have the potential to be used as alternative protein sources as they are a source of essential amino acids, their cultivation doesn't require arable land and doesn't conflict with other food chains. However, microalgae wild-type strains' high chlorophyll content grants microalgal biomass unpleasant organoleptic properties, which prevents its acceptance. Ethyl methanesulfonate random mutagenesis was used in this work to generate chlorophyll-deficient, and high protein-producing mutants of *Scenedesmus rubescens*. To isolate the randomly generated mutants a selection platform using nicotine and norflurazon, inhibitors of the carotenoid's biosynthetic pathway, and InChlo, an inhibitor of the chlorophyll's biosynthetic pathway, was developed. Four chlorophyll-deficient mutants were isolated: W1 (1.6 mg g^{-1}DCW), W2 (9.2 mg g^{-1}DCW), and W5 (0.6 mg g^{-1}DCW), isolated with nicotine, and Y1 (10.3 mg g^{-1}DCW) which was isolated with InChlo. Mutants W1 and Y1 had a decreased protein content (21 % and 19.1 %), while W2 and W5 maintained their protein content (27.7 % and 25.7 %). Additionally, three mutants, 200a, 300b and 300d, with increased chlorophyll (24.4 mg g^{-1}DCW, 34.4 mg g^{-1}DCW and 27.2 mg g^{-1}DCW) and protein contents (55.7 %, 61.0 % and 59.8 %) were also isolated without using metabolic inhibitors. Among all the isolated mutants, W2 and 300d displayed the highest potential for biotechnological applications, such as feedstock alternatives, cosmetics and nutritional applications.

Keywords: Microalgae, strain improvement, random mutagenesis, selection, chlorophyll, protein.

Resumo

Fontes proteicas comuns, como comida de origem animal, têm um elevado impacto ambiental, que será agravado pelo aumento na população mundial. Microalgas têm o potencial de serem utilizadas como fontes proteicas alternativas pois o seu cultivo não requer terrenos aráveis, não perturbam outras cadeias alimentares e são uma fonte de aminoácidos essenciais. No entanto, o elevado conteúdo de clorofila das estirpes selvagens atribui propriedades organoléticas desagradáveis à biomassa, o que restringe a sua aceitação. Mutagénese aleatória com metanossulfonato de etilo foi utilizada para gerar mutantes de *Scenedesmus rubescens* com deficiência de clorofila e elevado conteúdo proteico. Para isolar os mutantes aleatoriamente gerados, foi desenvolvida uma plataforma de seleção utilizando nicotina e norflurazon, inibidores da via dos carotenóides, e InChlo, um inibidor da via das clorofilas. Quatro mutantes deficientes em clorofila, W1 (1.6 mg g^{-1}DCW), W2 (9.2 mg g^{-1}DCW), e W5 (0.6 mg g^{-1}DCW), isolados com nicotina, e Y1 (10.3 mg g^{-1}DCW), que foi isolado com InChlo. Os mutantes W1 e Y1 tiveram um decréscimo no conteúdo proteico (21 % e 19.1 %), enquanto W2 e W5 o mantiveram (27.7 % e 25.7 %). Adicionalmente, três mutantes, 200a, 300b e 300d, com conteúdos de clorofila (24.4 mg g^{-1}DCW, 34.4 mg g^{-1}DCW e 27.2 mg g^{-1}DCW) e proteína (55.7 %, 61.0 % e 59.8 %) aumentados, foram isolados sem utilizar inibidores metabólicos. De todos os mutantes isolados, W2 e 300b têm elevado potencial para aplicações biotecnológicas, como rações alternativas, cosmética e aplicações nutricionais.

Palavras-chave: Microalgas, melhoria de estirpes, mutagénse aleatória, seleção, clorofila, proteína.

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Abbreviations

ALE – Adaptive Laboratory Evolution

ARTP – Atmospheric and Room-temperature Plasma

DCW – Dry Cell Weight

DNA – Deoxyribonucleic Acid

CEW – Cellulosic Ethanol Wastewater

CO_2 – Carbon Dioxide

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

EFSA – European Food Safety Authority

EMS – Ethyl Methanesulfonate

EU – European Union

FACS – Fluorescence-Activated Cell Sorting

FAME – Fatty Acid Methyl Esters

FDU – 5'Fluorodeoxyuridine Monophosphate

GMO – Genetically Modified Organisms

He-Ne – Helium-Neon

HPLC – High-Performance Liquid Chromatography

IC – Inhibitory concentration

LC-PUFA – Long-chain Polyunsaturated Fatty Acids

LD – Lethal Dose

MTP – Microtiter Plate

MUFA – Monounsaturated Fatty Acids

Nd:YAG – Neodymium-doped Yttrium Aluminum Garnet

NF – Norflurazon

Nic – Nicotine

NTG – Nitro-N-nitrosoguanidine

OD – Optical Density

PSII – Photosystem II

TAG – Triacylglycerol

TALEN – Transcription Activator-Like Effector Nucleases

UV – Ultraviolet

WT – Wild-type

ZFN – Zinc Finger Nucleases

γ – Gamma

1. Introduction.

1.1. Microalgae overview.

1.1.1. General Characteristics.

Microalgae are unicellular or simple multicellular eukaryotic microscopic organisms found both in seawater and freshwater, but also in hypersaline environments, moist soils and rocks.[1,2] They constitute a phylogenetically diverse group, that encompasses different phyla and classes of organisms, with an estimated 50000 existing species, out of which only around 25000 species have been isolated and identified.[1,3]

There is a growing interest in microalgae production, with 75 companies in Europe producing microalgae.[4] Just in Portugal, there are six microalgae production plants.[4] The increasing market interest for algae-derived products is due to the high versatility and applicability of microalgae in the biotechnology industry, represented by a compound annual growth rate of over 5.2 % and a market value foreseen to achieve US$ 44.6 billion by 2023.[5] The main commercial biotechnological applications (Figure 1) of microalgae biomass are related to human nutrition, nutraceuticals, cosmetics and feed (animal nutrition).[1,4]

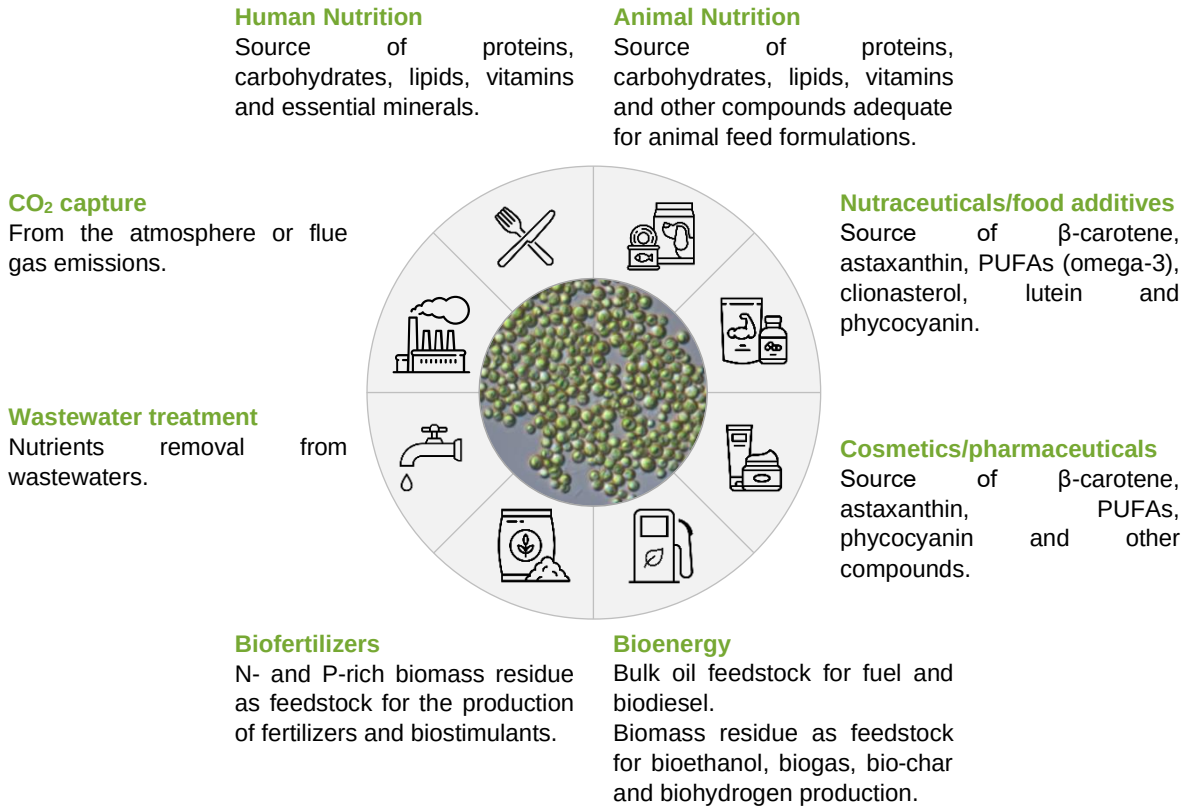

Figure 1. Main applications described for microalgae. (Adapted from Vale et al. 2020)[1]

Some of the most interesting microalgae-derived metabolites are: i) protein and amino acids, such as leucine and isoleucine, which are essential amino acids; ii) pigments, such as chlorophylls and carotenoids; iii) fatty acids, namely essential long-chain polyunsaturated fatty acids (LC-PUFAs), known as omega-3 and 6; and iv) vitamins and carbohydrates.[5] Besides algae-derived products, microalgae can also be used for wastewater treatment, carbon dioxide (CO_2) capture, and biofuel production. [2,6–8]

Microalgae are also a potentially environmentally sustainable feedstock.[9] The rise in world population is accompanied by an increase in demand for food production, particularly protein.[10] However, the food system should include more environmentally sustainable protein sources, given the rising consumer awareness for environmental protection and the impact that common protein sources, such as animal-based food, have.[9] In fact, animal-based food production represents 57 % of total greenhouse gas emissions related to food production, derived mainly from land usage, enteric fermentation and grazing land.[11] On the other hand, plant-based food products still represent 29 % of total greenhouse gas emissions related to the food system, whose impact derives mainly from land usage, which imperatively needs to be arable land.[11] This causes extra environmental pressure because it contributes to deforestation and restrains carbon sequestration.[11] It is noteworthy that the environmental impact reported for plant-based production is also related to meat production since most are forage crops and only a small portion for human nutrition.[12] Thus, more sustainable protein sources, such as microalgae, must be developed and included in the food system. Microalgae are a suitable alternative to include in food and feed production because their cultivation doesn't require arable land or freshwater and it doesn't conflict with other established food chains.[5,13] Additionally, microalgae can grow on minimal nutrient conditions, whether in fresh-, sea- or wastewater.[2,13,14]

Besides protein and essential amino acids, pigments, namely carotenoids and chlorophylls, are amongst the most interesting microalgae-derived products. Carotenoids are lipophilic pigments responsible for the characteristic yellow, orange or red colour found in microalgae, that participate in photosynthesis by absorbing light and quenching excessive energy. Additionally, the antioxidant properties of carotenoids grant them a protecting role, as they can quench triplet chlorophyll states, and neutralize free radicals and reactive oxygen species.[15,16] Due to this function, carotenogenesis is generally associated with specific environmental stresses, such as high temperature or nitrogen deficiency.[16] One of the most promising properties of carotenoids is their role in the immune system, where their antioxidant properties can be useful.[17] Regarding biotechnological applications, carotenoids can be used in food, feed, cosmetics, pharmaceutical and nutraceutical industries.[17] Chlorophylls are lipophilic green pigments found in most photosynthetic organisms, essential for light-harvesting and energy transduction in photosynthesis, functioning either as accessory or reaction centre pigments.[18] They have also been studied as a possible antioxidant, anticarcinogenic, antigenotoxic and antimutagenic compound, highlighting their potential as a pharmaceutical product. It has also been reported that chlorophyll has a beneficial role in digestion by stimulating gut microbiota and promoting an increase in short-chain fatty acids.[19]

Microalgae cultivation has to be optimized to maximize the production of specific compounds. Optimization starts with choosing a suitable species to produce the compounds of interest, but the

cultivation method and abiotic factors are also key factors that highly impact the productivity of target biomolecules.

1.1.2. Microalgae Cultivation.

Microalgae growth characteristics and composition are significantly dependent on their cultivation conditions. One of the most interesting aspects of microalgae is their metabolic flexibility, as some species possess the ability to grow on different metabolisms, such as photoautotrophic, heterotrophic and mixotrophic.[20,21] Table 1 summarizes the principal characteristics of the described metabolisms and cultivation methods.

Table 1. Comparison of the most common microalgae cultivation types. (Adapted from Chen et al. (2011) and Zhan et al. (2017)).[20,22]

	Photoautotrophic growth	**Heterotrophic growth**	**Mixotrophic growth**
Energy Source	Light (e.g., sunlight)	Organic (e.g., glucose, glycerol)	Light and Organic
Carbon Source	Inorganic (e.g., CO_2)	Organic	Organic and inorganic carbon sources simultaneously
Main Disadvantages	Low cell density. High capital and operational expenses. Variable environmental conditions. Risk of contamination. High ground surface occupation.	High cost of substrate. Low valuable metabolite contents. Skilled labour is required. Risk of contamination.	High cost of substrate. High contamination risk. Variable environmental conditions.
Main Advantages	Cheap (free) energy and carbon sources (sunlight and CO_2). High valuable metabolite accumulation.	Higher biomass productivity. Independent of environmental conditions. Strict control of cultivation conditions. Low ground surface.	Intermediate biomass productivity. High valuable metabolite accumulation.

1.1.2.1. Photoautotrophic cultivation.

Photoautotrophic cultivation is the most utilized cultivation method and occurs when microalgae use light, such as sunlight, as the energy source and inorganic carbon (e.g., CO_2) as the carbon source. The major advantage of this type of cultivation is the consumption of CO_2 from the atmosphere, which makes this process more environmentally sustainable. Additionally, when grown in the presence of light, biocompounds such as pigments and protein content are significantly enhanced, which further increases biomass' commercial interest.[23] Compared to other types of cultivation, photoautotrophic cultivation is more prone to contamination because the cultures are generally more exposed to the environment. Furthermore, photoautotrophic cultivation is generally characterised by low biomass concentrations due to poor light delivery, mutual shading of cells in dense cultures and overall slower growth of algal biomass, resulting in a long scale-up process.[20,23,24] Regarding the costs, photoautotrophic cultivation is associated with the usage of large volumes of freshwater (unless wastewaters are used instead) and

high energetic costs mainly derived from mixing, aerating and downstream processing of the low-density cultures. [25,26]

1.1.2.2. Heterotrophic cultivation.

Heterotrophic cultivation of microalgae consists in using organic carbon, such as glucose, as energy and carbon source. This type of cultivation doesn't require light, an advantage as it avoids dependency on weather conditions and problems associated with limited light, which hinder high cell density in large-scale photobioreactors.[20,24] Additionally, heterotrophic cultivation is carried out inside a fermenter, where it is possible to maintain axenic and controlled conditions. On the other hand, heterotrophic cultivation requires an organic carbon source, which increases the susceptibility to contamination, and that must be mitigated by performing any process carefully and aseptically by skilled workforce.[20,23] Moreover, a significant portion of the costs derives from the carbon source. Using inexpensive alternative carbon sources, such as glycerol or waste products with fermentable saccharides, that don't significantly impair microalgae productivity, can decrease the costs of heterotrophic cultivation. Combined with the higher growth rate, productivity and cell density, heterotrophic cultivation can become more economically sustainable than autotrophic cultivation. However, this is still a topic of discussion among the scientific community.[22,25,27–29] Smetana et al. (2017) pointed out that heterotrophic cultivation of *Chlorella vulgaris* is less environmentally impacting than its autotrophic cultivation, mainly due to the low microalgae concentration and high volumes of water that are obtained in autotrophic cultures.[28] Additionally, heterotrophic cultures have overall much higher productivity and require less land, water and electricity, which lowers the costs per weight of biomass produced.[28] These authors suggest that the most environmentally sustainable microalgae cultivation method is growing microalgae heterotrophically, using an alternative carbon source, such as food waste, and photovoltaic systems to provide the required energy.[28]

The present work was developed in heterotrophy with *Scenedesmus rubescens*, one of the few species that can grow heterotrophically, enabling it to reach high biomass productivity.

1.1.2.3. Mixotrophic cultivation.

Mixotrophic cultivation comprises the use of both inorganic and organic carbon simultaneously. This means that microalgae grow under both photoautotrophic and heterotrophic metabolisms.[20] Due to this metabolic flexibility, mixotrophic cultures are associated with higher growth rates and cell concentrations than autotrophic cultures.[21,22,24] The culture conditions that cause the change from photoautotrophic to heterotrophic metabolism are not yet fully understood but it has been reported that light accessibility and organic carbon concentration are important factors in determining the contribution of autotrophy and heterotrophy during mixotrophic cultivation.[30] Another advantageous phenomenon in mixotrophic cultures is CO_2 recycling, since the CO_2 released by microalgae via respiration is reused in the photoautotrophic metabolism.[20,21] However, mixotrophic cultivation of microalgae comes with some of the disadvantages mentioned: higher susceptibility to contamination and cost of the organic carbon source. Compared to autotrophic cultures, mixotrophic cultivation has considerably higher biomass

productivities, lower light requirements due to metabolism shifting and consequently a lower cost per kg produced.[31]

1.2. Microalgae Production and Strain Improvement.

1.2.1. Challenges in Microalgae Production.

Different microalgae species have different compositions and pigmentation, so their application in the biotechnology sector also differs. For example, a species with high saturated lipids contents would be more indicated for biofuel production[13], while species rich in LC-PUFAs, like docosahexaenoic acid (22:6) and eicosapentaenoic acid (20:5), would be more indicated for feed and food or pharmaceutical products, due to their significant health benefits.[5]

Despite the benefits of microalgal biomass and its biocompounds, the cost of microalgae-derived products still prevents many industries from making this desirable shift, since microalgae as feedstock are not economically competitive in several sectors.[6,32,33] Capital and operational expenses, such as the energetic costs associated with downstream processing and culture medium, make this process economically unviable.[34] Therefore, the selection of suitable microalgae species is of great importance to assure the productivity and economic feasibility of the industrial process, as well as the optimization of the whole process pipeline.

C. vulgaris is the most commercialized species in Europe, which is expected given its long history of safe use and approval for human consumption by the European Food Safety Authority (EFSA) (EU, 2017/2470)[35]. Other species, such as *Tetraselmis* sp., *Tisochrysis lutea*, *Isochrysis galbana*, *Dunaliella salina*, *Phaeodactylum tricornutum*, *Porphyridium* sp., and *Scenedesmus* sp. are also among the top produced species in Europe.[4] Nonetheless, a very limited number of species is approved for human consumption.[9]

Wild-type (WT) strains frequently don't possess the required characteristics for industrial production. In general, it is beneficial that microalgae species have a high growth rate and high tolerance to environmental factors, such as varying temperatures and salinities. Other advantageous characteristics are high tolerance to shear stress, such as those inside photobioreactors, and robustness to outgrow to contaminants, which are commonly found in open ponds and that might lead to culture to culture crashes.[36] Finally, there should be an easiness of harvesting, downstream processing, and manufacturing; and to meet the requirements for registration and approval by regulatory authorities when intended for human nutrition.[8]

Additionally, the characteristics of microalgal biomass, such as smell, taste and colour, hinder consumer's and even animal's acceptance.[37,38] Chlorophyll grants unpleasant organoleptic properties to microalgal biomass, described as a strong grassy taste, intense odour, dark-green colour, and unappealing texture. [35] Furthermore, high chlorophyll contents lead to low photosynthetic efficiencies in autotrophic cultures, resulting in lower biomass productivity.[39] Cells at the surface of the culture will absorb most of the light, creating a dark zone deeper into the culture. Because light doesn't reach this

dark zone, cells have a lower photosynthetic rate, resulting in lower growth. This process of impaired light penetration, leading to decreased photosynthetic efficiency, is termed self-shading.[39,40]

Finally, given the increasing demand for non-animal origin products worldwide, microalgae are considered an important alternative source of protein and lipids, particularly LC-PUFAs. However, the challenges derived from the low productivity and cost-effectiveness of microalgae cultivation, along with difficulties in harvesting and downstream processing, make the use of microalgae as an alternative feedstock challenging.[38,41] Besides optimizing biomass productivities, it is important to maximize compounds' productivities by studying and optimizing metabolic fluxes, which can lead to more interesting biochemical profiles, such as higher protein contents.

1.2.2. *Scenedesmus rubescens.*

Scenedesmus rubescens (Figure 2) is a green microalga, first described in 1965 by Dr. Pierre Jean Louis Dangeard (P. J. L. Dangeard) under the name *Halochlorella rubescens*, which is now regarded as a basionym of *Scenedesmus rubescens* after the work of Kessler *et al*. (1997)[42].[43,44] *S. rubescens* belongs to the *Scenedesmus* genus, the *Scenedesmaceae* family and the *Sphaeropleales* order (previously *Chlorococcales*). This microalga is mostly found in freshwater bodies, such as lakes and rivers. However, some studies have already demonstrated that it can grow in wastewater. [45–47] *S. rubescens* cells are nonmotile, spherical with a slight ellipsoidal shape, and a diameter of 6-10 µm.[47–49] Structurally, *S. rubescens* possesses a parietal chloroplast that contains one pyrenoid.[47] The cell wall of *Scenedesmus* is generally rigid and composed of three layers: an inner cellulosic layer delimiting individual cells, a thin middle algaenan-based layer, and an outer pectin layer which joins the cells into coenobium.[50] *Scenedesmus* genus can build colonies of four to eight cells, as an anti-grazing response which is desirable for cell sedimentation.[51] Reproduction is asexual, usually through autospores released by breakage of lateral cell walls.[49,52]

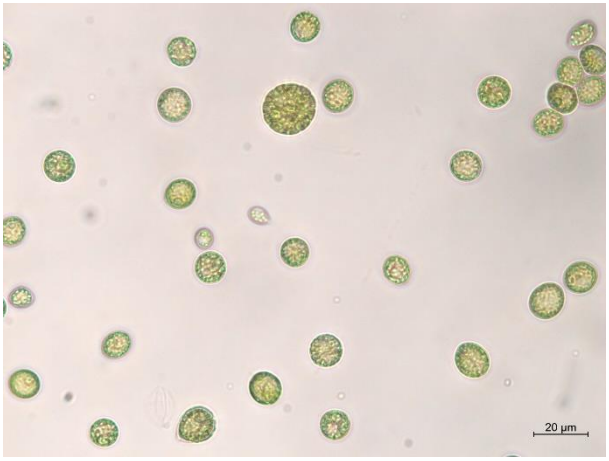

Figure 2. Heterotrophic culture of *Scenedesmus rubescens*. Scale Bar: 20 µm.

Regarding the nutritional content, *Scenedesmus* spp. are a rich source of bioactive compounds. The general macronutrient content of *S. rubescens* is represented in Table 2, where it is seen that carbohydrates represent the main biomass macronutrient, followed by protein and lipids.[53] *Scenedesmus* spp. are also found to contain all essential amino acids and vitamins B, C, and E.[54]

Table 2. Proximate composition of *Scenedesmus rubescens*. (Data retrieved from Espírito Santo, 2020)[53]

Macronutrient	Content (% Dry Cell Weight)
Proteins	15 – 33
Lipids	7 – 21
Carbohydrates	53 – 63
Ash	2– 3

As for their applications, *Scenedesmus* species are considered promising for biofuel production and wastewater treatment, since they have a high growth rate, high lipid content, and exhibit resistance to elevated CO_2 and ammonium concentrations as well as an adequate fatty acid profile.[47,51,55,56] Additionally, *S. rubescens*' capacity to grow heterotrophically can also be exploited.[57] Moreover, their rich composition in bioactive compounds, such as vitamins and essential amino acids, is interesting to apply in food supplements, feed and the pharmaceutical industry.[54,58,59] Up until now, no *Scenedesmus* species has been approved for human consumption.[9]

The objective of this work was to successfully improve the pigmentation and protein contents of *S. rubescens* by resorting to a well-established strain improvement method. This strategy aimed to develop improved strains that can be used in several biotechnological applications, such as sustainable feedstock alternatives.

1.2.3. Strain Improvement Methods.

Given all the challenges in the microalgae industry, besides choosing a suitable species for a specific purpose, improving WT strains is imperative to make microalgae cultivation an economically sustainable process. For this purpose, several genome-modifying techniques have been applied, such as random mutagenesis, adaptive laboratory evolution (ALE) and insertional and site-directed mutagenesis.

Random mutagenesis can be described as *"the exposure or treatment of biological material to a mutagen, i.e., a physical or chemical agent that raises the frequency of mutation above the spontaneous rate"* (Kodym and Afza, 2003)[60]. This methodology is well-established for microalgae strain improvement due to its robustness, accessibility and low cost (Figure 3).[36,61] Additionally, this method does not introduce any foreign genetic material into the cell, which is particularly useful if the biomass is intended for food applications because the generated strains are not considered genetically modified organisms (GMO).[37,62] Since the mutations induced by the mutagenic agents are random, little information about the target organism's genome or metabolism is necessary. However, this also means that mutations can be lethal or partially incapacitating.[36] The intrinsic deoxyribonucleic acid (DNA) repair mechanisms in microalgae's genome are also challenging, as they might undo the mutations induced by the mutagen, making the mutant phenotype often unstable and/or reversible.[63] Due to these challenges, it is important to establish a proper mutagenesis protocol by defining the optimal mutagen dose and developing an effective selection method.[36] The selection and screening step for mutants of interest is the most impactful step of random mutagenesis. Still, the lack of efficient selection and screening methodologies is the limiting step of this methodology.[36,64,65]

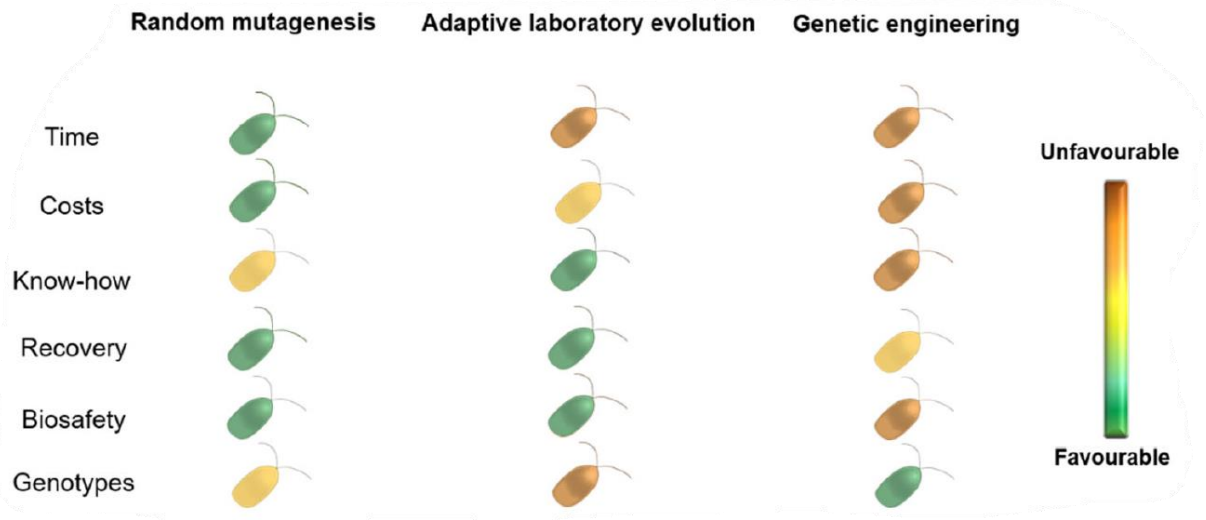

Figure 3. Comparison of several aspects of three methods of strain improvement: random mutagenesis, adaptive laboratory evolution and genetic engineering. Time—time required to perform the experiments and obtain results; Costs—general costs of using these methods; Know-how—level of knowledge required to implement the technology; Recovery—ease of selection and isolation of strains with the desired features; Biosafety—potential biosafety concerns for consumers and environment over the strains obtained; Genotypes—ability to attain the desired genotypes and phenotypes. (Retrieved from Trovão et al. 2022)[36]

Adaptive laboratory evolution is the incubation of cell cultures with continuous selective environmental stress over several generations. The adaptation of organisms to this selective pressure leads to the acquisition and/or recombination of genetic traits that confer resistance to the applied stress and that are inheritable through multiple generations.[36,66] Similarly to random mutagenesis, ALE also accelerates the process of natural evolution, as spontaneously generated mutants with non-specific mutations are selected by controlling the environmental stress applied.[66] This strain improvement approach doesn't require knowledge about the genome of the target organism and doesn't introduce exogenous genetic material into the cells, therefore, it is not considered a GMO-generating method either.[36] However, this method lacks efficiency, depends on the original strain, initial cell density, and more significantly on the stress strategy; and requires a longstanding process with a large number of generations.[67] The process that leads to the isolation of an optimized strain is long, and the stability and reversibility of ALE-selected strains are also challenging.[66] A strategy that has been proposed to optimize ALE efficiency is the integration of random mutagenesis in the process and the utilization of more efficient selection methods, such as flow cytometry.[67]

Insertional and site-directed mutagenesis are similar techniques based on the introduction of DNA fragments into the target organism's genome, differing only in whether the DNA fragment is introduced into a random location (insertional mutagenesis) or a specific site of the genome (site-directed mutagenesis).[68] Given the similarities, some of the disadvantages are also similar. Both methods require a DNA transformation protocol, which is only available for a few microalgae species (e.g. *Chlamydomonas reinhardtii*).[69] Additionally, as foreign DNA is introduced into the target organism's genome, the selected mutant is considered a GMO, which implies that it is under more strict regulations.[69] Site-directed mutagenesis also requires knowledge of the target genome and the definition of the target gene, which can be challenging given that some functions are not defined by a single gene and modifications might have a pleiotropic effect on other genes.[36,67,69] Additionally, genetic

engineering methodologies, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), Zinc Finger Nucleases (ZFN) or Transcription Activator-Like Effector Nucleases (TALENs), although allowing a specific genome modification, they require extensive expertise and come with increased associated costs.[69,70] Notwithstanding, the specific modification of genomes can be advantageous for studying gene functions and identifying novel genes.[69] Moreover, the mutants generated and the inserted mutations might be more easily identified through the use of specific selective markers and/or genetic or phenotypic tags.[36,69]

Overall, random mutagenesis is the cheapest and fastest method, requires little information about the genetic code and is a non-GMO generating method. Among the described methodologies (Figure 3), it is considered a suitable approach to improve microalgal strains for nutritional applications, with several successful reports (Table 5 – Appendix).[37]

1.3. Random Mutagenesis.

1.3.1. Historical Perspective.

Mutation breeding has a long history of success in culture improvement, not only in microalgae but also in plant cultivation. In the late 19th century, mutations as a source of plant variability were identified by Hugo de Vries, while "rediscovering" Mendel's laws of inheritance.[71] Since then, multiple reports have emerged describing the application of this technology. In 1928, Lewis John Stadler applied the mutagenic effect of X-rays in maize (*Zea mays*) and barley (*Hordeum vulgare*), setting a landmark in the application of mutagenesis for the improvement of essential food crops.[72] Then, in 1934, a mutant variety of the tobacco plant was the first commercialized mutagenesis-derived product.[71,72] Despite this, the systematic application of chemical mutagens to improve strains only started during the 1940s.[72] Before the work of Iosif Abramovich Rapoport in 1993, where he described the effect of more than 50 chemical mutagens, crop improvement was mainly performed using physical mutagens.[72,73] Nevertheless, advances in the application of random mutagenesis to improve important food crops were successful, as several barley varieties with improved traits such as chlorophyll profile, short stature, stiffness of straw and dense ears were developed.[72]

Besides plant mutagenesis, microalgae mutagenesis also has a history of success. Microalgae's pigmentation has been a subject of interest, dating back to the 1950s when pigment biosynthesis was studied by resorting to X-ray mutagenesis.[74] A colourless *Chlorella* mutant was also obtained in 1960 using radioactive isotopes.[75] Currently, chemical mutagenic agents, such as ethyl methanesulfonate (EMS) and nitro-N-nitrosoguanidine (NTG), are more frequently used for microalgae mutagenesis, representing more than 50% of the mutagenic agents.[36] For example, *Schüler et al.* (2020)[37] were also able to obtain a colourless (or white) mutant of *C. vulgaris* by using EMS as the mutagenic agent.

Within random mutagenesis, there are two categories: physical mutagenesis, where radiation induces mutations through direct physical and indirect physicochemical effects (e.g., ultraviolet (UV), gamma (γ) rays); and chemical mutagenesis, where a chemical reaction randomly induces mutations in a DNA molecule (e.g., EMS).[71]

Regarding microalgae mutagenesis, multiple reports describe its application on different species using a wide range of mutagens. The results and objectives of these experiments can also vary greatly. In Table 5 (Appendix), several studies are summarized, namely, regarding *Scenedesmus* spp., closely related *Desmodesmus* spp. and *C. vulgaris* mutagenesis, where it is possible to observe the wide variety of mutagenic agents used and results obtained.

1.3.2. Physical Mutagenic Agents.

Physical mutagenic agents comprise all nuclear radiations and sources of radioactivity; therefore they can be divided into non-ionizing radiation (e.g., UV light) and ionizing radiations (e.g., X-rays, γ rays, ion beam). Given its high energy levels, ionizing radiation can penetrate cells and damage DNA through two mechanisms: by direct physical impact, causing double-strand breaks, which results in DNA fragmentation, or by indirect physicochemical effects, derived from the reactive species formed during water radiolysis, such as hydroxyl radicals, free electrons, and hydrogen radicals.[63,76] Non-ionizing radiation, although less energetic compared to ionizing radiation, still possesses tissue penetrability capabilities and induces mutations directly by damaging DNA molecules. [68,82]

Amongst the wide variety of physical mutagens, UV radiation is the most frequently used for microalgae mutagenesis.[36] The drawback of most physical mutagenic agents is that they require specialized equipment and personnel, which is an obstacle as it leads to increased expenses.[77] Likewise, UV mutagenesis arises as a better alternative since it is simpler, cheaper and easier to apply. UV mutagenesis can be applied by exposing cells to standard UV lamps present in the sterile flow chambers, a basic equipment of most laboratories. Moreover, it facilitates the isolation of mutants in sterile conditions, which helps to avoid contaminations considerably. [78] However, mutants created with UV mutagenesis have been shown to have unstable phenotypes due to the existence of several DNA repair mechanisms that respond to UV-induced DNA damage, namely photoreactivation, excision and recombinational repair. These mechanisms can be light-dependent, such as photoreactivation, or able to repair DNA in the dark, such as excision. Given the possibility of DNA repair in the absence of light, even UV mutagenized strains cultured in the dark can revert the mutations induced by the UV radiation.[79,80]

1.3.3. Chemical Mutagenic Agents.

Compared to physical mutagens, chemical mutagenic agents are a much broader class of mutagens with variable mechanisms of action. Chemical mutagens act directly upon DNA and can cause errors in base pairing, deamination of purines, transcription and replication, transitions, transversions, frameshift mutations, and eventually strand breaks.[64] It has been generally considered that chemical mutagens only introduce point mutations, whereas physical mutagenic agents cause more significant lesions, such as chromosomal aberrations. However, it has been shown that chemical agents can also cause chromosomal rearrangements. This suggests that frequency and type of mutation are more directly correlated with dosage and rate of exposure rather than being a chemical or a physical mutagen.[71,81]

Given their variability, chemical mutagens are classified according to their mechanism of action and structure: intercalating agents, base analogues, non-alkylating agents or alkylating agents.[64]. Among these, alkylating agents are the most widely used chemical mutagens.[36] Alkylating agents act by transferring an alkyl group to the H-bonded oxygen at N^7 and N^3 positions of guanine and adenine base pairs, respectively, resulting in depurination and causing a mispair during DNA replication (Figure 4).[64] Amongst alkylating agents, the most used are EMS and NTG.[36,64,76,77]

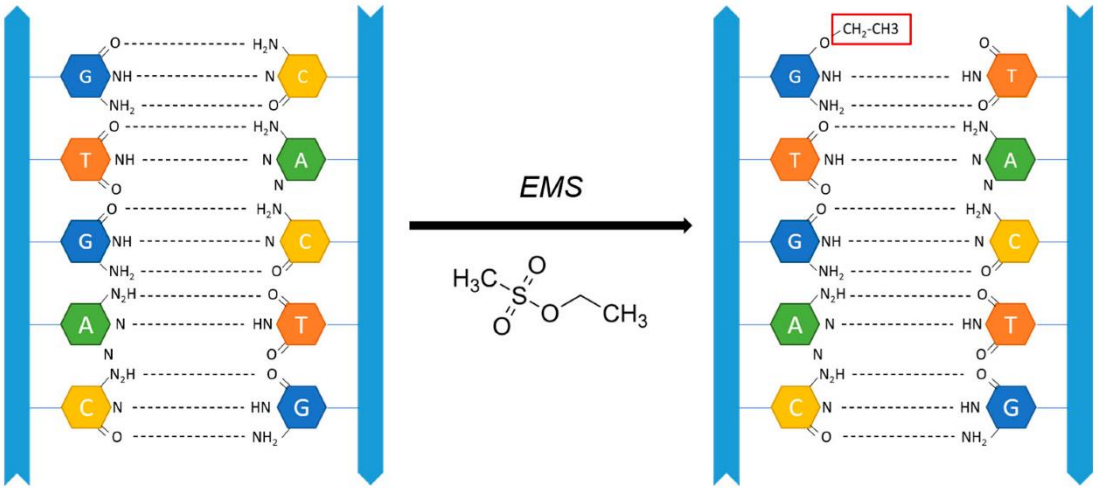

Figure 4. EMS structure and mechanism of mutagenic action. (Retrieved from Trovão et al. 2022)[36]

Compared to physical mutagens, the applicability of chemical mutagens is easier as it doesn't require any specialized equipment, being accessible in a standard laboratory setting. It also produces less chromosomal damage, resulting mainly in point mutations evenly distributed throughout the entire genome. The point mutations created can lead to loss-of-function and gain-of-function mutations if they originate a protein with modified activity or affinity.[35,71,77]

Regarding the technical limitations of chemical mutagens, the toxic properties of the mutagens raise significant health and safety concerns. Additionally, the high mutation rate can lead to several rounds of sub-cultivation to confirm the desired mutation and remove undesirable ones.[35,37]

EMS (Figure 4) represents 43% of the mutagenic agents used for microalgae mutagenesis.[36] EMS is a monofunctional alkylator, which means it has a single alkyl group that most commonly causes G/C to A/T transitions.[77] This agent has been applied in mutagenesis of different species, namely *Scenedesmus* sp.[82], *Desmodesmus* sp.[83] and *C. vulgaris*[84] (Table 5 – Appendix). The target of EMS mutagenesis varies, so that it has been used to improve tolerance to cellulosic ethanol wastewater (CEW)[82], lipid and biomass productivity[83], carotenoids content[84], photosynthetic efficiency[39,84,85], oxidative stress tolerance[85], violaxanthin productivity[86] and to decrease chlorophyll content[37,39]. Due to its extensive study and application in microalgae, EMS was chosen as the mutagenic agent to be used in this work to generate mutants of *S. rubescens*.[36]

1.3.4. Mutagen Dose and Mutant Selection.

As previously mentioned, the mutation frequency and type are a direct consequence of the mutagen applied, its dosage and the rate of exposure.[87] In general, the objective of a mutagenesis procedure is to obtain a modest number of mutations in a trait of interest, without significantly disrupting the genotypic integrity of the organism, which could result in loss of fitness. [76]

To carry out a mutagenesis experiment, the optimal mutagen dose must be previously established through the stipulation of a dose-response curve, which associates a specific mutagen dosage with the respective survival rate of a specific strain.[76] There are two main views on what the ideal dose should be. Some authors defend that the optimal mutagenesis dose should be LD_{50}, which is the dose that kills half of the treated population.[76,77] The authors that support this approach affirm that the probability of unintended induced deleterious alleles is reduced with this dose. However, this mutagen dose is mostly used in higher plants, such as rice. Most reports use a higher dose for microalgae mutagenesis, leading to survival rates between 5-10 % (Table 5 - Appendix).[37] This approach increases the probability that the surviving mutants contain at least one stable mutation and reduces the number of mutants to be screened.

Random mutagenesis generates an extensive, heterogeneous mutant library. However, only a limited portion of them express the phenotype of interest.[88] To isolate the desired mutants, a selection and/or screening step needs to be implemented. Although often used interchangeably, selection and screening are different concepts. Screening refers to the evaluation of every generated mutant for the desired property. Selection refers to the application of a selective pressure to the population in which the desired mutants have a survival advantage or a phenotypic change that distinguishes them from the remaining.[89,90] Given the high number of mutants and the complexity of qualifying/quantifying different properties, this is the limiting step of a mutagenesis protocol and it is where the most significant improvements can be made.[64,65]

Identifying the phenotype of interest is the first step of the screening process and will directly affect the chosen method. The phenotype of interest can be, for example, increased lipid or fatty acid contents, higher carotenoid production or lower chlorophyll contents. Therefore, the screening/selection method must be optimized for each of these examples.

Conventional screening methods for microalgae mutagenesis imply manually isolating mutants and analysing the mutant culture for the desired feature, which is a laborious and slow process.[87] To increase the efficiency of mutant screening, high-throughput methods have been developed. Some high-throughput methods for microalgae mutant screening include percoll density gradient centrifugation, fluorescence-activated cell sorting (FACS) and microtiter plate (MTP)-based screening.

Percoll density gradient centrifugation can be used to screen for microalgae mutants with a high lipid content, as this technique can separate mutant cells based on their relative densities. Mutants with a high lipidic content will have a lower density and, therefore, will be on the top of the gradient, while cells with a lower lipid content will remain at the bottom.[64] FACS is a specialized technique coupled to flow cytometry that can separate single cells according to their fluorescence. This fluorescence can be

intra- or extracellular and can originate from a biological source of fluorescence, like chlorophyll, or a synthetic one, such as Nile red or BODIPY505/515 (lipophilic dyes). This method is versatile as it can separate cells using a wide range of molecules collecting only the cells that surpass a given fluorescence threshold. Therefore, this method allows the identification and isolation of cells that are overproducing or underproducing a specific target compound.[64,65] MTP-based screening is a cost-effective, moderate-high throughput method that screens cells based on a screening indicator, such as pH or fluorescence. The method relies on inoculating a 96-well plate, or similar, and assessing the concentration or the variation on the target screening indicator. This technique is frequently applied to measure pH changes throughout a colourimetric assay.[65]

On the contrary, mutant selection encompasses the utilization of metabolic inhibitors that specifically target the biosynthetic pathways of the compounds of interest or the application of unspecific environmental stressors that inhibit the growth of susceptible mutants.[36] Mutants isolated in the presence of these metabolic inhibitors have mutations that grant them resistance to the applied stress. In contrast, mutants with undesired mutations are not able to grow in the presence of this inhibitor. [91]

To apply an effective selection approach, the system must be devised in a way that mutants with the desired properties have a growth advantage.[89] For example, to isolate mutants with a different pigmentation profile, metabolic inhibitors that target the carotenoids or the chlorophylls biosynthetic pathways are typically applied. Metabolic inhibitors such as norflurazon (NF), fluoridone, diphenylamine and nicotine (Nic) are commonly used to target the carotenoids' biosynthetic pathway (Figure 5). Likewise, acifluorfen, oxyfluorfen, gabaculine, levulinate and 2,2'-dipyridyl are metabolic inhibitors that specifically target the chlorophylls biosynthetic pathway, and that could possibly be used for microalgae mutant selection. (Figure 6).[36,91–96]

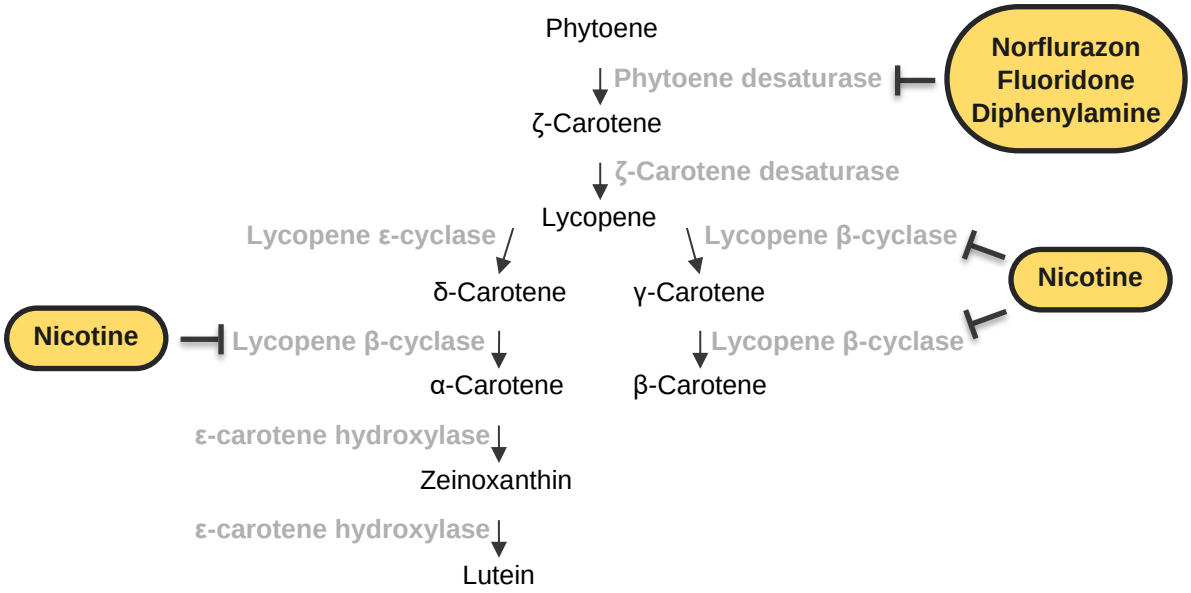

Figure 5. Carotenoids biosynthetic pathways. In grey are the enzymes that catalyze each reaction. Inhibitors of this pathway are highlighted in yellow boxes. Adapted from Ma et al. (2011).[149]

Specifically, NF, fluoridone, and diphenylamine target the phytoene desaturase enzyme, which converts phytoene into ζ-carotene, so when this reaction is inhibited, there is an accumulation of

phytoene and a decrease in the production of carotenoids.[97] On the other hand, Nic specifically inhibits the lycopene β-cyclase enzyme, which catalyses the conversion of lycopene to α- and β-carotene.[98] In

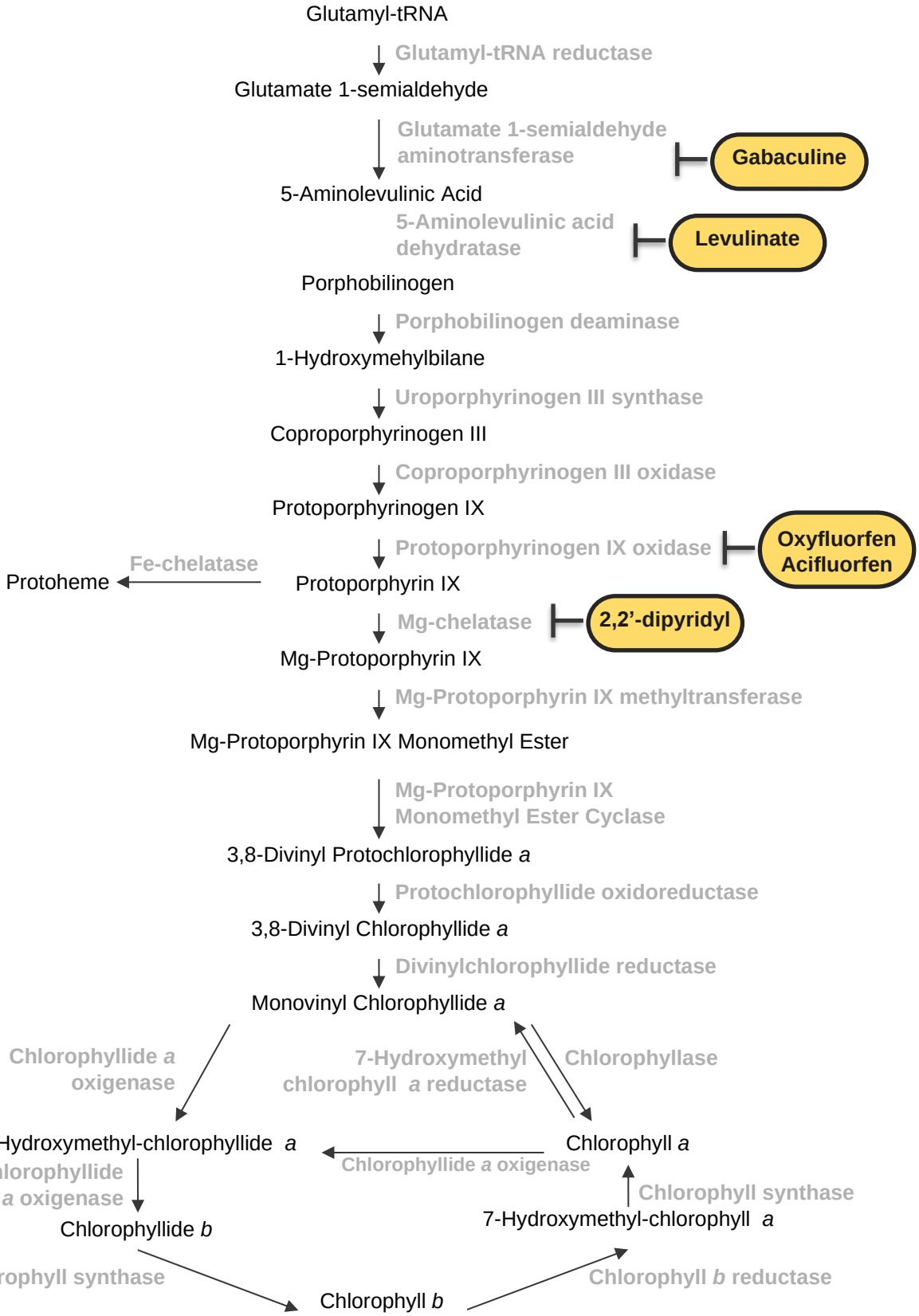

Figure 6. Chlorophylls biosynthetic pathway. In grey are the enzymes that catalyze each reaction. Inhibitors of this pathway are highlighted in yellow boxes. Adapted from Tanaka et al. (2010), Tanaka et al. (2007), Brzezowsky et al. (2015).[150–152]

the case of oxyfluorfen and acifluorfen, they are diphenyl ethers that inhibit the oxidation of protoporphyrinogen IX to protoporphyrin IX, by specifically targeting the protoporphyrinogen oxidase enzyme.[91,96] Gabaculine inhibits glutamate 1-semialdehyde aminotransferase, which converts glutamate 1-semialdehyde to 5-aminolevulinic acid.[95] As for levulinate, this compound inhibits the conversion of 5-aminolevulinic acid to porphobilinogen by inhibiting 5-aminolevulinic acid dehydratase.[94] Finally, it has been suggested that 2,2'-dipyridyl inhibits Mg-chelatase.[92,93]

In contrast to the examples shown above, environmental factors, such as the presence/absence of light, temperature, pH or CO_2, have also been used for microalgae mutant selection, aiming at different targets, namely tolerance to higher temperatures or adaptation to higher concentrations of CO_2, for example. [37,99,100]

Another strategy that can be applied to increase the effectiveness of a screening strategy is the combination of multiple screening and selection methods. For example, Yi et al. (2018) isolated carotenoid hyperproducing strains by combining the selection of strains resistant to diphenylamine, followed by an MTP-based screening for chlorophyll *a* and Nile red fluorescence.[61]

In summary, there are multiple ways to select mutants with improved characteristics being the results mainly dependent on the selective pressure that is applied. Nonetheless, there is still a need to develop new selection methods, mainly to identify phenotypes that are not distinguishable to the naked eye or through macroscopic properties, and to increase the efficiency of the selection process.

This work will focus on improving the organoleptic and nutritional characteristics of a *S. rubescens* strain resorting to EMS mutagenesis. To the author's knowledge, this is the first study regarding random mutagenesis with *S. rubescens* and one of the first reports on the use of chemical mutagenesis to mutate this genus. NF and Nic, inhibitors of the carotenoid's biosynthetic pathway, and InChlo[a], an inhibitor of the chlorophyll's biosynthetic pathway, combined with the absence of light, were also used in this work to select *S. rubescens* mutants with improved chlorophyll and protein contents. This is also the first study that applies a specific inhibitor of the chlorophyll biosynthesis pathway to select microalgal mutants with different pigmentation and protein content.

[a] InChlo will be used as a codified name for an inhibitor of the chlorophyll's biosynthetic pathway

2. Thesis Outline.

The main goal of this work was to generate novel *S. rubescens* mutants, through EMS random mutagenesis, with improved organoleptic and biochemical characteristics, such as decreased chlorophyll content and increased protein content. To achieve this objective, the experimental work was divided into four sections:

I. Dose-response study of the effect of EMS on the survival of *S. rubescens.* The objective of this study was to determine the susceptibility of this microalga to EMS and subsequently determine the concentrations of this mutagen to be used for random mutagenesis trials.

II. Metabolic inhibitors tests determined the effect of Nic, NF and InChlo on the growth of *S. rubescens*. These tests determined the concentration of each metabolic inhibitor that completely inhibits growth as well as the concentration right before that, which are the concentrations that should be tested for mutant selection.

III. Mutagenesis and mutant selection, in which mutants were generated using the concentrations of EMS previously determined and colonies with different characteristics were selected, using the metabolic inhibitors previously tested.

IV. Finally, mutants were characterized concerning heterotrophic growth, chlorophyll, carotenoids, and protein contents.

3. Material and Methods.

All the experiments herein described were carried out at Allmicroalgae Natural Products S.A., between the 3rd of March 2022 and the 30th of September 2022.

3.1. Inoculum and Culture Medium.

Scenedesmus rubescens axenic culture (0037SA) was obtained from Allmicroalgae Natural Products S.A. culture collection. Cultures were grown in the proprietary GM1 medium, containing glucose as carbon source, ammonium sulphate and urea as nitrogen sources, and supplemented with trace elements.

Culture manipulation was always conducted under sterile conditions, guaranteed by a laminar flow chamber (BioAir Top Safe® 1.5, Pero, Italy). Cultures were inoculated with a working volume of 50 mL, in 250-mL baffled Erlenmeyer flasks, previously sterilized by autoclaving at 121 °C for 40 minutes. Cells were grown heterotrophically in an orbital shaker (ArgoLab® shaker SKI 4, Carpi, Italy) at 28 ± 0.1 °C, under constant shaking (200 rpm) in the dark.

3.2. Growth Assessment and Sampling.

Cultures were sampled and analysed daily by measuring optical density (OD) at 600 nm (OD_{600}), using a UV-Vis spectrophotometer (Genesys 10S UV-Vis®; Thermo Fisher Scientific, Massachusetts, EUA). The culture was also analysed by optical microscopy (Axio Scope A1®, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The number of cells was determined using a Neubauer chamber (Hirschmann, Eberstadt, Germany) by direct observation under bright field microscopy. Images were acquired with a digital camera (Axiocam 503 color®, Carl Zeiss Microscopy GmbH, Oberkochen, Germany) assembled on the microscope.

Dry cell weight (DCW) was determined by filtering microalgal suspension using pre-weighed 0.7 µm glass microfibre filters (VWR International, Pennsylvania, USA) and washed with demineralized water to remove medium salts and cellular debris. Finally, the samples were dried at 120 °C and weighed using a moisture analyser (MA 50.R Moisture Analyzer, Radwag®, Radom, Poland).

The DCW was calculated by the difference between the weight of the filter with dried biomass (m_f) and the filter weight (m_i), divided by the volume of sample (V) (Equation 1). A correlation between OD and DCW was established (Figure 25 - Appendix).

$$DCW = \frac{(m_f - m_i)}{V} \quad (1)$$

The growth rate (µ) was obtained by Equation 2, whereas biomass productivity (P) was determined using Equation 3, where t_i and t_f represent the time when the exponential phase begins and ends, respectively, and DCW_i and DCW_f represent DCW at the beginning and end of the exponential growth phase, respectively.

$$\mu = \frac{\ln(OD_{600_f}/OD_{600_i})}{(t_f - t_i)} \quad (2)$$

$$P = \frac{(DCW_f - DCW_i)}{(t_f - t_i)} \quad (3)$$

3.3. EMS Dose-Response Curve.

To establish the dose-response curve of ethyl methanesulfonate (EMS; Sigma-Aldrich, St. Louis, USA) the *S. rubescens'* culture was exposed to different concentrations of this chemical mutagen. Wild-type (WT) inoculum in the early exponential phase (1.54×10^6 cells mL^{-1}) was concentrated 10-fold in GM1 medium diluted with sterile distilled water (1:2), by centrifuging the culture for 3 min at 3000 *g*, followed by supernatant discard and resuspension of the pellet. The concentrated culture was then treated with 100, 200, 300, 400, 500 and 600 mM of EMS and set under constant agitation (100 rpm), at 28 °C, for 1 h in the dark. The EMS reaction was stopped by adding sodium thiosulfate (5 % *w/w*), a neutralizing agent. Samples were then centrifuged for 1 min at 3000 *g* and the pellet was resuspended in the same volume of a solution of NaCl 0.9 %. The washing process with saline solution was repeated three times before resuspending the cultures again in GM1 medium, diluted with sterile distilled water (1:2). The mutagenized cells were incubated for 24 h in the dark at 28 °C. After this incubation time, cultures were serially diluted and plated in triplicates onto Plate Count Agar (PCA; HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated in the dark at 28 °C for 15-21 days. After this period, the colonies on each plate were counted to calculate the survival rate.

3.4. Metabolic Inhibitors Tests.

To select mutants with different pigmentation upon mutagenesis, metabolic inhibitors of either the carotenoids or chlorophylls biosynthetic pathways were applied, whose concentrations need to be determined previously.

To determine the optimal concentrations of each inhibitor, a culture in early-exponential phase was concentrated 10-fold in GM1 medium diluted with sterile distilled water (1:2), by centrifuging the culture for 3 min at 3000 *g*, followed by resuspension of the pellet. The concentrated culture was then spread in 6-well plates with PCA supplemented with different concentrations of each metabolic inhibitor, except for the control, which was PCA without metabolic inhibitors (plain-PCA). All the conditions were tested in triplicates.

Three different metabolic inhibitors were tested, norflurazon (NF; Sigma-Aldrich, St. Louis, USA) and nicotine (Nic; Tokyo Chemical Industry Co., Ltd, Tokyo, Japan), both inhibitors of carotenoid biosynthesis, and InChlo (Honeywell, Charlotte, USA), an inhibitor of chlorophyll biosynthesis. NF stock solution at 5.26 mM was prepared using acetone as a solvent and was filter-sterilized with 0.22 µm Branchia polytetrafluoroethylene (PTFE) filters (Labbox Labware, Barcelona, Spain). InChlo stock solution at 500 mg L^{-1} was prepared using methanol as a solvent, filter-sterilized with 0.22 µm Branchia polytetrafluoroethylene (PTFE) filters (Labbox Labware, SL, Barcelona, Spain). Nic was directly used at 6.23 M and filter-sterilized with 0.22 µm Branchia polyethersulfone (PES) filters (Labbox Labware, SL, Barcelona, Spain). Six concentrations of NF were tested: 5, 10, 15, 20, 25, and 30 µM, Nic was tested at 1, 2, 3, 4, 5 and 6 mM and InChlo was tested at 25, 50, 100, 150, 200, 300, 400, 500, 600 and 700 µg L^{-1}.

Cultures were incubated in the dark at 28 °C for 14 days, after which the growth in each well was observed and compared to the control. Since this visualization is difficult to rate, a sample was taken from each condition and streaked onto plain-PCA. After 3-5 days, the growth on these plates was

again visually analysed to determine the optimal concentration range of each inhibitor, which should be between the concentrations that allow minimal growth and no growth.

3.5. Random Mutagenesis and Mutant Selection.

The random mutagenesis protocol is similar to the establishment of the dose-response curve, differing on the concentrations of EMS to which the *S. rubescens* culture was previously exposed. For mutagenesis, only 150, 200 and 250 mM of EMS were used, according to the dose-response curve previously established, which correspond to a survival rate of 33.3 %, 8.9 % and 6.8 %, respectively. Mutagenized cultures were also serially diluted for the same EMS concentrations to verify if the cell counting was in accordance with the dose-response curve, as a control of the experiment. Furthermore, as a secondary control, non-mutagenized cells were also plated directly in plates with inhibitors to observe their inhibition/growth.

In order to identify mutants with different pigmentation upon mutagenesis, a selection step was applied by resorting to the metabolic inhibitors mentioned. The mutagenized cells were plated in PCA containing 20 or 30 µM of NF, 3 or 4 mM of Nic, or containing 100, 200 or 300 µg L^{-1} of InChlo. Plates were then incubated in the dark, at 28 °C, for 28 days. Colonies containing a different colour were picked and spread on PCA with and/or without metabolic inhibitors and re-streaked every week for ten generations to confirm phenotype stability.

3.6. Experimental Trials in Erlenmeyer Flasks.

After selecting and isolating the most promising mutants, their growth performance was characterized and compared in a lab-scale assay. All the experiments were performed in triplicate.

After selection and assessing mutants' stability in solid medium, mutants were transferred to liquid GM1 medium and grown at 28 °C, 200 rpm, with PIPES buffer, at pH 6.5 at 60 mM. The addition of buffer to the medium is important to avoid pH variability that might bias the results. The growth assay was conducted in 250-mL Erlenmeyer flasks, with a final working volume of 52 mL. Water evaporation throughout the assay was compensated according to the weight loss between different time points. The pH was measured by placing a drop of the culture on Metria universal pH test paper strips (Labbox Labware, SL, Barcelona, Spain). At the end of the assay, samples were centrifuged at 2000 *g* (VWR® Micro Star 12 microcentrifuge; Pennsylvania, USA) for 2 minutes, and the supernatant was retrieved to measure glucose. Glucose concentration was measured with Freestyle Precision Neo glucose kit (Abbott, Chicago, USA) and, if necessary, dilutions were performed with NaCl 0.9 % (*w/v*), accordingly.

3.7. Chlorophylls and Carotenoids Content.

To determine the chlorophylls and total carotenoid content of each mutant, pigment extraction and quantification was performed according to the Ritchie (2008)[101] method.

For each sample, a volume corresponding to 10 mg of biomass was centrifuged for 10 min, at 2547 *g* (Hermle® Z 300 centrifuge, Wehingen, Germany) in glass tubes, and the supernatant was discarded to remove the aqueous phase. Sequential zirconia bead milling was performed in acetone to extract the chlorophylls and carotenoids. For that, a mass of zirconia beads of approximately 2.85 g was

added to each sample, along with 6 mL of acetone. Samples were then agitated in a vortex for 15 min, followed by centrifugation for 10 min, at 2547 *g*. The supernatant of the samples was collected and kept in the dark until the absorbance was measured. The extraction step was repeated until the pellet became colourless to guarantee that all the pigments were extracted from the biomass. Quantification of chlorophylls and total carotenoids was performed by measuring the absorption spectrum of the supernatant (380 to 700 nm). Chlorophyll *a*, *b* and *a*+*b* were quantified by applying Equations 4, 5 and 6, as described by Ritchie (2008)[101]. Carotenoid total content was quantified with the Excel Add-In Solver on Windows 13 using an in-house developed approach.[102]

$$Chl_a = -0.3319Abs_{630} - 1.7485Abs_{647} + 11.9442Abs_{664} - 1.4306Abs_{691} \quad (4)$$

$$Chl_b = -1.2825Abs_{630} + 19.8839Abs_{647} - 4.8860Abs_{664} - 2.3416Abs_{691} \quad (5)$$

$$Chl_{a+b} = Chl_a + Chl_b \quad (6)$$

3.8. Determination of Protein Content.

Protein content was estimated by performing an elemental analysis (Vario EL®, Elementar Analyser System; GmbH, Hanau, Germany) according to the manufacturer's instructions and by multiplying the nitrogen content by a factor of 6.25.[103]

3.9. Statistical Analysis.

One-way ANOVA followed by Tukey HSD *post-hoc* multiple comparisons test, at a probability level of 0.05, was performed using GraphPad Prism version 8.0.1 for Windows, (GraphPad Software, San Diego, USA, www.graphpad.com). For each test, the mean and standard deviation were determined among biological triplicates. Graphical representation of the results was performed using GraphPad Prism version 8.0.1 for Windows.

4. Results and Discussion.

4.1. Dose-Response Study.

In general, the objective of a mutagenesis procedure is to obtain a modest number of mutations in a trait of interest, without significantly disrupting the genotypic integrity of the organism, which could result in loss of fitness.[76] Given this, the first objective of this work was to establish the survival rate of *S. rubescens* to different EMS concentrations. Different concentrations of the mutagen were tested, ranging from 100 to 600 mM. The effect of EMS on the survival rate of this species is represented by the dose-response curve in Figure 7.

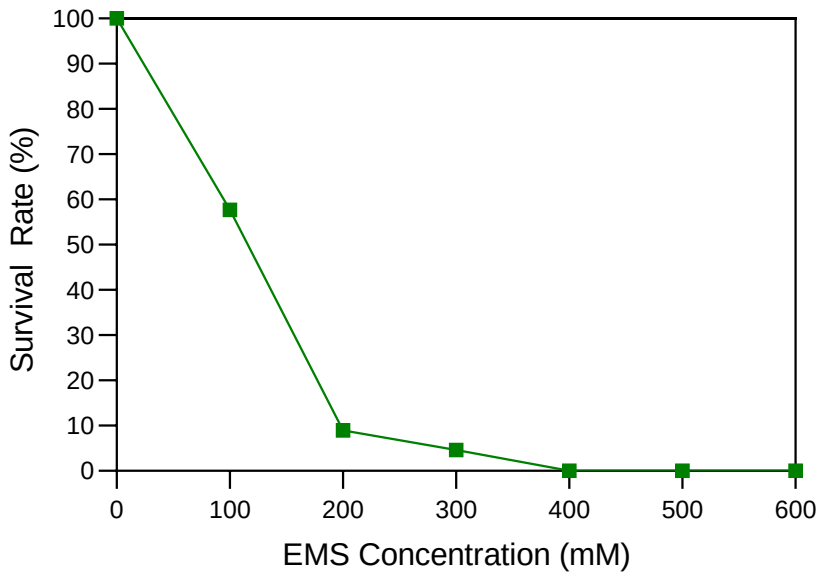

Figure 7. Survival rate of *Scenedesmus rubescens* exposed to different EMS concentrations.

In Figure 1, it is possible to observe that at 100 mM of EMS the survival rate was 58 %, at 200 mM was 9 %, at 300 mM was 6 % and at 400 mM was 0.1 %, while for the remaining concentrations, the survival rate was 0 %.

In a previous report, Zhang et al. (2018) determined the susceptibility of *Scenedesmus* sp. to EMS.[82] The authors incubated a *Scenedesmus* sp. (FACHB-489) culture with 200 mM EMS for 1 h, and directly plated on solid BG11 medium. Plating was followed by an incubation in the dark for 24 h and subsequent incubation under 46 μmol photons m^{-2} s^{-1}, at 25 °C, for 2 weeks. It was reported a survival rate of 46 %, 5-fold higher than the 9 % obtained with *S. rubescens* exposed to 200 mM of EMS in the present work. The difference in the results might be related to several reasons, such as the species used, cells' concentration, growth stage and culture medium.[87] Zhang et al. (2018) utilized a cell concentration that is 14 times higher than the one used in this work, 2.20×10^7 cells mL^{-1} versus 1.54×10^6 cells mL^{-1}, which is one of the justifications for the significant differences in the susceptibility to EMS. The presence or absence of light is also widely recognized as a significant factor since it might increase cells' susceptibility to the mutagenic agent and/or induce light-dependent DNA-repair mechanisms.[80,87] No more reports were found regarding the use of EMS to mutagenize

Scenedesmus spp. In addition, this is the first report concerning the application of EMS to mutagenize *S. rubescens*.

Since there are no more reports on the use of EMS on *Scenedesmus*, it is worth taking a look at *C. vulgaris*, a closely related example. For *C. vulgaris*, there are several reports using a wide range of EMS concentrations, that obtained different susceptibilities to EMS, such as 240 mM corresponding to <1 % survival rate, 300 mM corresponding to 5-10 % survival rate or 160 mM, almost half the concentration, corresponding to the same 5-10 % survival rate. [37,39,84] As previously mentioned, these differences can be associated with different mutagenesis protocols.[87] *Schüler et al.* (2020) applied a similar protocol to the one used in this work, to obtain *C. vulgaris* mutants with EMS, but instead using a cell concentration of 3.20×10^6 cells mL^{-1}.[37] In that study, *C. vulgaris* exhibited a 10 % survival rate under 300 mM of EMS. These results indicate that this *S. rubescens* strain might have an increased susceptibility to EMS, compared to that *C. vulgaris* strain. However, it is important to consider that a different cell concentration of *C. vulgaris* was used, of 3.20×10^6 cells mL^{-1}, which is 2-fold higher than the cell concentration used in this work.

The results obtained from the dose-response study were necessary to establish the conditions for random mutagenesis experiments. The survival rate should be between 5-10 %[37], for this reason, the optimal EMS concentrations to test on mutagenesis trials with *S. rubescens* were in the range of 200-300 mM in the conditions mentioned above.

4.2. Metabolic inhibitors Tests.

Random mutagenesis generates a large number of mutants with a wide range of phenotypes. To isolate the mutants with the desired phenotype, selection using metabolic inhibitors can be applied as a selective pressure, where only the mutants with increased resistance to this pressure can thrive.[88] For this purpose, the effect that different metabolic inhibitors have on the growth of *S. rubescens* was also studied. Three different inhibitors were tested at different concentrations, NF and Nic, inhibitors of carotenoid biosynthesis, and InChlo, an inhibitor of the chlorophyll biosynthetic pathway. In summary, the concentrated culture was plated with the respective inhibitor concentrations to analyse if the culture was able to grow or if that inhibitor concentration killed all the viable cells. The optimal inhibitor concentration to use for mutant selection should be between the concentrations that allow minimal growth and no growth, to assure that the surviving colonies acquire a suitable mutation that grants them resistance to these chemicals and a stable phenotype.[36,85] The results are displayed in Figure 8-10.

In the presence of Nic, the culture acquired a more yellowish colour (Figure 8, left), visible in every Nic concentration tested, when compared to the control. In addition, when each condition was streaked onto plain-PCA (Figure 8, right) it was possible to observe Nic's effect on the culture growth. *S. rubescens* was able to grow at 1, 2 and 3 mM of Nic. However, this growth was increasingly inhibited with an increase of Nic to 4 and 5 mM. At a Nic concentration of 6 mM the culture was no longer able to grow. Hereupon, the concentrations of Nic to be tested should be from 3 to 6 mM. In the literature, no report was found on the study of Nic as an inhibitor of *Scenedesmus* growth, nor as a selection strategy

for *Scenedesmus* spp. mutants. However, this inhibitor has been used for other microalgal species, to select mutants with improved carotenoid production after mutagenesis.[98,104,105]

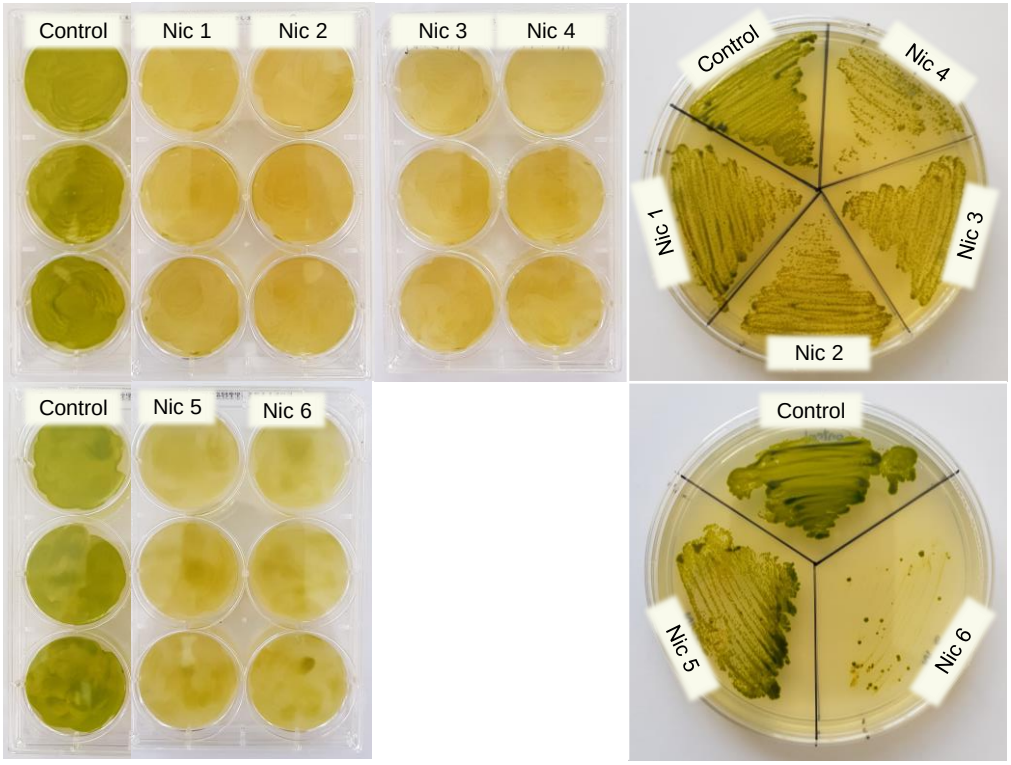

Figure 8. Nicotine (Nic) test. A *Scenedesmus rubescens* culture in exponential growth phase was first plated in PCA with nicotine (left), which after 14 days was streaked onto plain-PCA (right). Nicotine concentration is represented in mM.

Cordero et al. (2011) sought to isolate a high-lutein producing *Chlorella sorokiniana* by resorting to NTG random mutagenesis and selection of mutants resistant to inhibitors of the carotenogenic pathway, Nic and NF.[98] In their work, the minimum Nic concentration at which the growth of WT *C. sorokiniana* was inhibited was 0.4 mM. Similarly, Chen et al. (2017) also isolated high-lutein producing *C. sorokiniana* mutants, generated by NTG random mutagenesis, by resorting to 0.4 mM of Nic.[105] In another work, Chen et al. (2003), used 0.2 mM of Nic to isolate *Haematococcus pluvialis* mutants with increased astaxanthin production after EMS or UV mutagenesis.[104] The values found in the literature for mutant selection using Nic are significantly lower than here observed for *S. rubescens*, whose culture was able to grow up until 3 mM. The lower susceptibility observed for *S. rubescens* might be due to the difference in species stress resistance or in the different protocols used to determine resistance to Nic.

The yellow colour that is observed with increasing Nic concentrations might be due to an alteration in the pigmentation profile. Previous reports studying the effect of Nic have described not only an accumulation of acyclic carotenoids such as lycopene but also a decrease in chlorophyll content.[106,107] Nic is a known inhibitor of lycopene β-cyclase and prevents carotenoids cyclization (Figure 5). By hindering carotenoid cyclization, total carotenoid content decreases, more specifically α-carotene, β-carotene and lutein content.[106] The decrease in chlorophyll content might be related to lutein deficiency, which is essential for the assembly of active photosystem II units, leading to abnormal chloroplast formation.[106]

In the presence of NF (Figure 9), the colour of the culture changed from dark green to light green. From 5 to 30 μM of NF, it is clear that the culture was progressively inhibited. However, with increasing concentrations, up until 70 μM, the culture seemed to recover the ability to grow. When these cultures were streaked in the absence of NF, the culture maintained its ability to grow, regardless of the concentration of NF tested. Notwithstanding, it is possible to see a colour change in the cultures previously plated in 50, 60, and 70 μM of NF, indicating that cells are affected by this inhibitor at these concentrations. To the author's knowledge, NF has not been tested in *Scenedesmus* either.

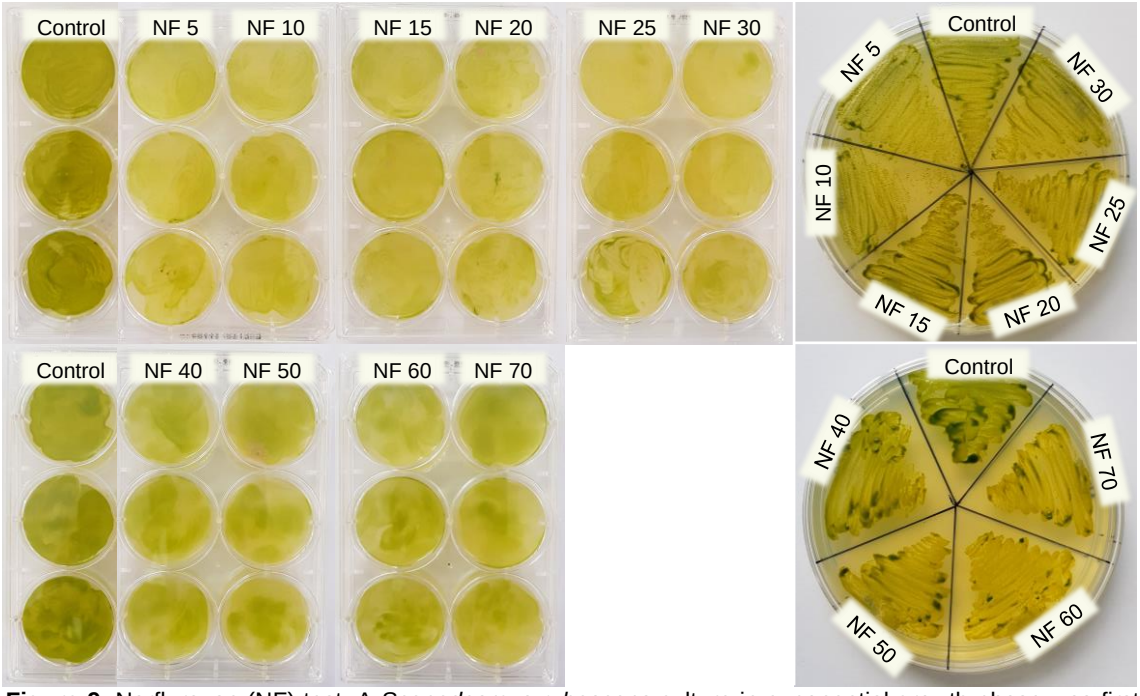

Figure 9. Norflurazon (NF) test. A *Scenedesmus rubescens* culture in exponential growth phase was first plated in PCA with norflurazon (left), which after 14 days was streaked onto plain-PCA (right). Norflurazon concentrations are represented in μM.

Other reports studying NF have described that this metabolic inhibitor effectively hinders the growth of *C. sorokiniana* at 4 μM[98] and the growth of *C. vulgaris* at 10 μM.[37] Given this, it would be expected that the concentrations tested would lead to inhibition of this microalgae's growth. Still, it always depends on other factors besides species, such as cell concentration, growth stage, and presence of light, among other protocol-related factors. [14]

It is possible that the culture might have survived either by using the inhibitor itself as a substrate, as it has been seen in other studies for Nic, where at low concentrations, it stimulated growth.[107] Another explanation for these results is apoptosis, where the cells that were more susceptible to NF underwent programmed cell death, which provided the necessary nutrients for other cells to remain healthy. These phenomena hindered the analysis of the results, since it was difficult to determine the range between survival and death and the optimal concentrations to test in mutant selection. However, additional studies would have to be done to confirm these hypotheses. Additionally, these results showed that *S. rubescens* is resistant to NF concentrations up to 70 μM. Despite being much higher than the values found in the literature, the NF concentrations tested were still below the threshold that kills *S. rubescens* cells, so higher concentrations could be tested. Nevertheless, since the culture acquired a different

yellowish colour, and also based on other reported *C. vulgaris* results obtained with the same protocol, 20-30 μM of NF were tested.

Regarding the lighter green colour that the cultures acquired in the presence of this inhibitor, reports have shown that NF can alter the pigment profile by inhibiting carotenoids biosynthesis (Figure 5). In a study performed by León et al. (2005), the incubation of a *Dunaliella bardawil* culture with NF led to an accumulation of phytoene and a proportional decrease in β-carotene. The authors also observed an increase in zeaxanthin, derived from a decrease in violaxanthin, along with the same chlorophyll contents.[108] Similarly, Xu and Harvey (2021) have also observed an increase in total carotenoids content and accumulation of phytoene in a *D. salina* culture treated with NF.[109]

As NF and Nic act on the carotenoid's biosynthetic pathway, they have been used to isolate randomly generated mutants with increased carotenoids production, such as accumulation of phytoene, lutein and astaxanthin, and decreased chlorophyll content.[37,98,104,105] These inhibitors have also been utilized to isolate mutants with increased eicosapentaenoic acid content, and whose accumulation is not directly related to the inhibition of the carotenoids biosynthetic pathway.[110] However, carotenoid and lipid production have been reported to be simultaneously enhanced in stress conditions, such as high light and nitrogen deficiency, which could be related to their antioxidant activity and indicate a link between the two metabolisms.[111]

Finally, when testing InChlo (Figure 10), the culture acquired a brownish colour, which was enhanced with increasing inhibitor concentrations. Additionally, when cultures were streaked to plain-PCA, it was possible to see that concentrations higher than 300 μg L^{-1} completely inhibited culture growth. Furthermore, the cultures that were plated in InChlo concentrations higher than 100 μg L^{-1} maintained the colours acquired in the presence of the herbicide. Likewise, concentrations between 100-300 μg L^{-1} were selected as the range to test in mutant selection.

As the use of inhibitors of the chlorophyll biosynthetic pathway in microalgae mutagenesis and selection is a novel approach developed in this work, no reports of microalgae resistance to this inhibitor on solid media were found. Nevertheless, Geoffroy et al. (2002) studied the effect of oxyfluorfen on an autotrophic liquid culture of *S. obliquus*.[91] Their study determined that the inhibitory concentrations (IC) of the growth of *S. obliquus* after a 24 h treatment were IC_{10} 3 μg L^{-1}, IC_{50} 15 μg L^{-1}, IC_{90} 22 μg L^{-1}.[91] Although not directly comparable due to significant differences in testing conditions, these values are much lower than the 300 μg L^{-1} of InChlo that were found to inhibit culture growth completely. The difference in the concentrations might be related to the species tested and the incubation with light, which increases photodynamic stress and non-enzymatic lipid peroxidation, promoted by protoporphyrin IX (Figure 6), a photosensitizing compound.[112] Cheng et al. (2015) also studied the toxicity of a different inhibitor of the chlorophyll biosynthetic pathway, acifluorfen, on the autotrophic growth of a liquid culture of *S. obliquus*.[113] The authors determined that the effective concentration of acifluorfen, resulting in a 50 % reduction in population growth relative to the control (after 96h) is 340.5 μg L^{-1}. Similarly to the previous comparison, as the testing conditions were different, it is not possible to make conclusions

directly from this report. However, the value reported by Cheng et al. (2015)[113] is closer to the value seen to inhibit *S. rubescens* growth in this work.

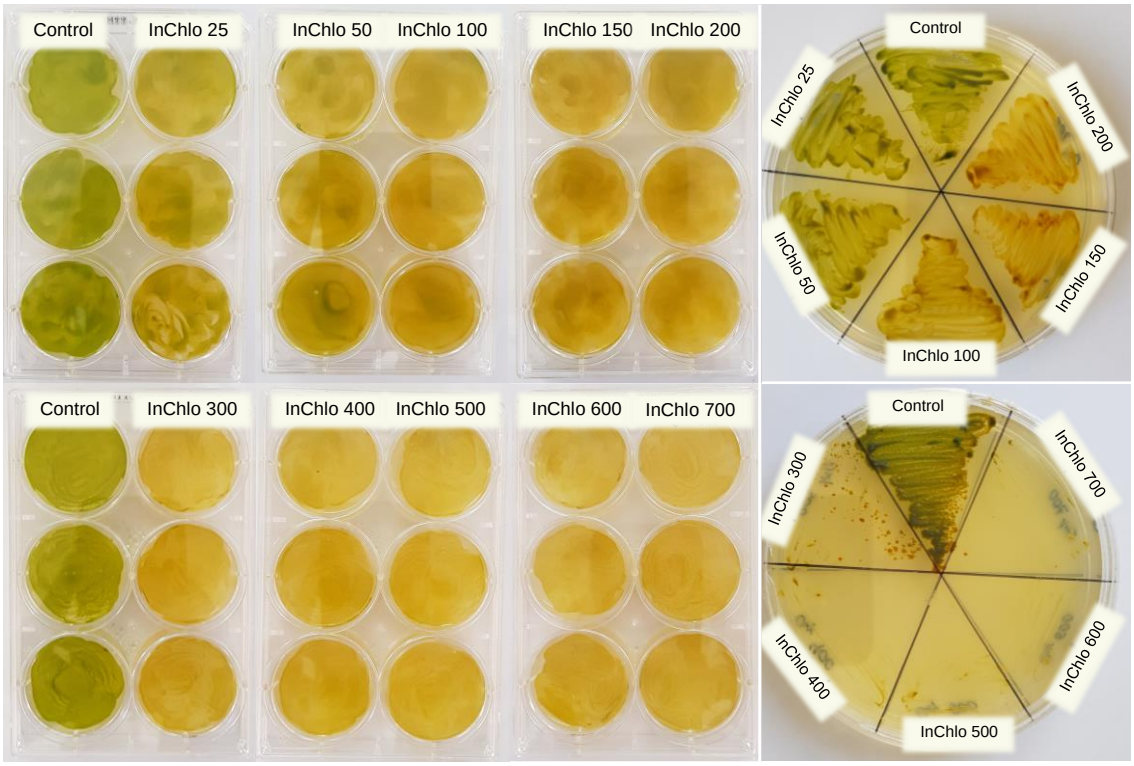

Figure 10. InChlo test. A *Scenedesmus rubescens* culture in exponential growth phase was first plated in PCA with InChlo (left), which after 14 days was streaked onto plain-PCA (right). InChlo concentrations are represented in μg L^{-1}.

The brown colour that was observed on the cultures incubated with InChlo might be related to a decrease in chlorophyll concentration, given the inhibition of the enzyme protoporphyrinogen oxidase of the chlorophyll biosynthetic pathway (Figure 6), which might explain the prominent brown/orange colour.[113,114]

4.3. Mutagenesis, Selection and Isolation of Mutants.

As previously mentioned, mutant selection represents the limiting step of a mutagenesis study. Due to the number of mutants generated and the complexity of identifying different phenotypes, multiple approaches have been developed to isolate the mutants of interest. In this work, three strategies were used with the objective of isolating *S. rubescens* mutants with improved protein content and pigmentation. The first strategy was isolating mutants obtained in the dose-response study by visual appearance (size and colour), without the use of metabolic inhibitors. After this, the second and third strategies consisted in isolating mutants through random mutagenesis, by plating the mutagenized cultures directly in PCA containing the metabolic inhibitor. After isolating the mutant colonies in metabolic inhibitors, the colonies were re-streaked in either plain-PCA, or PCA containing metabolic inhibitors for at least 10 generations.

The first approach used in this work was the isolation of mutants generated in the dose-response study. Although metabolic inhibitors increase the probability of isolating mutants with the intended

characteristics, mutants with alterations in their pigmentation can appear spontaneously after treatment with EMS. Besides pigmentation, this study also aimed to isolate mutants with improved growth performances, which have been associated with faster, bigger and darker colonies.[115] Figure 11 displays 3 mutants that were isolated from the dose-response study. Mutant 200a was generated using 200 mM of EMS from a large colony that arose before the remaining colonies on the plate. Mutants 300b and 300d were generated using 300 mM of EMS. Mutant 300b's colony had an orange colour, while mutant 300d was selected because it was a large colony.

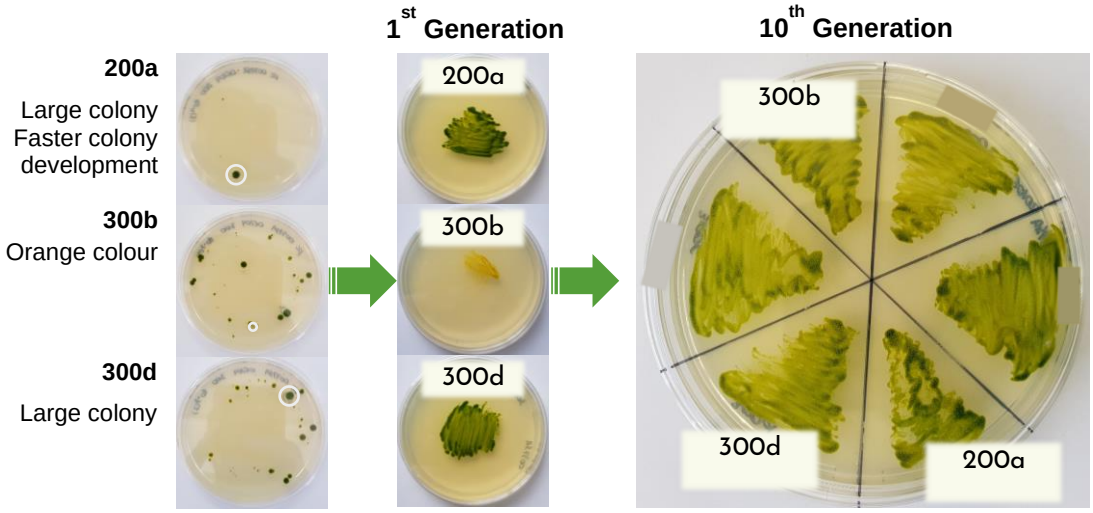

Figure 11. Diagram of the isolation of *Scenedesmus rubescens* mutants from the dose-response study, in the absence of metabolic inhibitors. 200a, 300b and 300d are the nomenclatures given to the mutants, where the number, 200 or 300, represent the concentration EMS that was used to generate them.

Mutant colonies were picked and re-streaked in plain-PCA for at least 10 generations to evaluate phenotype stability. In the 1st generation, it is possible to see that mutant 300b maintained its altered pigmentation. However, this phenotype was only stable for 4 generations, and by the 10th generation, it had a green colour similar to the WT. Phenotypic instability is a phenomenon that is common in microalgae mutagenesis, but that is improved by the use of effective selection methods, which in this case were absent.[36,116] Despite the green colour, the three mutants were selected for further studies, as they could have increased growth performance or protein content. These results show that this approach did not successfully result in the isolation of mutants with altered colours.

Two rounds of random mutagenesis were applied to isolate mutants with different colours, and the metabolic inhibitors previously tested were used as selection tools. Both the dose-response study and the metabolic inhibitors tests were essential for establishing the conditions to be used during mutagenesis and selection steps. However, before choosing the conditions to perform mutagenesis, it was important to consider that the combined detrimental effect of the metabolic inhibitors, together with the mutagenic agent, might be too harsh, even for colonies with mutations that grant them resistance to each of these stressors. Given this, it was important to work with a range of concentrations near and below the limit to account for the increased stress. A summary of the conditions tested is shown in Table ***3***. A representation of some of the plates obtained after mutagenesis, which contain the colonies that grew on metabolic inhibitors, is represented in Figure 12 and 13.

Figure 12 shows that the WT non-treated culture was viable (Control) and that multiple differently coloured mutant colonies appeared on the plates with inhibitors.

Table 3. Summary of the conditions for mutagenesis. Mutagenesis was performed with ethyl methanesulfonate (EMS). Mutant selection was performed in nicotine (Nic), norflurazon (NF), and InChlo.

EMS (mM)	Nic (mM)	NF (µM)	InChlo (µg/L)
1st Round			
150	3	20	100
200	4	30	200
2nd Round			
200	3	20	200
250	4	30	300

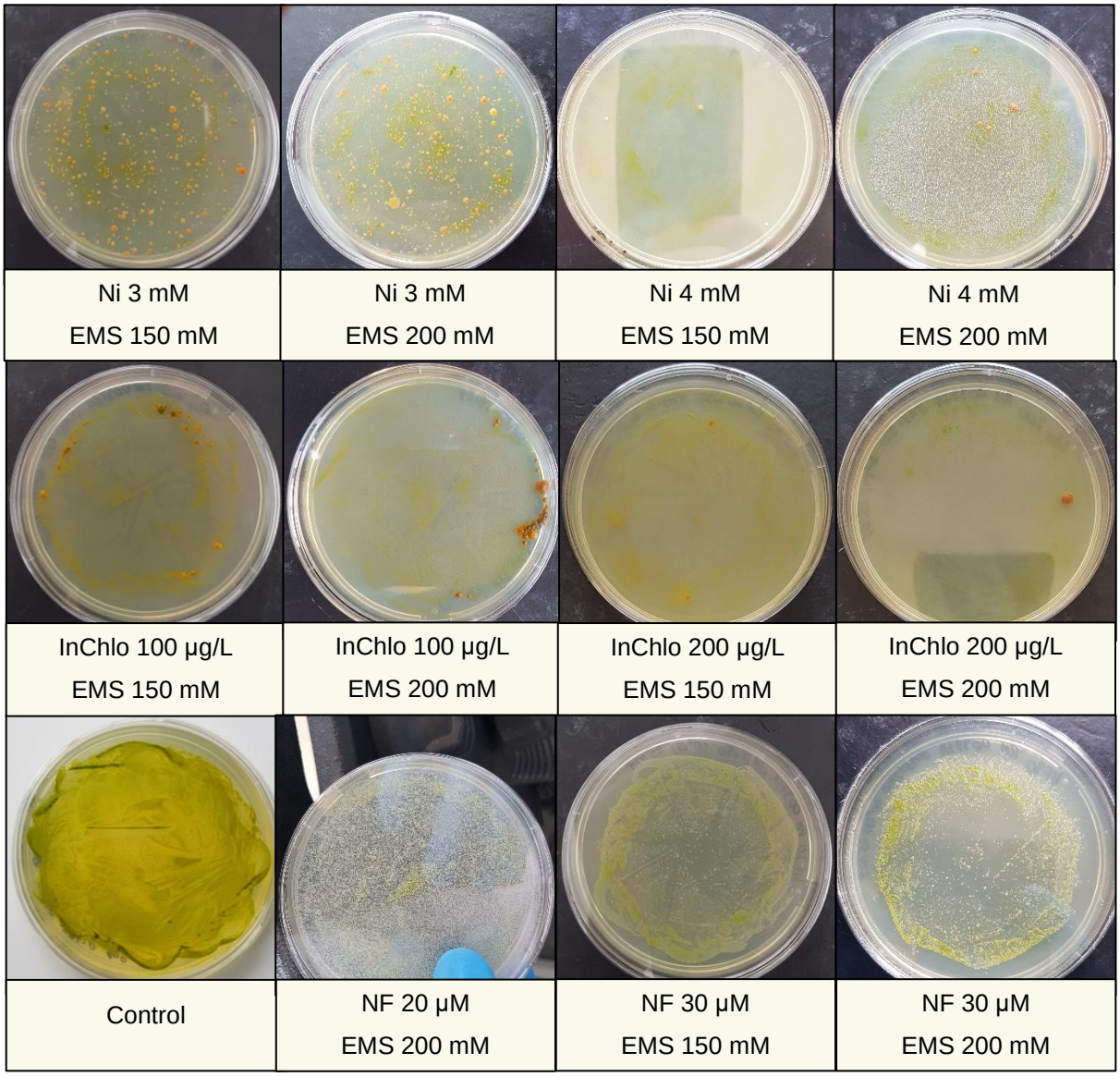

Figure 12. Plates of *Scenedesmus rubescens* cultures derived from the 1st round of mutagenesis. Control represents a non-mutagenized culture without any metabolic inhibitor. The remaining conditions represent an EMS mutagenized culture, which was plated in the presence of the corresponding metabolic inhibitor.

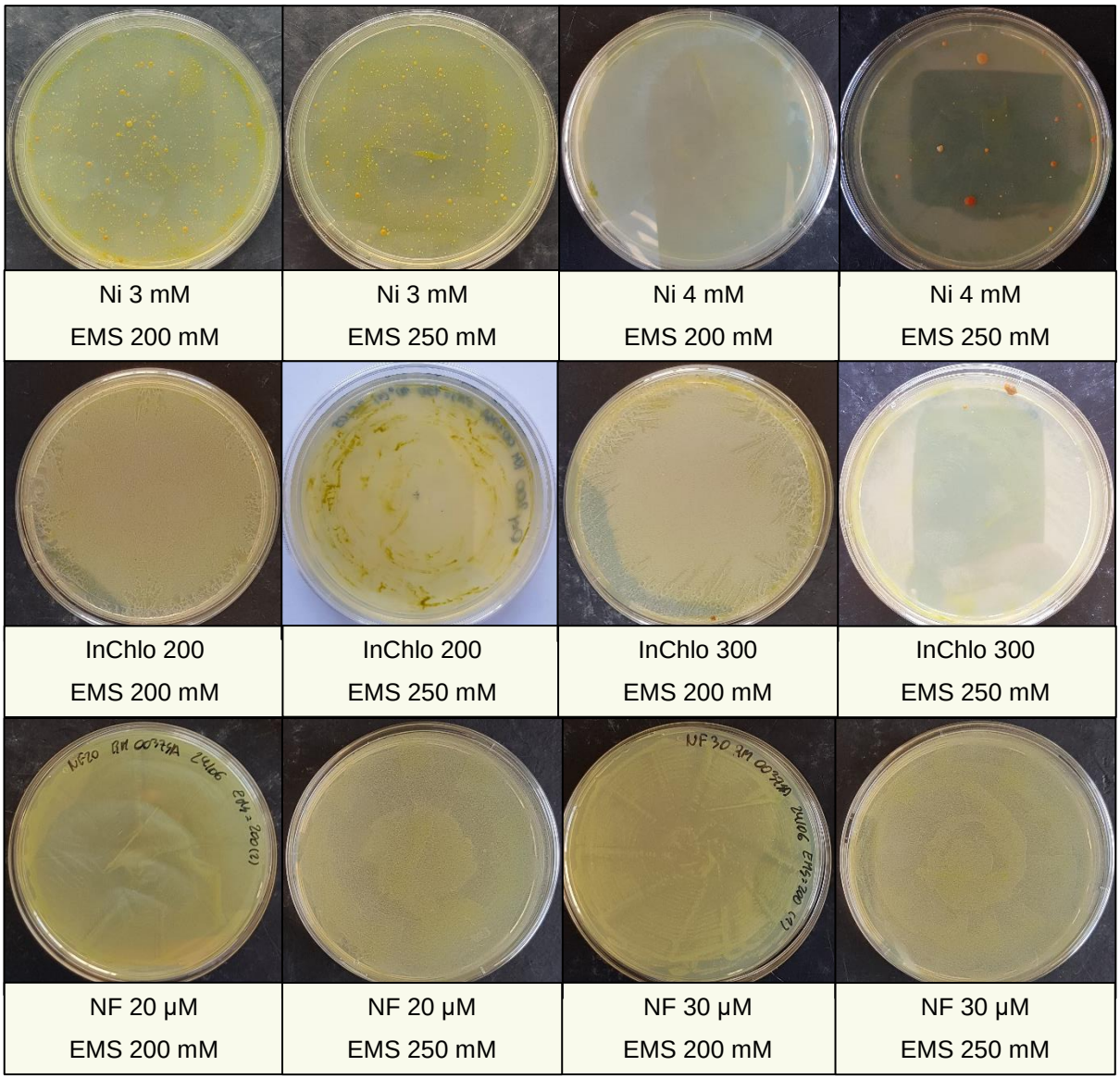

Figure 13. Plates of *Scenedesmus rubescens* cultures derived from the 2nd round of mutagenesis. Control represents a non-mutagenized culture without any metabolic inhibitor. The remaining conditions represent an EMS mutagenized culture, which was plated in the presence of the corresponding metabolic inhibitor.

Although not represented herein, other controls were performed, such as plating non-mutagenized culture in plates with the metabolic inhibitors and plating dilutions of the mutagenized culture in plain-PCA. These controls all performed according to what was seen in the dose-response study and the metabolic inhibitors' tests.

In the 1st round of mutagenesis, plates with Nic 3 mM showed a high number of colonies (uncountable) with many different colours, ranging from white to yellow, orange and red. The plates with Nic 4 mM also exhibited colonies with many different colours, as seen in the Nic 3 mM plates, but there were much fewer. Additionally, the colonies observed in the Nic 4 mM plates took considerably longer to appear than in the Nic 3 mM. Using a higher Nic concentration will result in a higher selective pressure, which can result in the isolation of more stable mutants.

The plate with InChlo 100 µg L^{-1} displayed few colonies with yellow, brown, orange and green colours. Similar to what was observed in the Nic plates, increasing the concentration of InChlo to

200 µg L^{-1} decreased the number of colonies, and they took longer to appear in the plates. However, the colonies appeared clumped together, probably due to an inefficient spreading technique, which complicated colony isolation.

The NF plates displayed uncountable small green and white colonies. The number of colonies in these conditions was too high, which hampered the isolation of single colonies with stable mutations. Taking into account these results, in the 2nd round of mutagenesis, the mutagen concentration was increased, as it would reduce the number of colonies generated. However, it is possible to observe in Figure 13 that the NF plates maintained a high number of resemblant colonies, which indicated that selection using NF was not as effective as with the other inhibitors. The colonies from the NF plates were streaked onto plain-PCA and they were not able to grow. Additionally, the isolated mutants were also observed under the microscope (Figure 14), where it was seen that the cells were dead, as they didn't possess any cellular content. This result again reflects the difficulty in tuning the optimal NF concentration to work with. Contrary to what was expected by the results attained in the inhibitors assays, which pointed out an insufficient NF concentration, it seems that the combination of NF and EMS concentrations was too harsh since the colonies that appeared on the NF plates were not able to grow after streaking in plain-PCA. Thus, for the same EMS concentrations, it could be interesting to test lower concentrations of NF. Additionally, these results highlight the importance of metabolic inhibitor tests. Furthermore, from analysing the results of Nic and InChlo plates, it seems that the concentration of metabolic inhibitor had a higher impact on the number of colonies than the mutagen concentration since the number of colonies and their viability was similar on the two mutagenesis rounds. Thus, the identification of the optimal metabolic inhibitor concentration for mutant selection is a critical step.

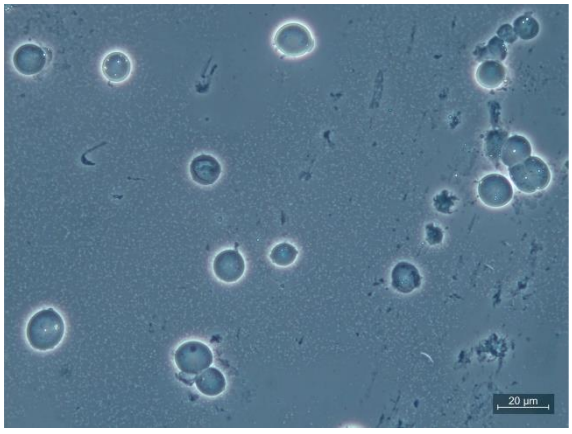

Figure 14. Phase contrast microscopy with a pH1 filter, of a mutagenized *Scenedesmus rubescens* culture isolated from an NF 30 µM plate. Scale bar: 20 µm.

From the Nic and InChlo plates of 1st round of mutagenesis, colonies with different colours were picked from the several plates and streaked onto plain-PCA. Additionally, the mutants were re-streaked for at least 10 generations to evaluate phenotype stability. As the interest of this work was mainly isolating mutants with altered pigmentation and stable phenotypes, the mutants that changed colour and/or turned green after plating in the absence of metabolic inhibitors were discarded.

With this methodology, it was possible to isolate three different mutants: W1 (avocado-green), W2 (army-green) and Y1 (brown-green) (Figure 15). Mutants W1 and W2 were obtained using 200 mM of EMS and selected using 3 mM of Nic. Similarly, Y1 was generated using 200 mM of EMS, but instead selected with 100 µg L^{-1} of InChlo.

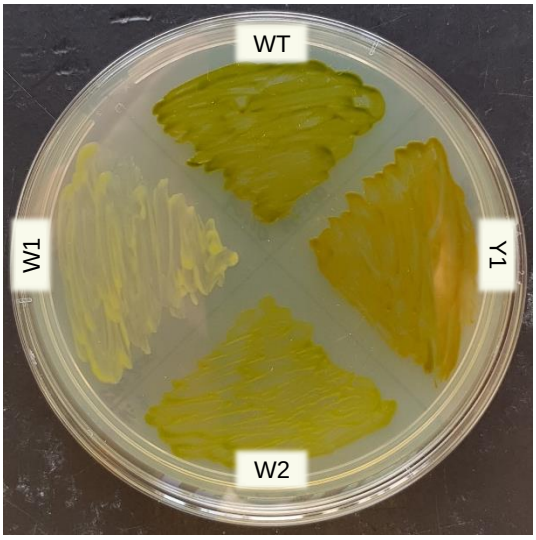

Figure 15. Wild-type and W1, W2 and Y1 mutants of *Scenedesmus rubescens*.

Instead of streaking the mutant colonies directly onto plain-PCA, streaking them onto plates containing the same concentration of metabolic inhibitors was also tested with colonies from the 2nd round of mutagenesis (Figure 16). By streaking colonies in the respective metabolic inhibitor concentration, colonies' selective pressure and subsequent phenotype of the colonies were maintained, allowing the isolation of mutants with different colours. After 10 generations, the mutants were then streaked onto plain-PCA to verify if their phenotypes were stable in the absence of the metabolic inhibitor.

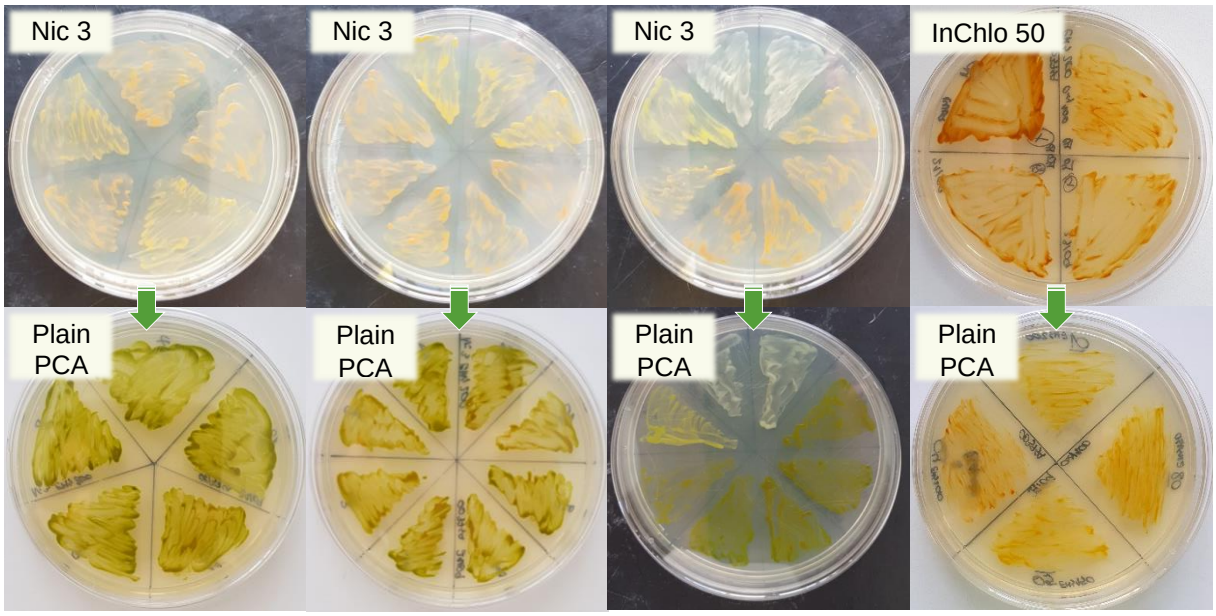

Figure 16. (Top) Isolated *Scenedesmus rubescens* mutant colonies after being streaked for 10 generations in PCA containing the metabolic inhibitors from which they were isolated. Nicotine (Nic) concentrations are in mM. InChlo concentrations are in µg L^{-1}. **(Bottom)** Mutant colonies isolated with metabolic inhibitors after one generation in plain-PCA.

By resorting to this approach, it was possible to obtain mutants with yellow, orange and white colours in the Nic plates. Additionally, since their responses to the same stress are different (different pigmentation profiles), it suggests that mutagenesis led to the introduction of mutations in the genome and that different genes were affected. However, when these mutants were plated in the absence of Nic, all of them, except for three, reverted their phenotype to green, which means that some mutations were not stable. Mutant W5, a white mutant on solid medium, was isolated and grown into a liquid culture (Figure 17), where it acquired a mint-green colour.

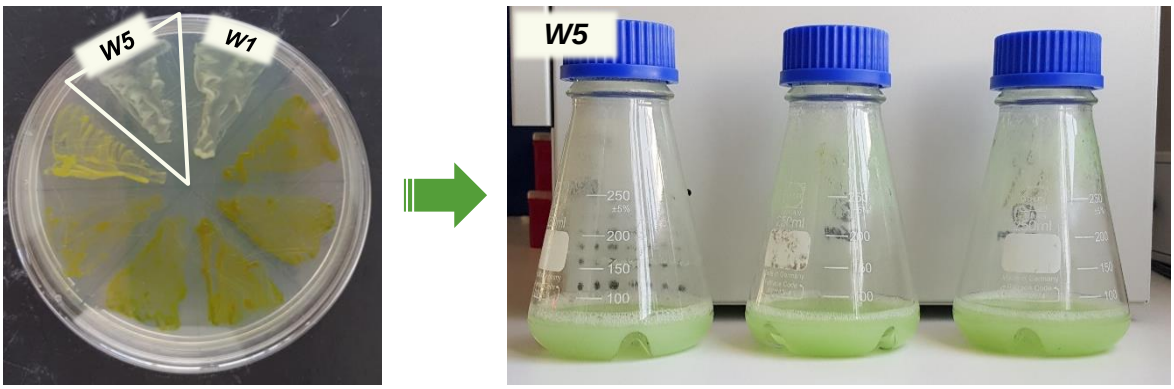

Figure 17. Isolation and scale-up of W5 mutant of *Scenedesmus rubescens*.

In the case of the InChlo mutants, they were picked from InChlo 100 µg L^{-1} plates and initially streaked in the same concentration. However, after a few generations, the colonies were growing very slowly, so the concentration of InChlo was decreased to 50 µg L^{-1}, after which the mutants started to grow again and kept their original colour. Although the mutants have increased resistance to InChlo, it still hinders their growth at high concentrations. After 10 generations, when these mutants were plated onto plain-PCA, they were able to maintain their orange colours. Afterwards, they were placed into liquid medium (Figure 18), where they weren't able to maintain the colour exhibited in the plates.

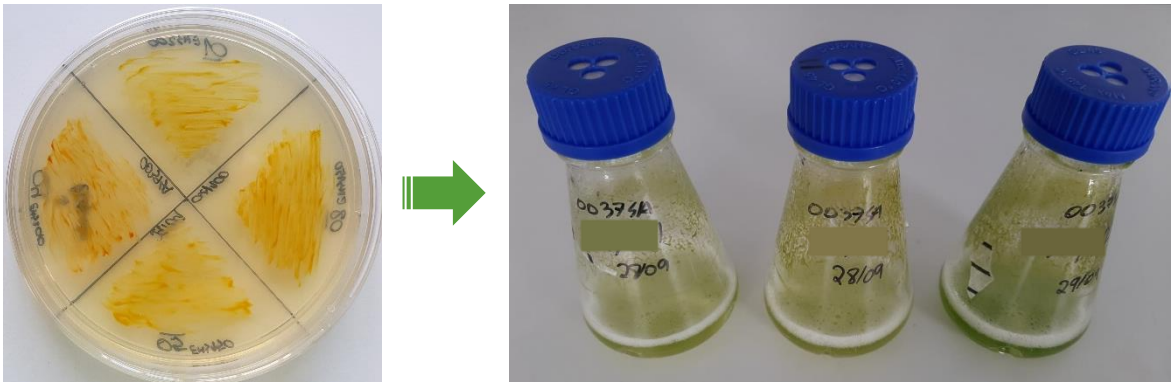

Figure 18. Isolation and scale-up of *Scenedesmus rubescens* mutants isolated from InChlo plates.

These colour alterations might have occurred for different reasons. Firstly, the mutation(s) could be unstable and, under altered conditions, the phenotype changed. Changing from a solid to a liquid medium comes with several alterations, namely much higher nutrient abundance, oxygenation and agitation, which might trigger phenotypic shifts and reversion of a mutation that remained stable in a solid medium.[117] On the other hand, nutrient availability has been reported as an impactful factor on the pigmentation of cultures of *S. rubescens*, which shifted from green to orange under N and P depletion.[53]

Thus, these mutants might have been able to keep their phenotype if the culture medium was optimized towards maintaining an orange colour.[53]

Overall, the results obtained with this isolation strategy showed that streaking the mutant colonies for 10 generations in the presence of the respective metabolic inhibitors allowed the maintenance of the selective pressure so that the mutants maintained their phenotype. Nonetheless, this approach was time-consuming and can also be misleading because the phenotypes may not be stable in the absence of the metabolic inhibitor, which takes some time to ensure (at least 10 generations). To improve this methodology, mutant colonies could eventually be streaked in the presence of the metabolic inhibitor for 5 generations, and the remaining 5 generations in plain-PCA, for example.

Through the multiple selection protocols used, 7 mutants were isolated for further studies: an avocado-green mutant, W1, an army-green, W2, a brown-green, Y1, a mint-green, W5, and 3 green mutants, 200a, 300b and 300d (Figure 19). All the mutants were generated using 200 mM of EMS. In addition, W1, W2 and W5 were isolated from Nic 3 mM, and Y1 was isolated from InChlo 100 µg L^{-1}. A summary of the applied selection strategies and the mutants isolated using each one of them is shown in Figure 20.

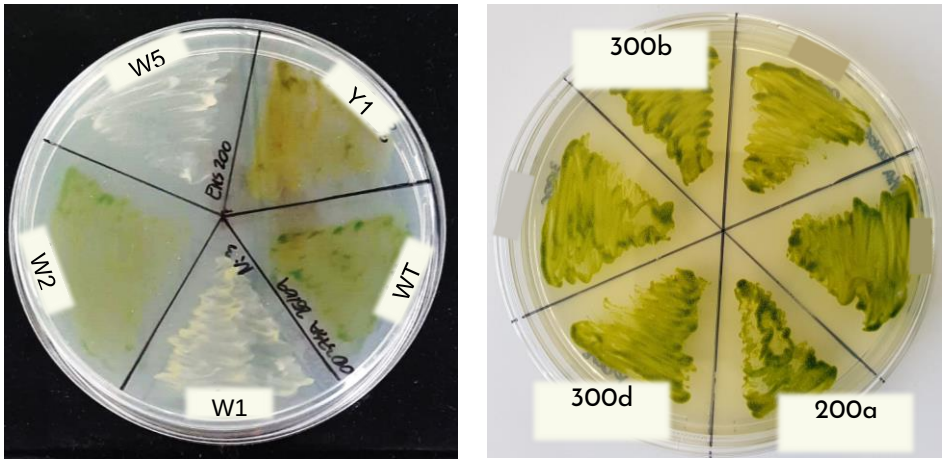

Figure 19. (Left) Wild-type and W1, W2, Y1 and W5 mutant strains of *Scenedesmus rubescens*. (Right) Mutants 200a, 300b and 300d of *Scenedesmus rubescens*.

This work marks the first successful application of EMS mutagenesis on *Scenedesmus* spp. Previously, only Zhang et al. (2018) reported the application and comparison of UV and EMS mutagenesis on *Scenedesmus* sp.[82] The results showed that the UV mutant had increased protein and lipid yield, along with increased tolerance to CEW. However, during the selection process, where the mutant cells were plated on undiluted CEW, no EMS mutants were obtained, leading the authors to suggest that UV mutagenesis is more suitable for generating *Scenedesmus* sp. mutants. No other report describing the application of EMS mutagenesis on the *Scenedesmus* genus was found.

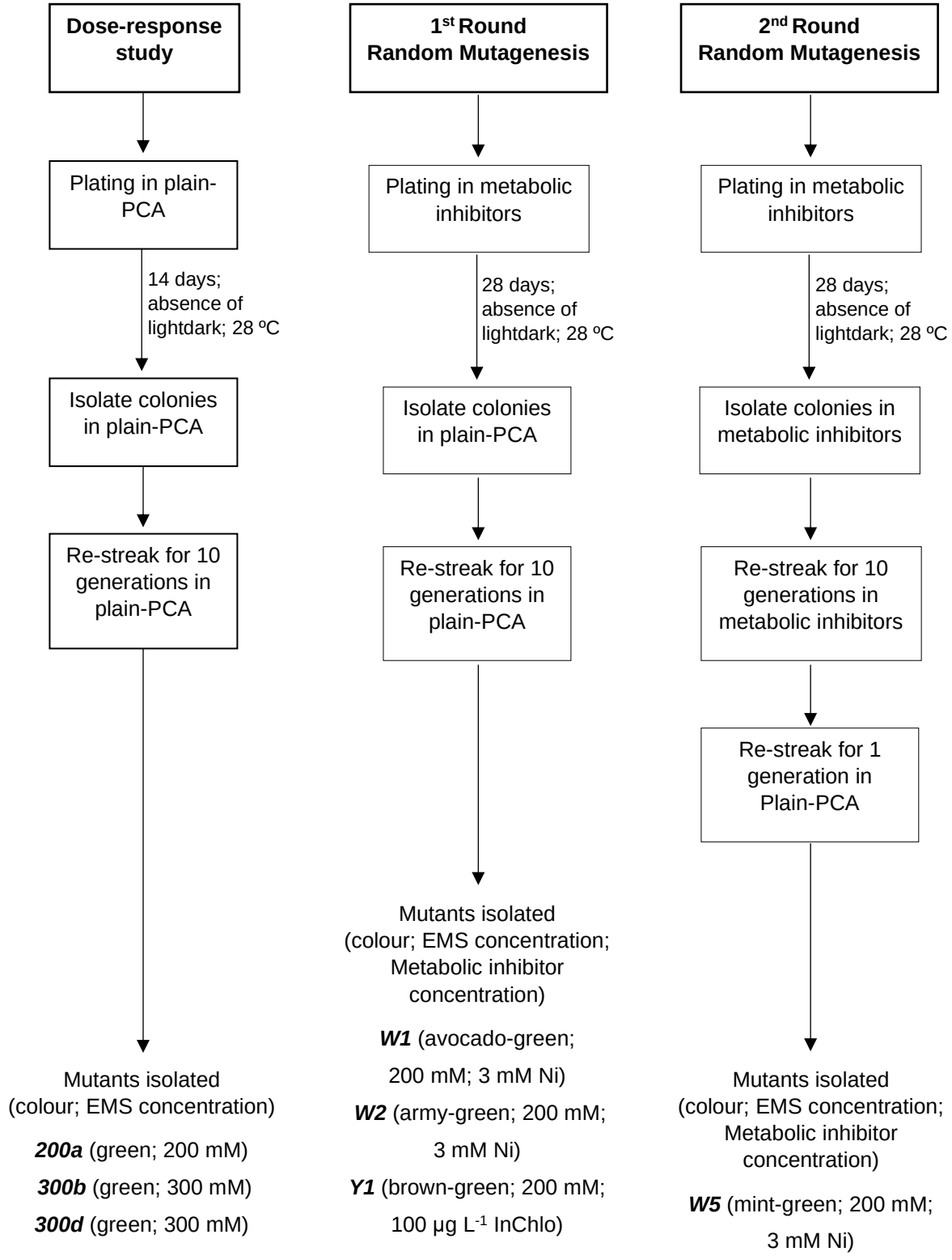

Figure 20. Schematic representation of the selection strategies applied in this work, depicting the several steps applied and summarizing the characteristics of the *Scenedesmus rubescens* mutants isolated.

Nevertheless, there are other reports on *Scenedesmus* spp. mutagenesis for other biotechnological applications, using different mutagenic agents and selection methods. *S. dimorphus* mutants with faster growth rates, higher lipid production, and increased triacylglycerol content were

isolated by using gamma radiation as a mutagenic agent and Nile red fluorescence for screening.[118] Screening for larger colonies of *Scenedesmus* sp. UV-C-generated mutants has also enabled the isolation of mutants with increased lipid production.[119] A combination of laser irradiation and UV radiation has also been applied in *S. obliquus* for the generation of mutants with increased biomass and lipid yields, and higher tolerance to wastewater, making the obtained mutants great candidates for the production of biodiesel in parallel with wastewater treatment.[120]

Further studies on this topic could explore high-throughput methods to isolate chlorophyll-deficient mutants more efficiently. Techniques such as FACS can quantify fluorescent molecules, such as chlorophyll *a*, and therefore be applied to isolate more efficiently randomly generated mutants with lower chlorophyll content. Additionally, combined with the use of metabolic inhibitors, the success of random mutagenesis applications could be improved.[64,65]

4.4. Mutants' Characterization.

After isolation, all the mutant and the WT were characterized regarding their growth performance, chlorophyll, carotenoids and protein content.

4.4.1. Growth Performance.

Mutants' heterotrophic growth was characterized in lab-scale Erlenmeyer flasks' trials, regarding their growth rate, biomass productivity and maximum DCW. The mutant strains were transferred to liquid GM1 medium, and cultures' conditions and growth were monitored daily. The results can be observed in Figure 21.

The growth curves depicted in Figure 21 and the values in Table 4 show that the WT strain reached the highest DCW_{max}, 9.92 g L^{-1}, followed by Y1 and W1, which both achieved a significantly lower DCW_{max} of 8.60 g L^{-1}, and W2, with 7.24 g L^{-1}. The lowest DCW_{max} were achieved by mutants 300b, W5, 300d and 200a, with 7.30 g L^{-1}, 6.85, g L^{-1}, 7.15 g L^{-1}, and 6.38 g L^{-1}, respectively. Similar to mutants 300b and 300d, the WT strain achieved the highest global productivity, 2.22 g L^{-1} day^{-1}, 2.20 g L^{-1} day^{-1} and 2.13 g L^{-1} day^{-1}, respectively. Mutant 200a achieved global productivity of 1.88 g L^{-1} day^{-1}, while W1 and Y1 attained 1.66 g L^{-1} day^{-1}, followed by W2 and W5, which reached 1.38 g L^{-1} day^{-1} and 1.25 g L^{-1} day^{-1}, all significantly inferior when compared to the WT. Finally, the WT strain also displayed the highest growth rate of 1.17 day^{-1}. However, contrary to what was observed with the global productivity, W2 has the second highest growth rate (1.09 day^{-1}), similar to 300d which reached a growth rate of 1.07 day^{-1}. Followed by 200a with 1.06 day^{-1} and Y1 with 1.05 day^{-1}, besides having different values, which don't have significant differences between them. The mutant W1 reached a growth rate of 1.02 day^{-1}, followed by mutant 300b, which achieved a growth rate of 1.00 day^{-1}. Among the studied strains, W5 reached the lowest growth rate of 0.82 day^{-1}.

When comparing the growth of the mutant strains to the WT, although they are all quite close, none of them achieved an improved growth performance. This can be due to a lack of optimization of growth conditions, as the culture medium, temperature and agitation were all previously optimised for the WT strain. If the conditions were optimised for each mutant, their growth could possibly reach similar

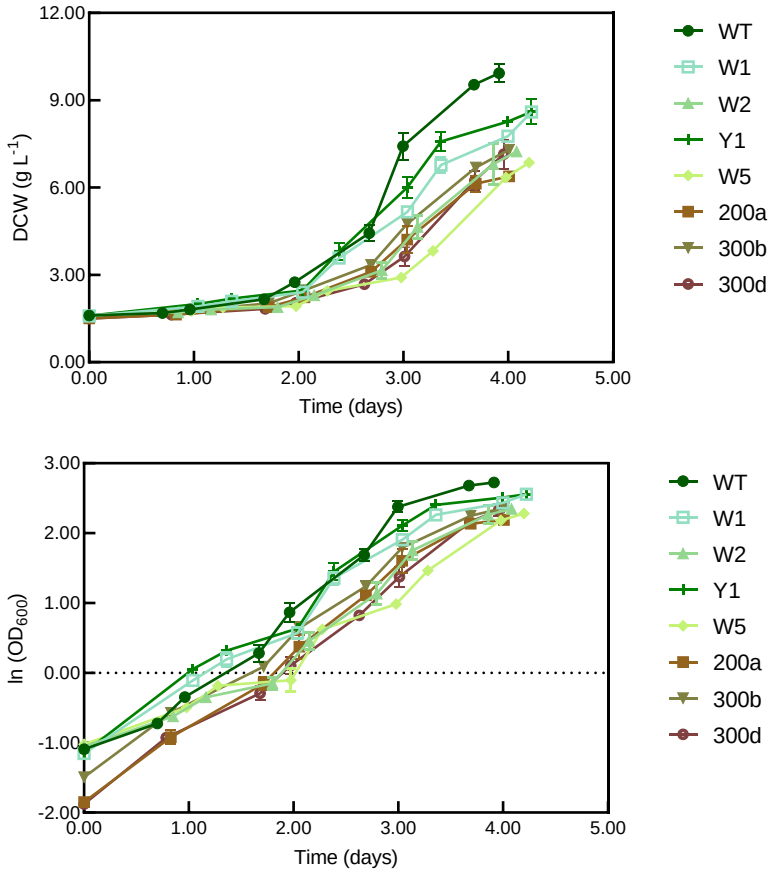

Figure 21. Growth assay. Wild-type and mutant strains were heterotrophically grown in 52-mL Erlenmeyer flasks, in GM1 medium supplemented with PIPES pH 6.5 buffer 60mM at 28 ºC and 200 rpm. **(Top)** Growth curve of the studied strains representing the DCW value, obtained by converting the OD_{600} values to DCW using an in-house developed calibration curve. **(Bottom)** Growth curve of the studied strains representing the neperian logarithm of OD_{600} against time. Data is represented as mean ± SD, for *n*=3.

Table 4. Summary of the results of the growth assay. Highest cell concentration (DCW_{max}), global productivity (P) and growth rate (μ) of the wild-type and mutant strains. Data is represented as mean ± SD, for *n*=3. Different letters indicate significant differences ($p < 0.05$) between strains and treatments.

	Highest cell concentration ($g\ L^{-1}$)	**Productivity ($g\ L^{-1}\ day^{-1}$)**	**Growth rate (day^{-1})**
WT	9.93 ± 0.32^{a}	2.13 ± 0.08^{a}	1.17 ± 0.03^{a}
W1	8.60 ± 0.04^{b}	1.66 ± 0.01^{b}	1.02 ± 0.01^{be}
W2	7.24 ± 0.17^{c}	1.38 ± 0.04^{c}	1.09 ± 0.01^{c}
Y1	8.60 ± 0.43^{b}	1.66 ± 0.10^{b}	1.05 ± 0.02^{b}
W5	6.85 ± 0.07^{cd}	1.25 ± 0.01^{c}	0.82 ± 0.01^{d}
200a	6.38 ± 0.08^{d}	1.88 ± 0.03^{b}	1.06 ± 0.01^{bc}
300b	7.30 ± 0.08^{c}	2.22 ± 0.03^{a}	1.00 ± 0.01^{e}
300d	7.15 ± 0.51^{cd}	2.20 ± 0.20^{a}	1.07 ± 0.02^{c}

or higher values than WT. Nevertheless, amongst the isolated mutants, 300d had the better growth performance, with the highest productivity and growth rate. Additionally, as random mutagenesis is often used as a tool to improve microalgae growth, many reports describe screening and selection methods

specifically applied for isolating mutants with increased growth rates and productivity (Table 5 - Appendix). However, when screening for other features, such as improved lipid productivity, other cell functions, such as growth, can be affected, leading to decreased growth rate, despite the improved lipid productivity.[121] Therefore, different pigment profiles might come with inferior biomass productivity and growth rate.

The heterotrophic growth of *Scenedesmus* spp. has been studied previously with reported values significantly lower than the data obtained in this study. Ren et al. (2013) studied the effect of carbon and nitrogen sources and initial pH on *Scenedesmus* sp., where at 10 g L^{-1} of glucose, in 250-mL Erlenmeyer flasks with a working volume of 150 mL, the highest cell concentration achieved was 3.47 g L^{-1}, along with a specific growth rate of 0.82 day^{-1}. These authors also observed an increase in the DCW_{max} to 4.12 g L^{-1} when using 30 g L^{-1} of glucose instead of 10 g L^{-1}.[122] In another study, heterotrophic cultivation of *S. obliquus*, in 500-mL Erlenmeyer flasks with a 250-mL working volume, resulted in a DCW_{max} between 3.90 g L^{-1} and 4.40 g L^{-1}.[123] These results are in accordance with a more recent report of heterotrophic cultivation of *S. obliquus*, using 10 g L^{-1} of glucose in 1-L Erlenmeyer flask, which resulted in productivity of 0.46 g L^{-1} day^{-1} and a maximum biomass concentration of 4.00 g L^{-1}.[124] Although comparable, these studies were all performed with different species and in different conditions, such as different glucose concentrations, culture media and reactors. Other reports studying microalgae heterotrophic cultivation in resemblant conditions to those reported in this work obtained similar results. Schüler et al. (2020) obtained DCW_{max} between 4.00 to 7.00 g L^{-1} for green, yellow and white *C. vulgaris* mutants, when growing this microalga in 250-mL Erlenmeyer flasks, with a working volume of 50 mL, at 30 ºC.[37] *Espírito Santo* (2020) achieved even higher values for *S. rubescens*, reaching a DCW_{max} of 11.50 g L^{-1}, a growth rate of 1.13 day^{-1} and a productivity of 1.94 g L^{-1} day^{-1}, using the same culture medium, temperature and reactor as in the present study.[53] Hereupon, the values reported for the WT and the mutants tested were higher than most of the values found in the literature for other *Scenedesmus* spp. and even other *C. vulgaris* mutants, which reinforces the potential of these species for industrial production. However, special attention must be given to the growth conditions to compare different reports.

4.4.2. Chlorophylls and Carotenoid Content.

Macroscopically, the cultures displayed different colours compared to the WT. As displayed in Figure 22, the WT culture displayed a dark-green colour, W1 had a very light green colour, described as avocado-green, W2 exhibited an army-green colour, Y1 had a yellowish-green colour that can be described as brown-green, and W5 had a mint-green colour. However, mutants 200a, 300b and 300d had a green colour, similar to the WT. The different colourations were evidenced by different pigmentation profiles, namely in the chlorophyll and carotenoid content of each mutant.

Chlorophyll was extracted and its content was quantified according to Ritchie (2008). The results (Figure 22) pointed out that the chlorophyll *a+b* content of the W1 strain was significantly reduced, corresponding to only 7 % of the WT chlorophyll *a+b* content. On the other hand, W2 and Y1, despite having a green colour, their chlorophyll *a+b* was also significantly reduced, accounting only for 40 %

and 45 % of the chlorophyll *a*+*b* content of the WT strain, respectively. The W5 had the lowest chlorophyll *a*+*b* content, with less than 3 % of the chlorophyll *a*+*b* content of the WT strain. Mutant 200a, isolated without the use of metabolic inhibitors, had a similar chlorophyll *a*+*b* content to the WT. Furthermore, mutants 300b and 300d, displayed the highest chlorophyll *a*+*b* contents, with a 49 % and 18 % increase, respectively. These results demonstrated that the use of metabolic inhibitors targeting the chlorophylls and the carotenoids biosynthetic pathways as a selection strategy was effective, as the mutants isolated with these approaches had significantly decreased chlorophyll *a*+*b* contents. On the other hand, mutants selected without using these inhibitors all had a similar or higher chlorophyll *a*+*b* contents, despite initially having a yellow colour (Figure 11).

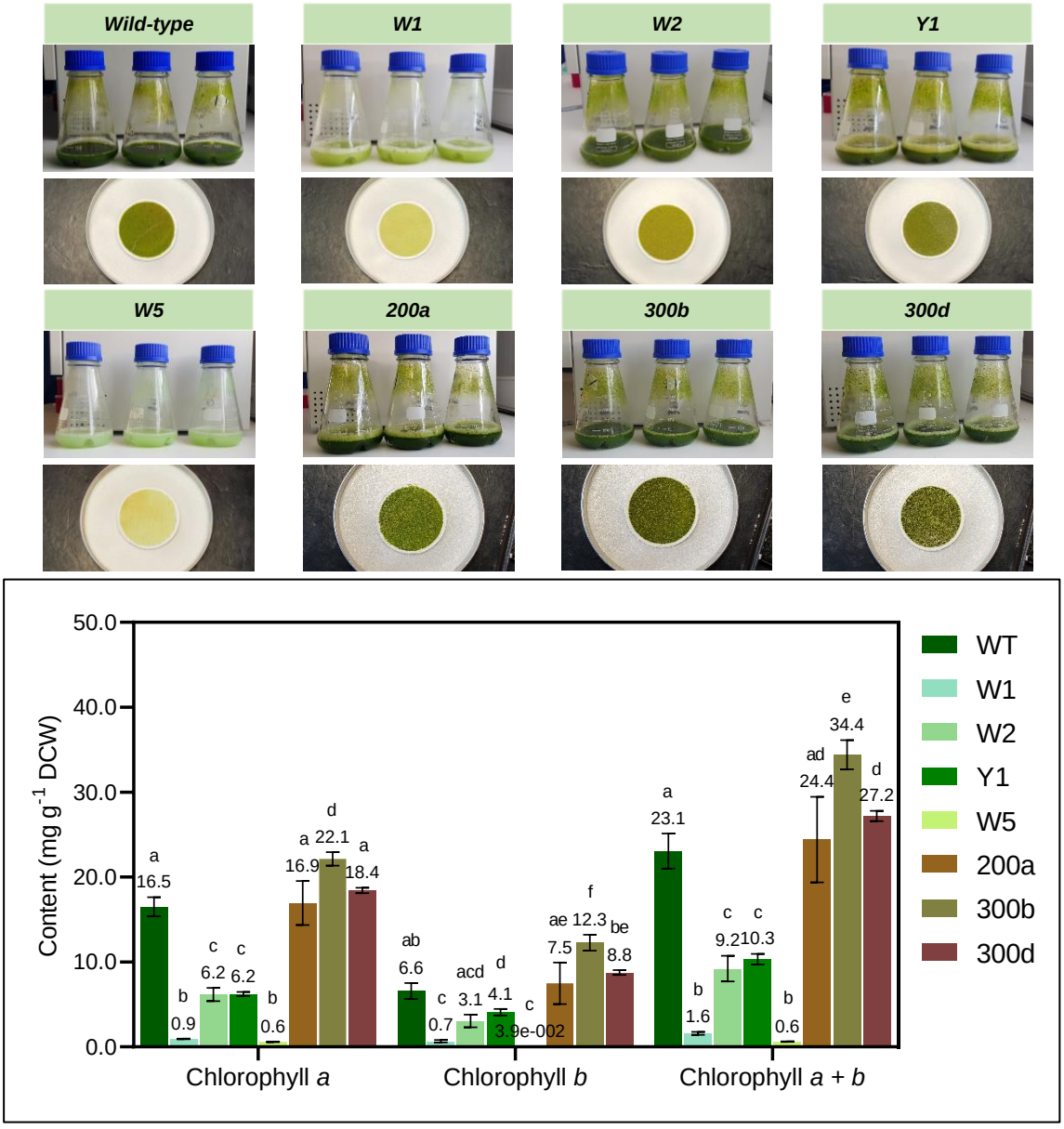

Figure 22. (Top) Macroscopic view of the cultures in 250-mLbaffled Erlenmeyer flasks at the end of the growth assay and dry weight filters with the corresponding cultures. **(Bottom)** Chlorophyll *a*, chlorophyll *b* and chlorophyll *a*+*b* content of *Scenedesmus rubescens* wildtype and mutans. Data is represented as mean ± SD, for *n*=3. Different letters indicate significant differences ($p < 0.05$) between strains, within each group.

Other random mutagenesis reports have been able to obtain microalgae with altered chlorophyll contents. However, within the *Scenesdesmus* genus, only two reports were found that have been able to isolate mutants with a modified chlorophyll profile. Xi et al. (2021) isolated a *S. obliquus* mutant with

increased chlorophyll *a* content, resorting to $^{12}C^{6+}$ ion beam mutagenesis, aiming at isolating strains with increased photosynthetic efficiency and lipid content.[125] On the other hand, Corcoran et al. (2018) isolated chlorophyll-deficient *Desmodesmus armatus* mutants, by applying UV mutagenesis and selection with fluazinam, a fungicide that inhibits energy production in fungal cells, intending to obtain mutant strains more resistant to this fungicide.[126] Reports of other closely related species, such as *C. vulgaris*, with reduced chlorophyll contents, were also found in the literature. Dall'Osto et al. (2019) have isolated chlorophyll-deficient *C. vulgaris* by screening EMS-generated mutants for colonies with a pale green colour and selection with red bengal, a photosensitizer which generates $^1O_2$ when exposed to white light.[85] Schüler et al. (2020)[37] sought to isolate chlorophyll-deficient mutants, while Guardini et al. (2021)[84] intended to isolate mutants with improved photoprotection and lower optical density. Both authors reported the isolation of *C. vulgaris* mutants with reduced chlorophyll content by resorting to EMS mutagenesis and selection with NF. These reports are in agreement with the results of this work, whereby using inhibitors of the carotenoids biosynthetic pathway enabled the isolation of mutants with decreased chlorophyll content.

Decreased chlorophyll contents have been associated with a decrease in light-harvesting proteins and a reduced functional antenna size of photosystem II, which results in increased photosynthetic efficiency and biomass productivity in photoautotrophic cultures.[39,85] The pale green phenotype obtained in this work could be associated with mutations that affect several different processes, such as translation of light-harvesting complexes[127,128], protein import into the chloroplast[129], light-harvesting complexes insertion in the thylakoids[130], and chlorophyll[129,131] or carotenoid[132] biosynthesis. Nevertheless, additional genetic analysis would have to be performed to confirm the cause of the decrease in chlorophyll content.

Besides increasing photosynthetic efficiency, it is also interesting to obtain low chlorophyll content to restrain microalgae's biomass unpleasant organoleptic chlorophyll-derived properties, described as a strong grassy taste, intense odour, dark-green colour, and unappealing texture.[37] Therefore, the described mutants are promising strains for several biotechnological applications, namely cosmetics and nutritional applications, such as animal nutrition. Moreover, the high growth rates and biomass productivities and robustness, grant them suitability for industrial production.

Total carotenoid content was also spectrophotometrically determined (Figure 23), where it was observed that WT, Y1 and 200a had statistically similar contents, 8.3 mg g^{-1} DCW, 6.0 mg g^{-1} DCW and 8.0 mg g^{-1} DCW, respectively. The remaining chlorophyll-deficient mutants displayed significantly decreased carotenoid content, compared to the WT. The W2 mutant had a 46 % decrease in total carotenoid content, which is smaller than the decrease in its chlorophyll content compared to WT. The W1 and W5 have the lowest total carotenoid contents, 0.9 mg g^{-1} DCW and 0.1 mg g^{-1} DCW, respectively. The mutants with increased chlorophyll content, 300b and 300d, also had increased carotenoid content, with 9.9 mg g^{-1} DCW and 10.3 mg g^{-1} DCW.

Decreased chlorophyll content, accompanied by a decrease in total carotenoid content has been previously seen in other random mutagenesis reports. With the objective of isolating chlorophyll-deficient mutants, Schüler et al. (2020)[37] generated *C. vulgaris* mutants with EMS and resorted to NF to

isolate mutants with decreased chlorophyll and carotenoid contents. The authors noted that despite a decrease in overall carotenoids content, the isolated mutant strains had accumulated phytoene, which is the pigment accumulated in the presence of NF, the selective pressure used in their work.[108] Similarly, as the selective pressure used in this work was Nic, acyclic carotenoids might have accumulated, despite the decrease in total carotenoid content.[106] Another EMS random mutagenesis report of *C. vulgaris*, aiming to obtain mutants with a truncated light-harvesting chlorophyll antenna, screened for light green colonies and isolated a chlorophyll-deficient mutant with a carotenoid content reduced to 75.3 %, compared to the WT.[39] In another report, the use of NF and Nic as a selection method for NTG-generated *C. sorokiniana* mutants resulted in the isolation of lutein hyper-producing strains.[98] To confirm if there was an accumulation of specific carotenoids, despite the overall decrease in total carotenoid content, other carotenoid quantification methods, such as high-performance liquid chromatography (HPLC), would have to be used to quantify and identify the carotenoids profile of each mutant. [7]

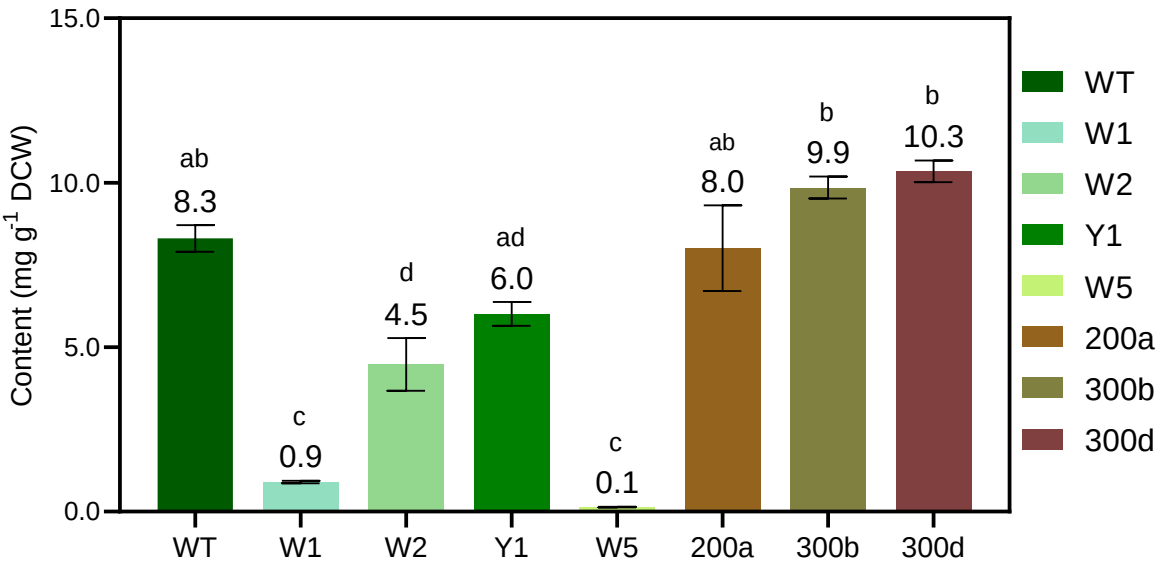

Figure 23. Total carotenoids content of *Scenedesmus rubescens* wildtype and mutants. Data is represented as mean ± SD, for *n*=3. Different letters indicate significant differences ($p < 0.05$) between strains.

An increase in chlorophyll *a* content followed by an increase in total carotenoid content has also been previously reported by Xi et al. (2021)[125]. The authors applied $^{12}C^{6+}$ ion beam random mutagenesis with the objective of increasing the lipid productivity of *S. obliquus*, and isolated mutants based on their photosynthetic efficiency. This increment in chlorophyll *a* and carotenoid content was associated with an increased expression of genes involved in photosynthesis, such as cytochrome b6, cytochrome b6/f and cytochrome c6, and upregulation of the lycopene epsilon-cyclase gene, which is related to carotenoids biosynthesis (Figure 5).[125] A similar transcriptomic analysis would have to be performed to confirm if overexpression of genes involved in photosynthesis-related processes and carotenoid metabolism is also occurring in the mutants 300b and 300b.

4.4.3. Protein Content.

Besides improved pigmentation, one of the other objectives of this work was to obtain mutants with increased protein content. Then, the protein content was determined through elemental analysis. The results are shown in Figure 24.

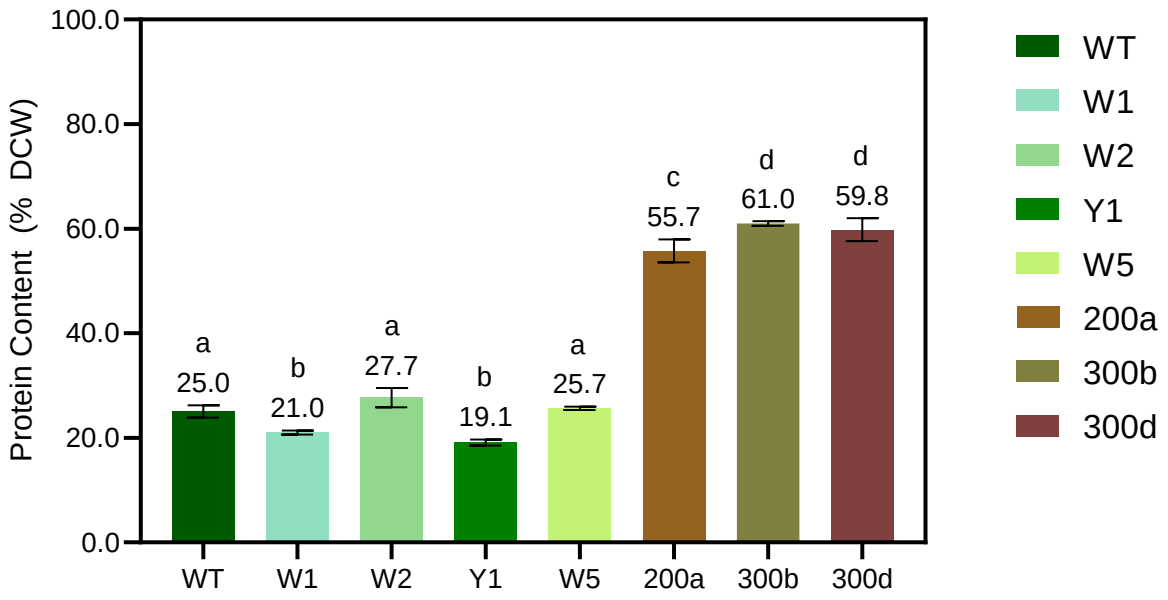

Figure 24. Total protein content of *Scenedesmus rubescens* wildtype and mutants. Data is represented as mean ± SD, for n=3. Different letters indicate significant differences ($p < 0.05$) between strains.

The results show that WT, W2 and W5 had similar protein contents, corresponding to 25.0 %, 27.7 % and 25.7 % of DCW, respectively, without significant differences. From the isolated mutants, only W1 and Y1 displayed decreased protein contents of 21.0 % and 19.1 %, respectively. The mutants with an increased chlorophyll content also had an increased protein content, namely mutant 200a with a 55.7 % protein content, 300b with 61.0 % and 300d with 59.8 %, which is more than twice of protein content of the WT.

Chlorophyll and protein content have been previously linked together, as chlorophyll-deficient mutants have been reported to have a truncated light-harvesting antenna size of the photosystem.[130] These findings are in accordance with other random mutagenesis reports, which have isolated *C.vulgaris* mutants with decreased protein and chlorophyll contents.[39,85] Similar results have been obtained for *Desmodesmus* sp., through atmospheric and room-temperature plasma (ARTP) mutagenesis, which generated mutants with lower chlorophyll and protein contents.[133] Given these reports, the lower protein contents of W1 and Y1 mutants might be related to a truncated light-harvesting antenna. Similarly, the higher protein content of the mutants with an increased chlorophyll content might be related to larger light-harvesting antenna sizes. Contrarily to these reports, Schüler et al. (2020)[37] isolated chlorophyll-deficient *C. vulgaris* mutants with increased protein contents, suggesting that the correlation between protein and chlorophyll contents is the opposite. Higher protein content, combined with decreased chlorophyll content, has been previously associated with higher expression of thylakoid

membrane proteins.[134] As the correlation between chlorophyll and protein content is not clear, future studies could employ a proteomic analysis to understand how the protein content varied in the mutants, and what proteins become over- and under expressed with different chlorophyll contents.

The chlorophyll-deficient mutant W2, which has improved growth compared to W5, seems to be the better strain for nutritional and cosmetic applications, given its protein content, similar to WT, with the advantage of a significantly reduced chlorophyll content that turns the biomass more appealing. Additionally, previous reports have shown that *S. rubescens* can reach protein contents of up to 60 % of the biomass, which means that the mutants' protein content could possibly be further increased.[135] Furthermore, mutant 300d has potential for use as a protein alternative, given that its protein content was significantly higher than the WT, and was the mutant with the best growth performance.

5. Conclusions and Future Perspectives.

This work described the first study of the application of random mutagenesis to develop novel *S. rubescens* mutants, as well as the first report to successfully use EMS to mutate this genus. The approach described in this work sought to improve this species' organoleptic features, namely by decreasing chlorophyll and enhancing protein contents. Nic and NF, inhibitors of the carotenoid's biosynthetic pathway, InChlo, an inhibitor of the chlorophyll's biosynthetic pathway, and absence of light, were used as selective pressures to isolate mutants with the desired characteristics. This work also described the first application of an inhibitor of the chlorophyll's biosynthetic pathway as a selection method in random mutagenesis with microalgae.

Firstly, 3 mutants with increased chlorophyll content, 200a, 300b and 300d, were isolated without the use of metabolic inhibitors. This increment in chlorophyll content was parallel to an increase in protein content to more than double of the WT, which was not observed in the chlorophyll-deficient mutants.

The use of metabolic inhibitors as a selection method allowed the isolation of 4 mutants with decreased chlorophyll contents, W1, W2, Y1 and W5. These mutants were selected using Nic, except for Y1, which was selected with InChlo. Likewise, Nic and InChlo were suitable inhibitors for selecting and isolating chlorophyll-deficient mutants. No mutants were isolated with NF, which indicates that its application should be further optimized to use this inhibitor as an effective selection tool. Chlorophyll-deficient mutants W2 and W5 maintained their protein content, which makes their nutritional profiles more interesting for nutritional applications.

Overall, by using this approach, two groups of *S. rubescens* mutants with different characteristics were isolated: a chlorophyll-deficient group of mutants, which were isolated using metabolic inhibitors that target the chlorophylls or the carotenoids biosynthetic pathway, with protein contents similar to the WT (around 25.0 %); and a protein-hyperproducing group of mutants with higher chlorophyll contents, which were selected by colony size, colour and growth, directly after the dose-response study, without the use of metabolic inhibitors.

Among all the isolated mutants, W2 displayed the highest potential to be used in cosmetic and nutritional applications, due to decreased chlorophyll content (9.2 mg g^{-1} DCW) maintained protein content (27.7 %) and growth rate (1.09 day^{-1}). On the other hand, due to the high protein (59.8 %) and chlorophyll (27.2 mg g^{-1} DCW) content, and high biomass productivity (2.20 g L^{-1} day^{-1}) and growth rate (1.07 day^{-1}), mutant 300d displayed the highest potential to be used as an alternative protein source.

Further application of genomics, transcriptomics and proteomics approaches to understand what caused the phenotypic changes could lead to the development of new selection platforms, which are currently the bottleneck of random mutagenesis studies. Furthermore, new selection tools should be tested, developed and optimized, particularly high-throughput technologies, such as FACS. Regarding the methodology applied, several conditions could be tested to optimize the mutagenesis protocol and improve the outputs of this technology, namely by testing different incubation times with EMS or even submitting the cells to other selective pressure, as other inhibitors and/or other

physicochemical conditions. The use of NF as a selection method should also be optimized by repeating the assays that were performed and/or testing mutant selection with lower NF concentrations. Further studies should test phenotype stability and growth performance during autotrophic growth, given that autotrophic growth has been associated with higher protein content. Carotenoid identification and quantification should also be performed to identify if there was an accumulation of carotenoids of interest, despite the overall decrease in carotenoid content. The proximate composition of the main macronutrients should also be defined. It could also be interesting to analyse and characterize some organoleptic features of the mutants, such as texture, flavour and smell. Additionally, growth conditions, namely culture medium and growth parameters (such as dissolved oxygen, agitation, pH, temperature, etc) could also be optimized for each mutant to further improve their growth performance and biochemical composition. Furthermore, these mutants should also be scaled-up to fermenters to validate the results and optimize the whole process at larger scales. Finally, it would also be interesting to study other applications for the mutant's biomass, such as their use in cosmetics, nutritional applications and agriculture (biostimulant and biopesticide).

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7. Appendix.

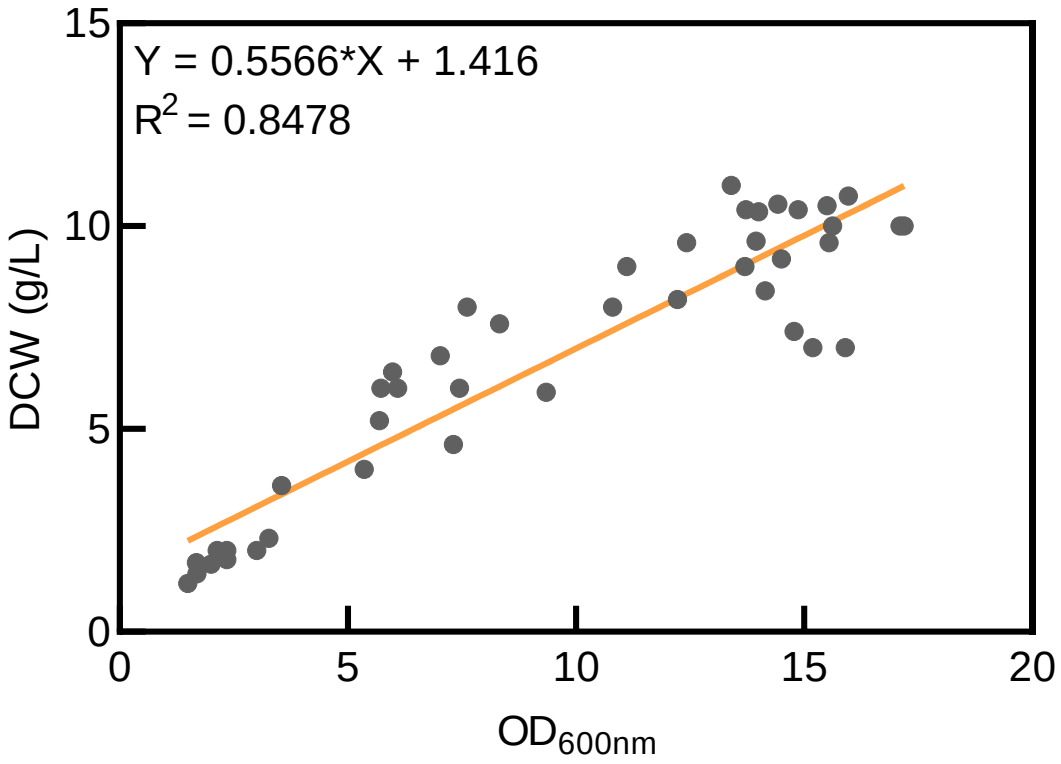

Figure 25. Calibration curve establishing a relationship between $OD600_{nm}$ and DCW for heterotrophically grown wild-type *Scenedesmus rubescens*.

Table 5. Reports of physical and chemical mutagenesis with *Scenedesmus* spp., *Desmodesmus* spp. and *Chlorella vulgaris*.

Microalga Species	Mutagen	Improvement Target	Selection/Screening Method	Results	Reference
Scenedesmus dimorphus	^{60}Co γ (800 Gy)	Lipid productivity	Visual screening for flask cultures exhibiting darker green colours and MTP-based screening for Nile red fluorescence.	-Higher growth rate and lipid accumulation.	118
Scenedesmus obliquus	$^{12}C^{6+}$ Ion Beam (120 Gy)	Lipid productivity	MTP-based screening for photosynthesis efficient mutants (determined by chlorophyll fluorescence), and a second round of screening for lipid contents and photosynthetic efficiency.	-Improved biomass and lipid yield. -Increased growth rate. -Increased chlorophyll *a* and carotenoids content. -Improved photosynthetic efficiency.	125
Scenedesmus obliquus	UV (254 nm)	Biomass productivity, CO_2 fixation	MTP-based screening for higher growth rate and genetic stability under 15% (v/v) CO_2.	-Improved growth performance and light conversion efficiency. -High CO_2 tolerance and fixation efficiency.	136
Scenedesmus obliquus	N^+ ion beam ($1.8x10^5$ ions/cm)	Lipid productivity, wastewater tolerance	Screening for higher lipid content (ratio of relative Nile Red fluorescence intensity and OD_{680nm}).	-Enhanced lipid contents. -Increased tolerance to municipal wastewater. -Improved removal efficiency of Total Phosphorus, Total Nitrogen, Ammonia-nitrogen and Chemical oxygen demand.	137
Scenedesmus obliquus	Semiconductor laser (473 nm) + UV (254 nm)	Lipid productivity	Screening for highest biomass productivity and growth rate in artificial wastewater.	-Higher dry weight, lipid content and biomass productivity in real wastewater.	120
Scenedesmus obliquus	UV (254 nm)	Lipid productivity	MTP-based visual screening for dark green cultures and Nile red fluorescence.	-Higher TAG and lipid contents. -Elevated cell size.	138
Scenedesmus obliquus	UV (254 nm)	Starch deficiency	Selection of non-purple strains with iodine staining.	-Decreased starch content. -Increase in TAG accumulation rate. -No substantial decrease in biomass productivity. -Increased total fatty acids and neutral lipid productivity.	139
Scenedesmus sp.	UV (254 nm)	Tolerance to CEW	Exposure to increasing concentrations of CEW.	-Improved tolerance to CEW. -Higher biomass, protein and lipid yields.	82
	UV (254 nm) + EMS (200-300 mM)			-No growth.	
Scenedesmus sp.	UV-C (254 nm)	Lipid and biomass productivity	Visual screening for colonies with bigger size, followed by screening for lipid content and biomass productivity.	-Increased lipid production.	119
Scenedesmus sp.	^{60}Co γ (50 Gy) + ^{60}Co γ (100 Gy)	Lipid productivity	Nile red fluorescence screening for highest lipid content and biomass content.	-Higher lipid content. -Darker green cells. -Higher biomass accumulation.	140
Desmodesmus armatus	UV (254 nm)	Fluazinam tolerance	Selection with ~100 ppm fluazinam, followed by MTP-based screening for improved growth rate in the presence and absence of fluazinam. Additionally, screening for growth performance in Oswald-style raceways and in 33 kL raceways, in the presence of fluazinam and other herbicides.	-Increased resistance to fluazinam (commercially known as Omega 500F®). -Increased growth rate and productivity. -Decreased chlorophyll fluorescence. -Increased quantum efficiency.	126

Table 1. *Cont.*

Desmodesmus armatus	UV (254 nm)	Lipid and biomass productivity	FACS of top 1% BODIPY505/515 fluorescence and MTP-based screening for Nile Red fluorescence.	-Increased biomass productivity. -Increased fatty acid production.	141
Desmodesmus sp.	ARTP	Lipid productivity, growth rate	Preliminary screening based on high lipid productivity (high specific growth rate and high relative lipid fluorescence), followed by a secondary screening for productivity (the product of specific growth rate and relative lipid fluorescence).	-Improved lipid productivity. -Low photosynthetic capacity. -Decreased protein and carbohydrate levels.	133
Desmodesmus sp.	EMS (800 mM) EMS (600 mM)	Lipid and biomass productivity	Preliminary screening for fast-growing colonies with high neutral lipid fluorescence (by Nile red staining). Additionally, screening for improved biomass and lipid production (biomass yield, total lipid content and biochemical stability).	-Improved growth rate and enhanced lipid accumulation. -Stable biomass yield and lipid production. -Reduced PUFAs and glycol lipids contents. -Increased MUFAs and neutral lipids contents.	83
Desmodesmus sp.	$^{12}C^{6+}$ Ion Beam (60-120 Gy)	Lipid productivity	Preliminary screening by light microscopy. Re-iterative MTP-based screening for quantum yield and Nile Red fluorescence.	-Higher photosynthetic efficiency and lipid contents. -Higher lipid productivity.	142
Chlorella vulgaris	2.2% EMS (*w/v*)	Carotenoids content, photosynthetic efficiency	Selection of colonies exhibiting a pale green colour using 4 µM Norflurazon.	-Reduced chlorophyll content and functional antenna size of PSII. -Increased photosynthetic efficiency and biomass productivity (under high light intensity). -Increase in carotenoids content and phototolerance. -Reduced oil content.	84
Chlorella vulgaris	EMS (300 mM)	Chlorophyll deficiency	Selection of colonies exhibiting a lighter colour with 10 µM Norflurazon.	-Yellow and white strains. -Decrease in chlorophyll contents, presence of lutein and phytoene (when grown heterotrophically in the dark). -Improved protein contents and organoleptic characteristics.	37
Chlorella vulgaris	UV (254 nm)	CO_2 tolerance	MTP-based screening for better growth under 15% (*v/v*) CO_2.	-High CO_2 tolerance, high CO_2 requirements and high genetic stability. -Increased light conversion efficiency and carbohydrate content. -Decreased lipid content.	143
Chlorella vulgaris	UV (254 nm)	Lipid and biomass productivity	FACS with 1% BODIPY505/515 fluorescence and MTP-based screening for Nile Red fluorescence.	-Increased biomass productivity and specific growth rate. -Increased fatty acid production.	141
Chlorella vulgaris	UV (254 nm) FDU (0.25 and 0.50 mM)	Lipid productivity	Selection with Streptomycin (200 µg/ml), erythromycin (200 µg mL^{-1}), atrazine (3 µM), photosystem-II inhibitor, and norflurazon (2 µM) and screening for high lipid content.	-Increase in the biosynthesis of saturated fatty acids. -Higher lipid accumulation. -Decline in the overall growth rate.	121
Chlorella vulgaris	UV-C (254 nm)	Lipid, TAG, and biomass productivity	Selection of non-purple strains with iodine staining and visual screening for larger and greener colonies.	-Increased cell concentration and growth rate. -Decreased cell size and distorted cell shape. -Increased lipid and TAG content.	115

Table 1. *Cont.*

Chlorella vulgaris	NTG (500 µg/mL)	CO_2 and temperature tolerance	w-zipper pouch method (MTP- based screening for better growth at 40ºC, 15% CO_2/air).	-Thermo-tolerant and high-CO_2 tolerant mutants. -Increased photosynthetic activity.	99
Chlorella vulgaris	Nd:YAG laser (1064 nm) He-Ne laser (808 nm) Semiconductor laser (632.8 nm)	Lipid and biomass productivity	Screening for higher growth rate (Flask cultures).	-Enhanced accumulation of biomass and lipids. -Unstable phenotype.	144
Chlorella vulgaris	UV-B (312 nm)	Lipid and biomass productivity	Visual screening for large colonies exhibiting a darker colour.	-Increased biomass yield and lipid contents (lab-scale conditions). -Lower biomass and lipid productivities (large-scale conditions).	145
Chlorella vulgaris	EMS	Photosynthetic efficiency, oxidative stress tolerance	Visual screening for colonies exhibiting a pale green colour.	-Increased specific growth rate and biomass productivity. -Reduced chlorophyll content and functional antenna size of PSII. -Enhanced photosynthetic productivity.	85
	EMS		Selection with 12 µM Red Bengal.	-Increased specific growth rate and biomass productivity. -Reduced chlorophyll content and functional antenna size of PSII. -Enhanced photosynthetic productivity. -Similar growth (as WT) in limiting-light conditions. -Higher resistance to photooxidative stress.	
Chlorella vulgaris	UV (254 nm) EMS (25 mM)	Lipid productivity	MTP-based screening for Nile red fluorescence (elevated neutral lipid levels) and screening for higher lipid productivity (Flask cultures).	-Increased lipid productivity and growth rate.	146
Chlorella vulgaris	UV (254 nm) + EMS (25 mM)	Lipid productivity	MTP-based screening for Nile red fluorescence and screening for higher lipid productivity (Flask cultures).	-Higher lipid productivity. -FAME composition suitable for biodiesel production.	147
Chlorella vulgaris	EMS (240 mM)	Photosynthetic efficiency (Chlorophyll deficiency)	Visual screening for colonies exhibiting a lighter colour.	-Reduced chlorophyll content, light-harvesting proteins, and functional antenna size of PSII. -Increased photosynthetic activity, electron transport rate and biomass productivity (under high light condition).	39
Chlorella vulgaris	EMS (200 mM-400 mM)	Violaxanthin productivity	Screening for low fluorescent colonies (low xanthophylls-producing colonies).	-Increased violaxanthin content. -Improvement of the growth rate (higher photosynthetic efficiency).	86
Chlorella vulgaris	UV	Lipid productivity	MTP-based screening for Nile red fluorescence.	-Increased neutral lipid content and productivity.	148

UV – Ultraviolet, CO_2 – Carbon Dioxide, MTP – Microtiter plate, OD – optical density, TAG – Triacylglycerol, EMS – Ethyl metanesulfonate CEW – Cellulosic ethanol wastewater, FACS – Fluorescence-activated cell sorting, ARTP – Atmospheric and room-temperature plasma, PUFA – Polyunsaturated fatty acids, MUFA – Monounsaturated fatty acids, PSII – Photosystem II, FDU – 5′Fluorodeoxyuridine monophosphate, Nd:YAG - a neodymium-doped yttrium aluminum garnet, He-Ne – helium-neon, WT – Wild-type, FAME – Fatty acid methyl ester.