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

Lucas Barata Cardoso[1,2]

Supervisors: Specialist Mafalda Trovão dos Santos[2], Prof. Marília Clemente Velez Mateus[1]

[1] iBB – Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa

[2] Allmicroalgae Natural Products S.A., R&D Department, Rua 25 de Abril s/n, 2445-413 Pataias, Portugal

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, random mutagenesis, strain improvement, selection, chlorophyll, protein.

Introduction

World's population is expected to reach 9.7 billion people in 2050[1]. The rise in world population is accompanied by an increase in demand for food production, particularly protein.[2] However, the food system must shift to more sustainable protein sources, given the rising consumer awareness for environmental protection and the impact of common protein sources, such as animal-based food.[3] Microalgae are a suitable alternative to include in the food system because their cultivation doesn't require arable land or freshwater, doesn't conflict with other established food chains and are a source of essential amino acids.[4,5]

However, 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. Additionally, the characteristics of microalgal biomass, such as high chlorophyll content, grants biomass unpleasant organoleptic properties, described as a strong grassy taste, intense odour, dark-green colour, and unappealing texture, that hinder consumer and even animal acceptance.[6,7]

Given all the challenges in the microalgae industry, the improvement of 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, 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)[8]. This methodology is well-established for microalgae strain improvement due to its robustness, accessibility and low cost.[9,10] 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 (directive 2001/18/ec).[6,11]

When the target cells are incubated with mutagens, either physical (e.g., ultraviolet (UV)) or chemical (e.g., Ethyl Methanesulfonate (EMS)), an extensive, heterogeneous mutant library is generated.[12] However, only a limited portion of them express the phenotype of interest.[13] To isolate the desired mutants, a selection and/or screening step needs to be implemented. 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.[14,15] Metabolic inhibitors that target the carotenoids' biosynthetic pathway such as norflurazon (NF), fluoridone, diphenylamine and nicotine (Nic), or the chlorophylls biosynthetic pathway, could be applied to isolate mutants with improved pigmentation.[10]

The focus of this work will be on improving the organoleptic and nutritional characteristics of a *Scenedesmus rubescens* strain by resorting to EMS mutagenesis. To the author's knowledge, this is the first study regarding random mutagenesis with *S. rubescens*, as well as 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, 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 decreased chlorophyll content and improved protein content. This is also the first study that applies a specific inhibitor of the chlorophyll biosynthesis pathway to select mutants with different pigmentation and protein content.

Materials and Methods

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 a carbon source, ammonium sulphate and urea as nitrogen sources, and supplemented with trace elements.

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.

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). Additionally, the number of cells was determined using a Neubauer chamber

(Hirschmann, Eberstadt, Germany) by direct observation under bright field microscopy.

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 analyzer (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).

$$DCW\ (g\ L^{-1}) = \frac{(m_f - m_i)}{V} \qquad (1)$$

The growth rate (µ) was obtained by Equation 2 and 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.

$$\mu = \frac{\ln (DCW_f / DCW_i)}{(t_f - t_i)} \qquad (2)$$

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

Mutagenesis and EMS Dose-Response Curve.

To establish the dose-response curve of ethyl methanesulfonate (EMS; Sigma-Aldrich, St. Louis, USA) and *S. rubescens* survival rate, the culture was exposed to different concentrations of this chemical mutagen. Wild-type 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.

For mutagenesis, only 150, 200 and 250 mM of EMS were used. 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.

Metabolic inhibitors Tests.

To determine the optimal concentrations of each inhibitor to be used for mutant selection, a culture in the 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 any metabolic inhibitor (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 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 and 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).

Cultures were incubated in the dark at 28 °C for 14 days. 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.

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. 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 growth assay was conducted in 250-mL Erlenmeyer flasks, with a final working volume of 52 mL. All the experiments were performed in triplicate.

Chlorophylls and Carotenoids Content.

To determine the chlorophylls and total carotenoid content of each mutant, pigment extraction was performed according to the Ritchie (2008)[16] 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. To extract the pigments, sequential zirconia bead milling was performed in acetone. 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. Quantification of chlorophylls and total carotenoids was performed by measuring the absorption spectrum of the supernatant (380 to 700 nm) (Genesys® 10S UV-Vis spectrophotometer; Thermo Scientific, Massachusetts, USA). Chlorophyll *a*, *b* and *a+b* were quantified by applying Equations 4, 5 and 6, as described by Ritchie (2008)[16]. Total carotenoids' content was quantified with the Excel Add-In Solver on Windows 13 using an in-house developed approach.[17]

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

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

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

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.[18]

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.

Results and Discussion

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. 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 (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 %.

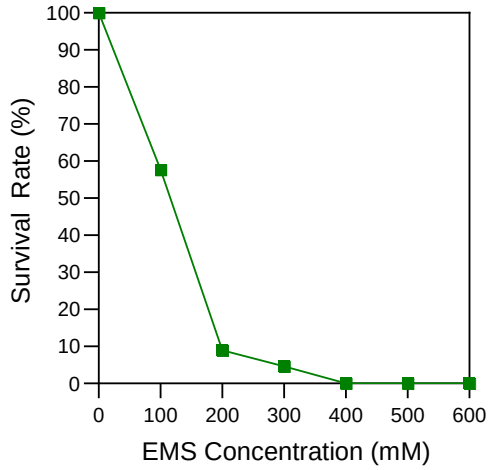

Figure 1. Survival rate of *Scenedesmus rubescens* exposed to different EMS concentrations.

In a previous report, Zhang et al. (2018) determined the susceptibility of *Scenedesmus* sp. to EMS.[19] The authors 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 the protocol used because it differed in presence of light and cell number, and the survival rate is affected by different factors such as species, cells' concentration and growth stage.[20] In a study performed by Schüler et al. (2020), using the same protocol reported in the present work, the resistance of *C. vulgaris* to EMS was also tested.[6] *C. vulgaris* exhibited a 10 % survival rate under 300 mM of EMS, which indicates that *S. rubescens* might have an increased susceptibility to EMS when compared to *C. vulgaris*. However, it is important to consider that the cell concentration used by Schüler et al. (2020) was different.

For the random mutagenesis experiments, the survival rate should be between 5-10 %, so that the probability of the surviving mutants having at least one stable mutation and that mutants with detrimental mutations are eliminated is higher.[6] For this reason, the optimal EMS concentrations to use on mutagenesis trials with *S. rubescens* should be between 200-300 mM.

Metabolic Inhibitors Tests

To isolate the mutants with the desired phenotype, metabolic inhibitors can be applied as a selective pressure. For this purpose, the effect that NF, Nic and InChlo have on the growth of *S. rubescens* was also tested. The optimal inhibitor concentration should be between the concentrations that allow minimal growth and no growth at all.[10,21]

Nic was tested at 1, 2, 3, 4, 5 and 6 mM. In the presence of Nic, the culture acquired a more yellowish colour, which might be due to an accumulation of acyclic carotenoids such as lycopene, but also a decrease in chlorophyll content.[22,23] *S. rubescens* was able to grow up to 3 mM of Nic, however, this growth was increasingly inhibited with an increase of Nic concentration to 5 mM. At a Nic concentration of 6 mM the culture was no longer able to grow.

These values are higher than what was found for *C. sorokiniana*, where it has been reported that Nic inhibited its growth just after 0.4 mM, which suggests that *S. rubescens* was more resistant to this stress than *C. sorokiniana*.[24,25]

In the presence of NF (5, 10, 15, 20, 25, 30 µM), the colour of the culture changed from dark green to light green. This change might be due to an alteration in the carotenoids profile, such as an accumulation of phytoene coupled with a decrease in total carotenoid content.[26,27] The inhibitory effect of NF in culture growth was seen from 5 to 30 µM of NF. 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, which made it difficult to determine the range of concentrations to test. Other reports have described an effective inhibition of the growth of *C. sorokiniana* with NF at 4 µM[25] and the growth of *C. vulgaris* at 10 µM.[6] Given this, it would be expected that the concentrations tested would lead to inhibition of this microalgae's growth, but it always depends on other factors besides species, such as cell concentration.[14]

Finally, when testing InChlo (25, 50, 100, 150, 200, 300, 400, 500, 600 and 700 µg L^{-1}), the culture acquired a brownish colour, which might be explained by decreased chlorophyll content.[28] Additionally, it was possible to observe that at concentrations higher than 300 µg L^{-1}, the growth was completely inhibited.

Hereupon, the concentrations range of each metabolic inhibitor to be tested was 3-6 mM of Nic, 20-30 µM of NF, and 100-300 µg L^{-1} of InChlo.

Mutagenesis, Selection and Isolation of Mutants.

Due to the number of mutants generated and the complexity of identifying different phenotypes, multiple approaches have been developed to isolate mutants of interest. In this work, three strategies were used to isolate *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 resulted in the isolation of 3 mutants. Mutant 200a was generated using 200 mM of EMS and was selected because it was identified as a larger and faster colony that arose before the remaining colonies. Mutants 300b and 300d were generated using 300 mM of EMS. Mutant 300b's colony displayed an orange colour, while mutant 300d was selected due to the size of the colony. Mutant colonies were picked and re-streaked for at least 10 generations in plain-PCA to evaluate phenotype stability, and 300b maintained its orange colour until the 4^{th} generation, but it acquired a green colour by the 10^{th} generation, similar to WT. These results show that this approach was not successful in isolating mutants with altered pigmentation.

Given this, to isolate mutants with different colours, two rounds of EMS random mutagenesis were applied and the metabolic inhibitors previously tested were used as selection

methods. When choosing the conditions to perform mutagenesis, it is important to consider that the combined detrimental effect of the metabolic inhibitors, together with the mutagenic agent, might be too harsh. Given this, it is important to work with a range of concentrations, to account for the increased stress. A summary of the conditions tested is shown in Table 1. Experimental controls, such as plating non-mutagenized culture in plain-PCA and PCA with the metabolic inhibitors, and plating dilutions of the mutagenized culture in plain-PCA were also performed.

Table 1. Summary of the conditions for mutagenesis. Mutagenesis was performed with ethyl methane sulfonate (EMS). Mutant selection was performed in nicotine (Nic), norflurazon (NF), and InChlo.

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

Several colonies with different characteristics appeared on the plates with inhibitors. The plates with Nic exhibited colonies with many different colours, ranging from white to yellow, orange and red. Plates with InChlo treatment displayed few colonies with yellow, brown, orange and green colonies. Finally, in the plates with NF, uncountable small, green and white colonies appeared, which had lost their ability to grow, meaning that the conditions used to isolate mutants with NF were too harsh. This result reflects once again the difficulty in tuning the optimal NF concentration to work with.

From these plates, colonies were picked and streaked for 10 generations in the absence or the presence of the respective metabolic inhibitor. This methodology led to the isolation of four *S. rubescens* mutants with different phenotypes: an avocado-green mutant, W1, an army-green, W2, a brown-green, Y1, and a mint-green, W5. All the mutants were generated using 200 mM of EMS, W1, W2 and W5 were isolated from Nic 3 mM, and Y1 was isolated from InChlo 100 µg L^{-1}. To note that W5 was the only mutant streaked for 10 generations in the presence of the metabolic inhibitor from where it was isolated, the remaining were streaked in plain-PCA.

Growth Performance

Firstly, mutants' heterotrophic growth was characterized in lab-scale Erlenmeyers' trials (Table 2). When comparing the growth of the mutants to the WT, none achieved an improved growth performance. Additionally, the W5 mutant was the slowest mutant, having the lowest growth rate and productivity, 0.82 day^{-1} and 1.25 g L^{-1} day^{-1}, respectively. The mutants with the higher growth rates were W2 and 300d, with 1.09 day^{-1} and 1.07 day^{-1}, respectively. The mutants 300b and 300d had the with highest productivities, similar to the WT, with 2.22 g L^{-1} day^{-1} and 2.20 g L^{-1} day^{-1}, respectively.

Table 2. 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}

Compared to other reports of heterotrophic *Scenedesmus* spp. growth on different conditions, most describe lower growth rates and productivities.[29–31] However, in a study

where the growth of *S. rubescens* was optimized, higher cell density, growth rate, and productivity were achieved. Similarly, as the conditions in this work were also optimised for WT *S. rubescens*, the lower growth values achieved for the mutant strains might be due to a lack of optimisation, which could make the mutants achieve a growth performance similar to WT.

Chlorophylls and Carotenoid Content

Macroscopically, the cultures displayed different colours compared to the WT. As it can be observed in Figure 2, the WT culture displayed a dark-green colour, W1 had a very light green colour, described as avocado-green, W2 displayed an army-green colour, Y1 had a brown-green colour, and W5 was mint-green. The remaining mutants, 200a, 300b and 300d were dark-green, similar to WT. The alteration in colour can be due to alterations in the pigmentation profile, especially in the chlorophyll content of each mutant.

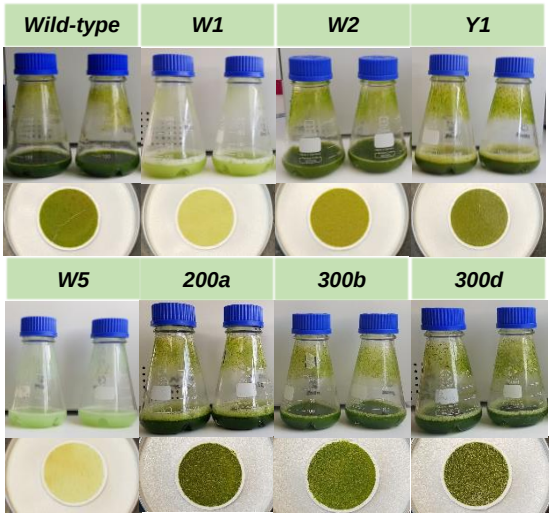

Figure 2. Macroscopic view of the cultures in 250-mL baffled Erlenmeyer flasks at the end of the growth assay and dry weight filters with the corresponding cultures.

Chlorophyll content was then characterized according to the protocol reported by Ritchie (2008)[16]. In Table 3 it is possible to see that the chlorophyll *a+b* content of the W1 strain was significantly reduced, corresponding to only 7 % of the WT. On the other hand, W2 and Y1, despite having a green colour, their chlorophyll *a+b* content was also significantly reduced by 60 % and 55 %, respectively. W5 exhibited the lowest chlorophyll *a+b* content, 0.6 mg g^{-1} DCW. Regarding the remaining green mutants, 200a has a similar chlorophyll content to WT, while 300b and 300b had a 49 % and a 5 % increase in chlorophyll *a+b* content, respectively.

Table 3. Chlorophyll a+b and carotenoids 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.

	Chlorophyll *a+b* (mg g^{-1}DCW)	**Carotenoids (mg g^{-1}DCW)**
WT	23.0 ± 2.0^{a}	8.3 ± 0.4^{ab}
W1	1.6 ± 0.1^{b}	0.9 ± 0.0^{c}
W2	9.2 ± 1.5^{c}	4.5 ± 0.8^{d}
Y1	10.3 ± 0.6^{c}	6.0 ± 0.4^{ad}
W5	0.6 ± 0.0^{b}	0.1 ± 0.0^{c}
200a	24.4 ± 5.0^{ad}	8.0 ± 1.3^{ab}
300b	34.3 ± 1.7^{e}	9.9 ± 0.3^{b}
300d	27.2 ± 0.6^{d}	10.3 ± 0.3^{b}

As for total carotenoids content, WT, Y1, 200a, 300b and 300d had statistically similar contents. The W2 mutant had a 46 % decrease in total carotenoid content, compared to WT, which was a smaller decrease than in its chlorophyll content. The W1 and W5 displayed the lowest total carotenoid contents, 0.9 mg g^{-1} DCW and 0.1 mg g^{-1} DCW, respectively.

The decreased chlorophyll content has 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, but also improves biomass organoleptic features.[6,21,32] However, the increased chlorophyll content can also be useful for applications in the cosmetic and pharmaceutical industries.[33]

A decrease in chlorophyll content accompanied by a decrease in carotenoids total content has also been previously described.

Schüler et al. (2020)[6] used NF to isolate chlorophyll-deficient *C. vulgaris* mutants whose total carotenoid content was reduced, despite having accumulated phytoene.[6] Similarly, the mutants isolated in this work might have also accumulated acyclic carotenoids.

Protein Content.

Another objective of this work was to obtain mutants with increased protein content. Given this, the protein content was determined through the elemental analysis of each of the isolated strains (Table 4).

Table 4. Protein content (% DCW) 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.

	Protein content (% DCW)
WT	25.0 ± 1.2^{a}
W1	21.0 ± 0.4^{b}
W2	27.7 ± 1.8^{a}
Y1	19.1 ± 0.6^{b}
W5	25.7 ± 0.4^{a}
200a	55.7 ± 2.2^{c}
300b	61.0 ± 0.5^{d}
300d	59.8 ± 2.2^{d}

WT, W2 and W5 had similar protein contents, corresponding to 25.0 %, 27.7 % and 25.7 %, respectively. From the isolated mutants only W1 and Y1 had a decrease in protein content of 16 % and 24 %, respectively. The mutants with higher chlorophyll content also had an increased protein content. Chlorophyll content and protein content have previously been linked together, as chlorophyll-deficient mutants have been reported to have a truncated light-harvesting antenna.[34] Given these reports, the alterations in protein content might be related to size differences in the light-harvesting antenna. However, contrarily to these reports, Schüler et al. (2020)[6] have previously isolated chlorophyll-deficient *C. vulgaris* mutants with increased protein content. The higher protein content, conjugated with a decrease in chlorophyll content, has been related to higher expression of thylakoid membrane proteins.[35] The application of a proteomic analysis would be useful to understand what caused the variations in protein content observed in the mutant strains.

Conclusion and Future Work

This work describes the first application of a random mutagenesis methodology to develop novel *S. rubescens* mutants with improved pigmentation and protein contents. This work also describes the first application of an inhibitor of the chlorophyll's biosynthetic pathway as a selection method. Two groups of mutants were isolated: a chlorophyll-deficient group of mutants, (W1, W2, Y1 and W5), which had their protein content maintained or reduced, and a protein-hyperproducing group (200a, 300b and 300d), with higher chlorophyll contents. Among all the isolated mutants W2 displayed the highest potential for nutritional and cosmetic applications, due to decreased chlorophyll content (9.2 mg g^{-1} DCW), maintained protein content (27.7 %) and growth rate (1.09 day^{-1}). 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. Future studies should focus on studying the isolated mutants, such as optimizing growth conditions and understanding what caused the phenotype changes. Additionally, new selection tools should be developed and optimized for mutant selection.

References

1. United Nations Department of Economic and Social Affairs PD (2022). *World Population Prospects 2022: Summary of Results*.
2. Smith P, Clark H, Dong H, et al. Agriculture, forestry and other land use (AFOLU). In: *Climate Change 2014: Mitigation of Climate Change. IPCC Working Group III Contribution to AR5*. Cambridge University Press; 2014:811-922.
3. Mendes M, Navalho S, Ferreira A, et al. Algae as food in Europe: An overview of species diversity and their application. *Foods*. 2022;11(13). doi:10.3390/foods11131871
4. Hossain N, Mahlia TMI, Saidur R. Latest development in microalgae-biofuel production with nano-additives.

Biotechnol Biofuels. 2019;12(1). doi:10.1186/s13068-019-1465-0
5. Kusmayadi A, Leong YK, Yen HW, Huang CY, Chang JS. Microalgae as sustainable food and feed sources for animals and humans – Biotechnological and environmental aspects. *Chemosphere*. 2021;271. doi:10.1016/j.chemosphere.2021.129800
6. Schüler L, Greque de Morais E, Trovão M, et al. Isolation and characterization of novel *Chlorella vulgaris* mutants with low chlorophyll and improved protein contents for food applications. *Front Bioeng Biotechnol*. 2020;8. doi:10.3389/fbioe.2020.00469
7. Amorim ML, Soares J, Coimbra JS dos R, Leite M de O, Albino LFT, Martins MA. Microalgae proteins: production, separation, isolation, quantification, and application in food and feed. *Crit Rev Food Sci Nutr*. 2021;61(12):1976-2002. doi:10.1080/10408398.2020.1768046
8. Kodym A, Afza R. Physical and Chemical Mutagenesis. In: Grotewold E, ed. *Plant Functional Genomics*. Humana Press; 2003:189-204. doi:10.1385/1-59259-413-1:189
9. Yi Z, Su Y, Xu M, et al. Chemical mutagenesis and fluorescence-based high-throughput screening for enhanced accumulation of carotenoids in a model marine diatom *Phaeodactylum tricornutum*. *Mar Drugs*. 2018;16(8). doi:10.3390/md16080272
10. Trovão M, Schüler LM, Machado A, et al. Random mutagenesis as a promising tool for microalgal strain improvement towards industrial production. *Mar Drugs*. 2022;20(7). doi:10.3390/md20070440
11. Zimny T, Sowa S, Tyczewska A, Twardowski T. Certain new plant breeding techniques and their marketability in the context of EU GMO legislation – recent developments. *N Biotechnol*. 2019;51:49-56. doi:10.1016/j.nbt.2019.02.003
12. Oladosu Y, Rafii MY, Abdullah N, et al. Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnology & Biotechnological Equipment*. 2016;30(1):1-16. doi:10.1080/13102818.2015.1087333
13. Arora N, Philippidis GP. Microalgae strain improvement strategies: Random mutagenesis and adaptive laboratory evolution. *Trends Plant Sci*. 2021;26(11):1199-1200. doi:10.1016/j.tplants.2021.06.005
14. Arora N, Yen HW, Philippidis GP. Harnessing the power of mutagenesis and adaptive laboratory evolution for high lipid production by oleaginous microalgae and yeasts. *Sustainability*. 2020;12(12). doi:10.3390/su12125125
15. Yu Q, Li Y, Wu B, Hu W, He M, Hu G. Novel mutagenesis and screening technologies for food microorganisms: advances and prospects. *Appl Microbiol Biotechnol*. 2020;104(4):1517-1531. doi:10.1007/s00253-019-10341-z
16. Ritchie RJ. Universal chlorophyll equations for estimating chlorophylls *a*, *b*, *c*, and *d* and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. *Photosynthetica*. 2008;46(1):115-126. doi:10.1007/s11099-008-0019-7
17. Correia N, Pereira H, Silva JT, et al. Isolation, identification and biotechnological applications of a novel, robust, free-living *Chlorococcum (Oophila) amblystomatis* strain isolated from a local pond. *Applied Sciences*. 2020;10(9). doi:10.3390/app10093040
18. Krul ES. Calculation of nitrogen-to-protein conversion factors: A review with a focus on soy protein. *J Am Oil Chem Soc*. 2019;96(4):339-364. doi:10.1002/aocs.12196
19. Zhang Q, Chang C, Bai J, Fang S, Zhuang X, Yuan Z. Mutants of *Scenedesmus* sp. for purifying highly concentrated cellulosic ethanol wastewater and producing biomass simultaneously. *J Appl Phycol*. 2018;30(2):969-978. doi:10.1007/s10811-017-1311-3
20. Bleisch R, Freitag L, Ihadjadene Y, et al. xxxStrain Development in Microalgal Biotechnology—Random Mutagenesis Techniques. *Life*. 2022;12(7):961. doi:10.3390/life12070961
21. Dall'Osto L, Cazzaniga S, Guardini Z, et al. Combined resistance to oxidative stress and reduced antenna size enhance light-to-biomass conversion efficiency in *Chlorella vulgaris* cultures. *Biotechnol Biofuels*. 2019;12(1). doi:10.1186/s13068-019-1566-9
22. Ishikawa E, Abe H. Lycopene accumulation and cyclic carotenoid deficiency in heterotrophic *Chlorella* treated with nicotine. *J Ind Microbiol Biotechnol*. 2004;31(12):585-589. doi:10.1007/s10295-004-0179-9
23. Fazeli MR, Tofighi H, Madadkar-Sobhani A, et al. Nicotine inhibition of lycopene cyclase enhances accumulation of carotenoid intermediates by *Dunaliella salina* CCAP 19/18. *Eur J Phycol*. 2009;44(2):215-220. doi:10.1080/09670260802578526
24. Chen JH, Chen CY, Chang JS. Lutein production with wild-type and mutant strains of *Chlorella sorokiniana* MB-1 under mixotrophic growth. *J Taiwan Inst Chem Eng*. 2017;79:66-73. doi:10.1016/j.jtice.2017.04.022
25. Cordero BF, Obraztsova I, Couso I, Leon R, Vargas MA, Rodriguez H. Enhancement of lutein production in *Chlorella sorokiniana* (Chorophyta) by improvement of culture conditions and random mutagenesis. *Mar Drugs*. 2011;9(9):1607-1624. doi:10.3390/md9091607
26. León R, Vila M, Hernánz D, Vílchez C. Production of phytoene by herbicide-treated microalgae *Dunaliella bardawil* in two-phase systems. *Biotechnol Bioeng*. 2005;92(6):695-701. doi:10.1002/bit.20660
27. Laje K, Seger M, Dungan B, Cooke P, Polle J, Holguin F. Phytoene Accumulation in the Novel Microalga Chlorococcum sp. Using the Pigment Synthesis Inhibitor Fluridone. *Mar Drugs*. 2019;17(3):187. doi:10.3390/md17030187
28. Sandmann G, Böger P. Comparison of the bleaching activity of norflurazon and oxyfluorfen. *Weed Sci*. 1983;31(3):338-341. http://www.jstor.org/stable/4043717
29. Ren HY, Liu BF, Ma C, Zhao L, Ren NQ. A new lipid-rich microalga *Scenedesmus* sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. *Biotechnol Biofuels*. 2013;6(1). doi:10.1186/1754-6834-6-143
30. EL-Sheekh MM, Bedaiwy MY, Osman ME, Ismail MM. Mixotrophic and heterotrophic growth of some microalgae using extract of fungal-treated wheat bran. *International Journal Of Recycling of Organic Waste in Agriculture*. 2012;1(1). doi:10.1186/2251-7715-1-12
31. Shen XF, Gao LJ, Zhou SB, et al. High fatty acid productivity from *Scenedesmus obliquus* in heterotrophic cultivation with glucose and soybean processing wastewater via nitrogen and phosphorus regulation. *Science of The Total Environment*. 2020;708. doi:10.1016/j.scitotenv.2019.134596
32. Shin WS, Lee B, Jeong B ryool, Chang YK, Kwon JH. Truncated light-harvesting chlorophyll antenna size in *Chlorella vulgaris* improves biomass productivity. *J Appl Phycol*. 2016;28(6):3193-3202. doi:10.1007/s10811-016-0874-8
33. Li Y, Lu F, Wang X, Hu X, Liao X, Zhang Y. Biological transformation of chlorophyll-rich spinach (*Spinacia oleracea* L.) extracts under in vitro gastrointestinal digestion and colonic fermentation. *Food Research International*. 2021;139. doi:10.1016/j.foodres.2020.109941
34. Polle JEW, Kanakagiri SD, Melis A. tla1, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size. *Planta*. 2003;217(1):49-59. doi:10.1007/s00425-002-0968-1
35. Gu J, Zhou Z, Li Z, Chen Y, Wang Z, Zhang H. Rice (*Oryza sativa* L.) with reduced chlorophyll content exhibit higher photosynthetic rate and efficiency, improved canopy light distribution, and greater yields than normally pigmented plants. *Field Crops Res*. 2017;200:58-70. doi:10.1016/j.fcr.2016.10.008