DEPARTMENT OF CHEMISTRY

DEVELOPMENT OF NOVEL MICROALGAL MUTANTS WITH IMPROVED PIGMENTATION AND PROTEIN CONTENTS FOR INDUSTRIAL WASTE-BASED FERMENTATION

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Master in Biotechnology

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ABSTRACT

Microalgae are a promising alternative feedstock to achieve the second goal of the 2030 Agenda established by the United Nations: to end hunger, achieve food security and improved nutrition and promote sustainable agriculture. However, microalgal production and commercialisation still face several hurdles, namely the high production costs and the unappealing sensory properties of the biomass.

To overcome the abovementioned limitations, improved strains of *Chlorella vulgaris* and *Scenedesmus rubescens* were generated by chemical random mutagenesis. At this stage, two novel selection methods were developed: one to select chlorophyll-deficient mutants with a novel metabolic inhibitor, oxyfluorfen, which led to the selection of a yellow and a white mutant of *C. vulgaris* and a brownish mutant of *S. rubescens*; and another using fluorescence-activated cell sorting to select protein-rich mutants, that allowed to select a green mutant of *C. vulgaris*, 8GF4. Additionally, nicotine was also used to select a yellow mutant (7Y) of *C. vulgaris* and an orange mutant of *S. rubescens* for food and feed applications.

From the mutants generated, mutant 8GF4 displayed higher biomass and protein productivity, and was further validated as a biostimulant for agricultural applications, improving the germination of garden cress seeds. Moreover, a novel optimisation pipeline was developed to optimise the latter and a yellow (7Y) and white (31W) *C. vulgaris* mutants. This methodology allowed to improve the biomass productivity, colour, and protein content and productivity by up to 70, 20, 61 and 94%, respectively. Finally, a waste-based medium was formulated to cultivate mutant 7Y, reaching a biomass productivity of 22 g L^{-1} d^{-1} at industrial scale.

Overall, this study optimised the whole production pipeline, from strain improvement to bioprocess optimisation and validation of cultivation under a waste-based medium, to overcome the challenges of the biobased industry, allowing to improve the attractiveness, cost-effectiveness and nutritional profiles of microalgal products.

Keywords: Biomass sensory properties; Heterotrophic cultivation; Microalgae; Protein content and productivity; Strain improvement; Waste-based fermentation.

RESUMO

As Nações Unidas estabeleceram como segundo objetivo da Agenda 2030: acabar com a fome, atingir a segurança alimentar e nutrição melhorada, e promover a agricultura sustentável. As microalgas são uma matéria-prima alternativa com muito potencial para este efeito. Contudo, a produção e comercialização de microalgas enfrenta ainda grandes desafios, como os elevados custos de produção e processamento, e as propriedades sensoriais pouco apelativas da biomassa.

As estirpes selvagens de *Chlorella vulgaris* e *Scenedesmus rubescens* foram então submetidas a um processo de mutagénese aleatória, de forma a gerar estirpes melhoradas das mesmas. Além disso, foram desenvolvidos dois novos métodos de seleção: um para selecionar mutantes deficientes em clorofila, através de um novo inibidor metabólico, o oxyfluorfen, estratégia que permitiu isolar dois mutantes *C. vulgaris (*amarelo e branco), e um mutante *S. rubescens* (castanho); e outro para selecionar mutantes ricos em proteína, através de separação celular ativada por fluorescência, o que permitiu selecionar um mutante verde de *C. vulgaris*, 8GF4. Adicionalmente, foi também usada a nicotina para selecionar um mutante amarelo (7Y) de *C. vulgaris* e um mutante laranja de *S. rubescens* para aplicações na alimentação humana e animal.

Entre os vários mutantes gerados, o mutante 8GF4 apresentou uma produtividade de biomassa e proteína superiores, sendo posteriormente validado o seu potencial bioestimulante para agricultura, cuja aplicação melhorou significativamente a germinação de sementes de agrião. Por outro lado, foi também desenvolvida uma nova estratégia de otimização para otimizar este último mutante, bem como um mutante amarelo (7Y) e outro branco (31W) de *C. vulgaris*. Esta metodologia permitiu melhorar a produtividade de biomassa, a cor, e o conteúdo e produtividade de proteína até 70, 20, 61 e 94%, respetivamente. Por fim, foi ainda formulado um meio à base de resíduos para o cultivo do mutante amarelo, cujo crescimento à escala industrial permitiu atingir 22 g L^{-1} d^{-1}.

Assim, no decorrer desta tese foi otimizado todo o processo de produção, desde a melhoria das estirpes, à otimização do bioprocesso, até à validação do cultivo com meio à base de resíduos à escala industrial, com o objetivo de ultrapassar os desafios associados a esta indústria, contribuindo para melhorar a atratividade, a rentabilidade e valor nutricional dos produtos à base de microalgas.

Palavras-chave: Conteúdo e produtividade de proteína; Cultivo heterotrófico; Fermentação à base de resíduos; Melhoria de estirpes; Microalgas; Propriedades sensoriais da biomassa;

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ACRONYMS

DoE	Design of Experiments.
DW	Dry Weight.
EMS	Ethyl Methanesulfonate.
FACS	Fluorescence-Activated Cell Sorting.
Glu	Glucose.
ICP-AES	Inductively coupled plasma - Atomic emission spectrometry.
OD	Optical density.
Oxy	Oxyfluorfen.
Ni	Nicotine.
NF	Norflurazon.
SD	Standard deviation.
PC	Protein content.
PP	Protein productivity.

SYMBOLS

μ	Growth rate.
r_p	Biomass productivity.
X_{max}	Maximum dry weight.

1

INTRODUCTION

1.1 Background

Algae are an undervalued and underexploited resource with great potential for several biotechnology applications, namely production of novel food and feed products, nutritional supplements, cosmetics, pharmaceutics, bioremediation, agricultural bioproducts, biofuels, textile industry and novel materials (bioplastics and construction) [1]. Nonetheless, the algae biomass sector has been growing in the last few years, representing an "economic value" of 1.7 billion €/year in Europe (750 million € - microalgae) by 2018 (https://www.what-are-algae.com/). Additionally, algae are a promising feedstock to address two important goals, established by the United Nations in the 2030 Agenda for sustainable development: to end hunger, achieve food security and improved nutrition and promote sustainable agriculture (Goal 2) and to ensure sustainable consumption and production patterns (Goal 12) (https://sdgs.un.org/goals).

The term "algae" does not possess a taxonomic value; still, it is essential to agree on a definition to create standards and handle regulatory issues. Algae have been defined as a diverse group of oxygen-producing organisms that evolved from different lineages but share some common ground, namely containing the pigment chlorophyll *a* (Figure 1.1., retrieved from https://www.what-are-algae.com/) (https://algaevision.myspecies.info/node/3506). This term might include prokaryotic (absence of membrane-bound organelles) organisms, closely related to bacteria (cyanobacteria), and eukaryotic (presence of internal membranes and/or organelles as chloroplast and nuclei) organisms, closely related to plants, capable of performing photosynthesis (transform $C0_2$ and water into sugar and oxygen), though there are exceptions [2] (https://algaevision.myspecies.info/node/3506). Although most algae are photoautotrophic organisms, there are some exceptions, as some species are also capable of growing in the dark by resorting to an organic carbon source (heterotrophic conditions) [1, 2]. Microalgae are also commonly called phytoplankton in the field of aquatic ecology, which in spite of

overlapping sometimes, differ from macroalgae (or seaweeds) by being microscopic (vs macroscopic) and mostly unicellular (vs complex multicellular forms) [1, 2]. In addition, algae are primary producers in several ecosystems (mostly aquatic) and do not present complex differentiated structures or tissues (https://www.what-are-algae.com/).

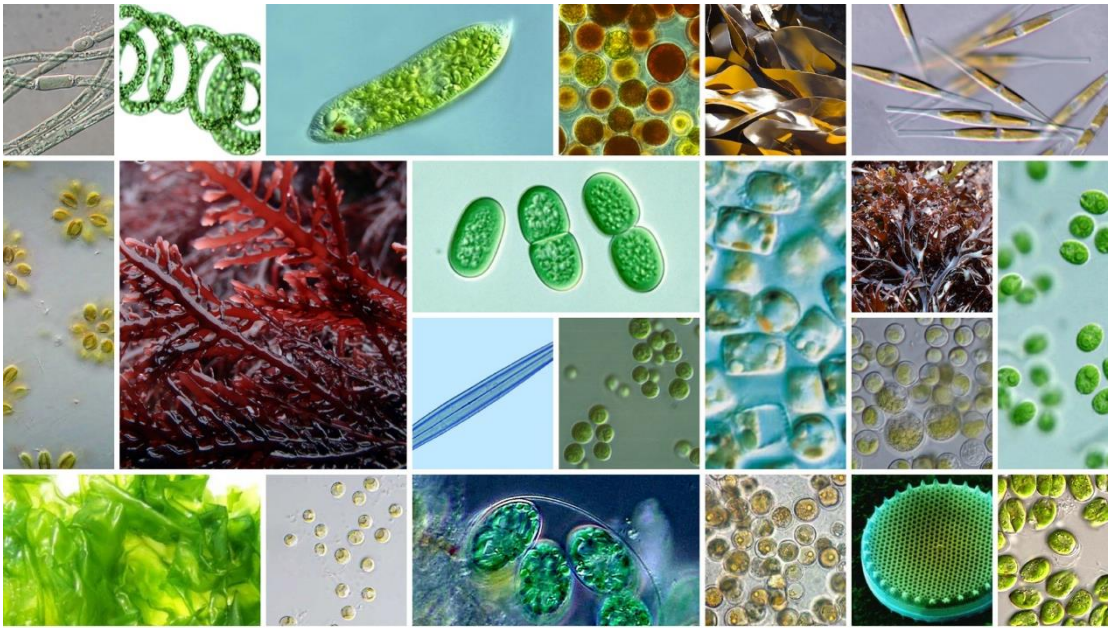


Figure 1.1 - Algae diversity. In https://www.what-are-algae.com.

Regarding food, feed and nutritional supplements, some microalgal species have been consumed for a long time, namely *Chlorella vulgaris* and *Arthrospira platensis*. *Chlorella vulgaris* belongs to the Phylum Chlorophyta as well as the genus *Scenedesmus*. In this phylum, also known as the phylum of green algae, cells present one to several green chloroplasts and accumulate mostly starch as storage material [3].

Despite the lengthy history of safe consumption of microalgae, many challenges have emerged lately concerning algae production, processing and commercialisation. In the present work, a number of these challenges will be discussed and addressed through different approaches.

1.2 Motivation

Although the potential of microalgae as a feedstock has been widely recognized, the implementation of its usage and commercialisation remains a challenge for several reasons, as it will be further discussed.

The global demand for food and feed is growing, as is the demand for algal biomass for those purposes, given their nutritional value, health benefits, and functional properties [4]. However, it is essential to demonstrate such benefits by studying and validating the great value of algae, namely by comparing it with other feedstocks. In addition, the interaction of these nutrients and/or compounds with human, plant and animal metabolism should also be studied, as well as their bioavailability and biodigestibility. The cultivation strategies and culture medium used, the adjustment of abiotic factors, and processing and storage methodologies should be optimised according to the intended application of a specific product, since these factors will affect biomass composition and functionality.

The use of microalgal biomass in food and feed applications is already a reality, with several microalgae-based products in the market. Nevertheless, the widespread potential of microalgae in these markets is still restricted due to the high costs and the low attractiveness of biomass and products. The high cost of microalgal products is one of the main hindrances to their commercialisation. The use of industrial sidestreams/effluents has been applied as a strategy to reduce production costs, reducing also, at the same time, the potential environmental and financial discharge impacts of these residues [5, 6]. In addition, this approach comes with benefits regarding the sustainability of the product, as well as a marketing leverage with increasing consumers' awareness. On the other hand, the optimisation of the cultivation and processing strategies to attain higher productivity and high-quality products, will not only reduce costs but also increase the value of the product. Finally, to create more appealing products, the sensory attributes of the biomass, such as colour, texture, flavour and smell, as well as nutritional value, have a high potential to be improved, either by strain improvement and/or optimisation of cultivation and processing conditions [7–10].

Therefore, the goal of this project was to develop novel microalgal strains with improved sensory characteristics and nutritional value, *i.e.*, particularly pigmentation, and subsequently taste, odour, and protein content, after which the selected strains cultivation conditions were optimised towards maximum biomass and protein productivities, faster growth and improved colour. In addition, the set of optimised conditions was then tested and adapted to similar conditions by replacing the inorganic media with sidestreams, which are commonly more sustainable and cheaper than traditional nutrient sources. Finally, this process was further optimised and scaled up to validate the results obtained and develop a strategy for future industrial production.

1.3 Thesis Outline

This PhD thesis is divided into seven chapters. Besides the first and last chapters, which correspond to the introduction and the main conclusions of this work, respectively, the other chapters are based on papers that have been either published or submitted for publication in different journals. To facilitate the understanding of the structure of this work, a brief description of each chapter and the thesis outline is given below:

- **Chapter 1 - Introduction**:

A brief contextualization of the theme, motivation and aim of the study, as well as the thesis layout;

- **Chapter 2 - Random mutagenesis as a promising tool for microalgal strain improvement towards industrial production**:

A published review paper which explains the concepts of strain improvement, the advantages and drawbacks of each approach, as well as an extensive review and discussion of what is reported in the literature and future prospects;

- **Chapter 3 - Random mutagenesis and selection with metabolic inhibitors to isolate microalgal chlorophyll-deficient mutant strains for nutritional applications**:

In this chapter, the strategies that have been attempted to generate chlorophyll-deficient mutants are summarized, and it is explained which mutants were selected to carry out the other tasks and why.

This chapter encompasses 3 subchapters:

- a published research paper entitled "**Oxyfluorfen: a novel metabolic inhibitor to select microalgal chlorophyll-deficient mutant strains for nutritional applications**", carried out with both *Chorella vulgaris* and *Scenedesmus rubescens*;

- an extra section entitled "**Isolation and characterisation of chlorophyll-deficient mutants of *Chlorella vulgaris***" with other results, namely the isolation of mutants of *C. vulgaris* with the same strategy as in the previous subchapter and with other metabolic inhibitors besides oxyfluorfen;

- a submitted research paper entitled **"Isolation of novel *Scenedesmus rubescens* mutants with high-quality protein and improved sensory properties**";

- **Chapter 4 - Isolation and selection of protein-rich mutants of *Chlorella vulgaris* by Fluorescence-Activated Cell Sorting with enhanced biostimulant activity to germinate garden cress seeds**:

A published research paper that describes a novel high-throughput strategy to isolate mutants with enhanced protein contents as well as the application of the selected strain as a biostimulant in plants;

- **Chapter 5: Improving the heterotrophic media of three *Chlorella vulgaris* mutants toward optimal color, biomass and protein productivity**:

The strains that were selected for further work in Chapter 3 and 4, a green, a yellow and a white strain of *C. vulgaris*, were submitted to an optimisation process by a Design of Experiments (DoE) strategy. The optimal cultivation conditions were established for each. This work gave rise to another submitted research paper.

- **Chapter 6: Heterotrophic cultivation of *Chlorella vulgaris* yellow mutant on industrial sidestreams: medium formulation and process scale up:**

  A research paper, also already submitted, in which a waste-based medium is developed to meet the nutritional requirements of the yellow mutant of *C. vulgaris* established in Chapter 5. The results achieved at laboratory scale were validated in 7-L and 200-L reactors to create an industrial production pipeline.

- **Chapter 7: Conclusions and future work**:

  Conclusions and future perspectives regarding the work developed.

Hence, the work developed during this PhD thesis resulted in 6 papers, 3 of which have been published, and the other 3 have been submitted.

2

RANDOM MUTAGENESIS AS A PROMISING TOOL FOR MICROALGAL STRAIN IMPROVEMENT TOWARDS INDUSTRIAL PRODUCTION

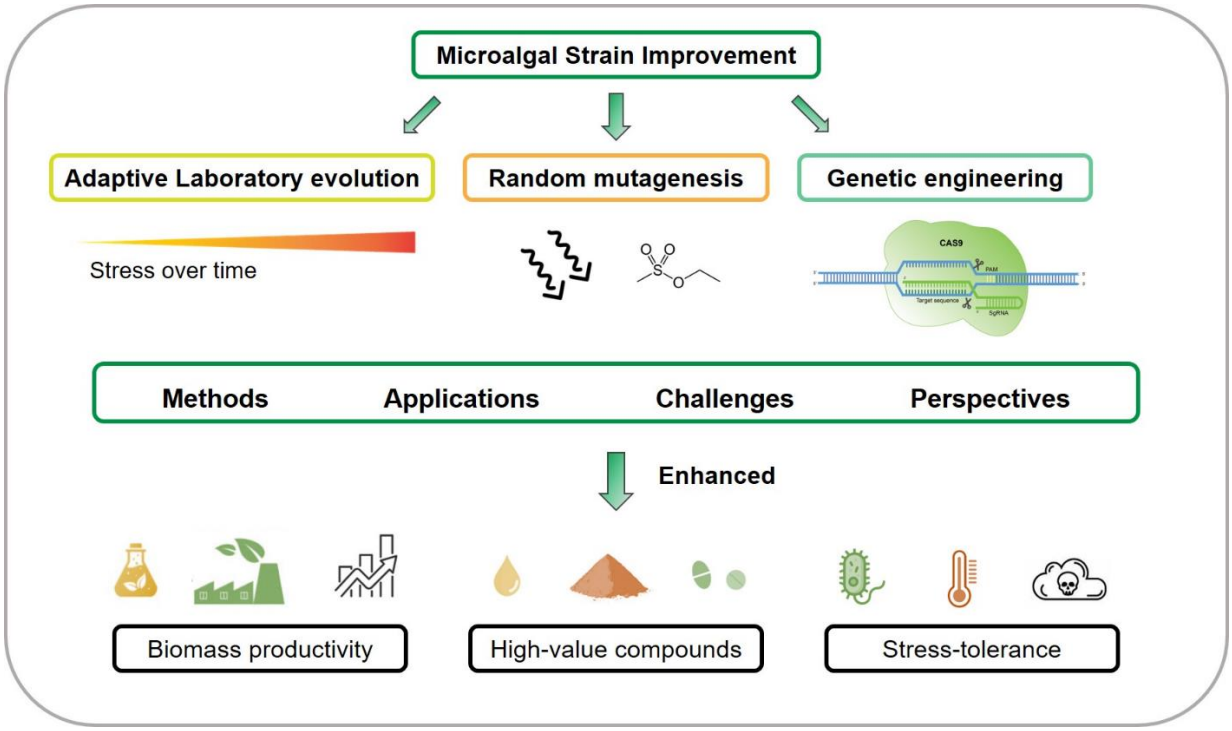
This chapter was adapted from the following published review:
M. Trovão, L. M. Schüler, A. Machado, G. Bombo, S. Navalho, A. Barros, H. Pereira, J. Silva, F. Freitas and J. Varela, "Random Mutagenesis as a Promising Tool for Microalgal Strain Improvement towards Industrial Production," *Mar. Drugs*, vol. 20, no. 7, 2022, doi: 10.3390/md20070440.

ABSTRACT

Microalgae have become a promising novel and sustainable feedstock for meeting the rising demand for food and feed. However, microalgae-based products are currently hindered by high production costs. One major reason for this is that commonly cultivated wildtype strains do not possess the robustness and productivity required for successful industrial production. Several strain improvement technologies have been developed towards creating more stress tolerant and productive strains. While classical methods of forward genetics have been extensively used to determine gene function of randomly generated mutants, reverse genetics has been explored to generate specific mutations and target phenotypes. Site-directed mutagenesis can be accomplished by employing different gene editing tools, which enable the generation of tailor-made genotypes. Nevertheless, strategies promoting the selection of randomly generated mutants avoid the introduction of foreign genetic material. In this paper, we review different microalgal strain improvement approaches and their applications, with a primary focus on random mutagenesis. Current challenges hampering strain improvement, selection, and commercialisation will be discussed. The combination of these approaches with high-throughput technologies, such as fluorescence-activated cell sorting, as tools to select the most promising mutants, will also be discussed.

Keywords: Adaptive laboratory evolution; Fluorescence-activated cell sorting; Genetic engineering; Reverse and forward genetics; Selection methods.

GRAPHICAL ABSTRACT



2.1 Introduction

The world's population is estimated to reach 9 billion people in 2050 [11, 12], which raises significant concerns about the future energy, food, and feed demand, as well as waste management and dependency on limited resources [13–15]. In this context, microalgae are widely recognized as promising alternatives to conventional feedstocks, and represent a potential part of the solution to address these worldwide issues.

Microalgae are the major contributors to CO_2 fixation and O_2 production across the globe, with the ability not only to mitigate the rising CO_2 levels, but also to utilize nutrients from effluents that otherwise would be discharged into the environment. Thus, the production of microalgae makes a large contribution to the field of bioremediation and leveraging circular economy [12, 16–18]. In addition, microalgae might play a key role as part of alternative feedstocks to face global food and feed scarcity, since they do not require arable land to be cultivated, and possess very rich nutritional profiles, being a source of proteins, lipids, carbohydrates, polyunsaturated fatty acids (PUFAs), vitamins, and bioactive compounds [4, 10, 19, 20].

Despite the outstanding potential of microalgae for different biotechnological applications, the commercialisation of microalgae-based products, such as the biomass itself or added-value compounds (e.g., pigments and PUFAs), is still restricted to high-value niche markets. Current high production costs and unsuccessful attempts at marketing and selling these products has hindered the development of the microalgal industry and market [16, 21]. Concerning cultivation, the prevailing bottlenecks include low biomass conversion efficiencies, low target biocompound productivities, low light delivery in concentrated cultures under photoautotrophic cultivation, wide environmental variations under outdoor conditions, contamination of cultures, and costly inputs, namely culture media and energy demand [22–25]. Open systems arose as cheaper cultivation systems, as compared to closed systems. Although the former have enabled a reduction in energy costs [26, 27], they are prone to contamination, and if the microalga is not robust enough to outgrow grazers and competitors, culture crashes generally ensue. Moreover, the exposure to abiotic stress factors (e.g., temperature and salinity) might impact overall productivity, unless a robust and stress-tolerant strain is used [27]. Additionally, downstream processing usually implies high energy consumption with concomitant high operating expenses (OPEX), often with a low recovery of biomass and production surpluses [22, 28–30].

Overcoming these hurdles requires a multistage optimisation approach in the whole microalgae production and processing pipeline, to tackle the current bottlenecks of industrial-scale cultivation of microalgal biomass [22]. Rethinking and revamping the whole pipeline under a biorefinery and circular economy approach would contribute to more economically feasible industrial-scale processes [20, 31, 32]. On the other hand, strain selection and improvement are crucial stages in generating industrial strains and facilitating large-scale microalgal production [21, 31, 33]. However, this area has not received enough attention and a more effective research effort is still required.

Currently, only a few naturally occurring microalgal strains meet the required traits for economically viable industrial production for use in diverse biotechnological applications [16, 34]. Thereby, it is important to isolate and create novel strains able to face the challenges mentioned above. The first step towards this purpose begins with bioprospection and selection of strains with improved features, and enhanced biomass and target biomolecule productivities. High-throughput technologies, such as methods based on fluorescence-activated cell sorting (FACS), are powerful tools to mine and isolate improved strains [21, 35–37]. Nonetheless, to pursue large-scale production and profitability, such strains require further improvement.

Traditional industries, such as agriculture, cattle farming, and even pharmaceutical companies, have undergone significant development through investing in breeding, as well as in random mutagenesis and targeting strategies, such as the generation of genetically modified organisms (GMOs) to create more resilient and productive strains, rather than using their wildtype counterparts [15, 38–40].

Classical forward genetic studies with microalgae allow the identification of genes, the assignment of phenotypes to a gene sequence/genotype, and the expansion of our current understanding of the biology and metabolism of several microalgal species [21, 41, 42]. Throughout the years, this acquired knowledge has shed light on metabolic pathways and genotypes, which are now used by the scientific

community to perform reverse genetics experiments that target specific gene sequences to improve microalgal strains and generate novel phenotypes [42, 43].

Genetic variability and species evolution occurs naturally and randomly by exposure to UV irradiation from sunlight, reactive oxygen species (ROS), or other agents that cause spontaneous mutations in the genetic material [43–47]. Since these processes are slow and untargeted, strategies such as random mutagenesis and adaptive laboratory evolution have been applied to accelerate these naturally occurring processes, which enable the generation and the selection of mutant organisms with properties that meet the needs of industry [12, 39, 43]. A different strategy used to improve the key characteristics of microalgae is the promotion of site-directed mutagenesis by resorting to gene-editing tools, such as, clustered regularly interspaced short palindromic repeats associated protein 9 (CRISPR-Cas9) and zinc finger nucleases (ZFN). Moreover, RNA interference (RNAi), together with progression in the synthetic biology field, has been used to develop tailor-made genotypes by targeting specific genes to increase both the biomass and the yields of added-value compounds, thereby reducing production costs [12].

As several technologies have been applied to microalgal strain improvement to overcome the main hindrances of microalgae production, diverse overviews of this topic have been published. Spicer and Molnar [40] have published a useful discussion about gene editing in microalgae, and its correspondent challenges and perspectives. Other review papers have reported advances regarding the application of adaptive laboratory evolution [18, 48, 49], high-throughput techniques, and genetic engineering to microalgae [12, 16, 17, 21, 50]. In turn, Aklilu [42], Torres-Tiji et al. [15], and Hlavova et al. [43] provided a general overview on the importance of microalgal strain improvement and the different technologies available.

In this review, different strain improvement approaches will be discussed and compared, focusing on the current pipelines that combine random mutagenesis with high-throughput technologies, based on the combined use of FACS and metabolic inhibition, as a tool to improve microalgal strains for industrial purposes. Specific case studies, as well as the advantages and disadvantages of using such pipelines, will be discussed. Future perspectives for fast-tracking the improvement of microalgal strains will also be provided. Lastly, the current regulatory frameworks on GMOs applied to microalgae will be summarized and discussed.

2.2 Strategies for microalgal strain improvement

Classical genetics, also known as forward genetics, is based on the observation of phenotypes and the identification of the gene sequences responsible for specific phenotypic features, within which, diversity might arise through naturally occurring or induced mutations [42, 51]. In forward genetics, the target phenotype is selected, while the genotype is unknown (Figure 2.1A) [43]. Conversely, reverse genetics starts by introducing alterations in a known gene sequence through random or site-directed mutations, which are then translated into an observable phenotypic change, elucidating the respective function of the gene or set of genes (Figure 2.1A) [43, 51, 52]. Over recent years, whole genomes have been sequenced; this has resulted in an increased focus on reverse genetics, since scientists are now

able to change, or even disrupt, specific target genes in order to observe and study the effects of these alterations on the phenotype [42].

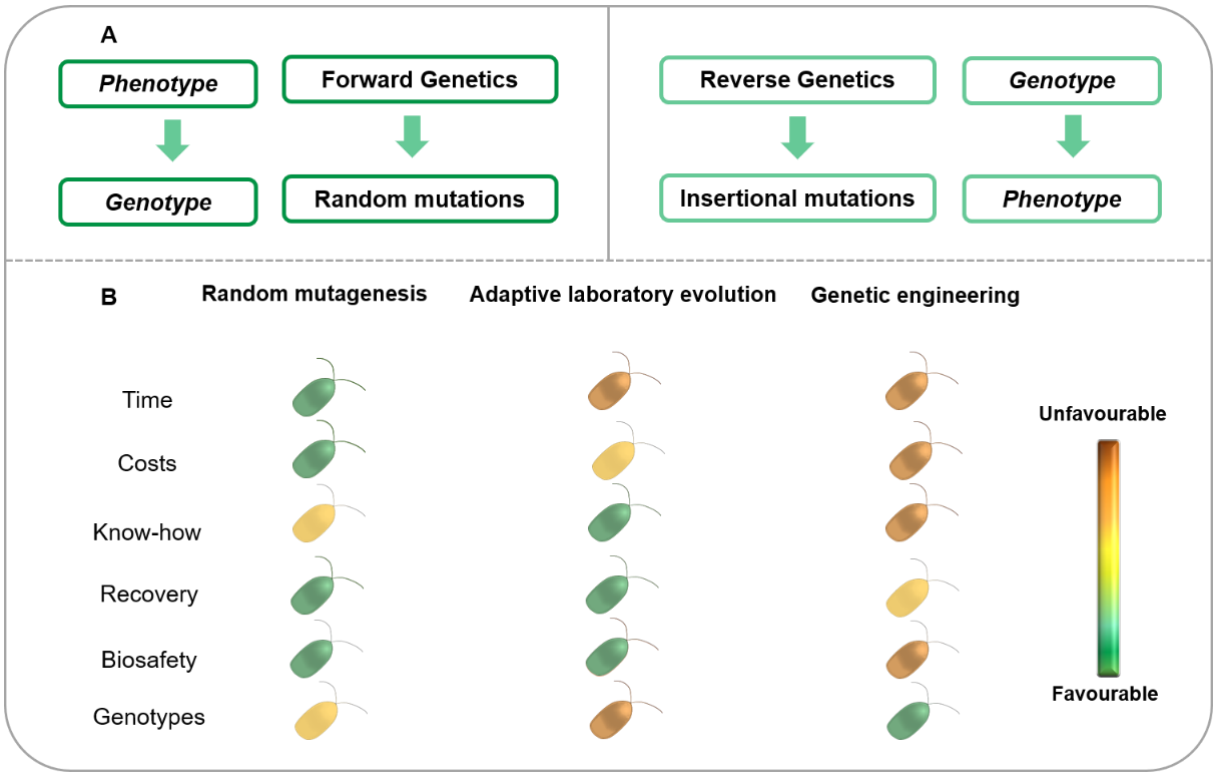


Figure 2.1 - Strain improvement approaches by forward and reverse genetics strategies (**A**). Comparison of several aspects of three methods of strain improvement: random mutagenesis, adaptive laboratory evolution, and genetic engineering (**B**). Time—time required to perform the experiments and obtain 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.

Forward genetics is also particularly useful, since it comprises tools such as random mutagenesis and adaptive laboratory evolution, which allow the generation of large pools of mutant phenotypes without any previous knowledge about the genetics and metabolism of the target organism, and without the need for the development of molecular tools, which can be time-consuming and more expensive than the application of random mutagenesis and adaptive laboratory evolution strategies (Figure 2.1B) [15]. The most desired features, namely higher biomass and target compound productivities, as well as higher tolerance to specific growth conditions, are selected first, while the respective mutation is identified afterwards [42, 43, 51].

Random mutagenesis is a robust, well-established, easy-to-perform, and cost-effective tool used to generate mutants (Figure 2.1B) [53, 54]. As mentioned above, random mutagenesis is merely an acceleration of naturally occurring processes, achieved by exposing organisms to a potent physical or chemical mutagenic agent, followed by the selection of mutants with the desired features [19, 21, 39, 43]. The mutations are non-specific; however, there is no introduction of foreign genetic material, which

allows for the production of these mutants at industrial facilities without the restrictions imposed on GMOs that contain heterologous DNA sequences [15, 19, 39, 54]. Although a great variety of mutants can be generated by random mutagenesis, the mutations are often lethal, or at least detrimental, to the mutagenized organisms. In addition, mutant characteristics can also be unstable and reversible, which highlights the importance of developing effective selection methods and ensuring phenotypic stability. Indeed, the lack of adequate selection methods is the most significant limitation of this technology, which has been addressed through the use of high-throughput methods, such as FACS, as discussed below in this review [21, 33, 53, 55].

Adaptive laboratory evolution is another cost-effective approach that does not require previous knowledge of the genetics of the microalgal strain under investigation (Figure 2.1B). Furthermore, it also avoids the introduction of foreign genetic material into the target cells; thus, the improved strains pose no regulatory issues due the long biosafety record of such technologies (Figure 2.1B) [56, 57]. In adaptive laboratory evolution experiments, cell cultures are subjected to a continuous selective pressure over a long period of time [33, 48, 49, 58]. Consequently, selection can act on all traits responsible for a suitable improvement under the environmental regimes of interest [33, 48, 49, 57, 59]. Thus, spontaneous, adaptive, and non-specific mutations are introduced into the genome and passed down from mother to daughter cells [49, 59, 60]. However, adaptive laboratory evolution is a highly laborious and time-consuming strategy that requires many generations to obtain the desired phenotype (Figure 2B) [58].

As an alternative technique to forward genetics, insertional mutagenesis has also been used to generate and select microalgal mutants of interest. In contrast with random mutagenesis and adaptive laboratory evolution, this technology requires the availability of a DNA transformation protocol for each organism, and exogenous DNA is introduced via insertional mutagenesis. This approach places DNA fragments in coding or non-coding regions of the genome, promoting gene disruption and/or insertion, and thus yielding novel mutants, in which the mutation will be more easily identified by the presence of selective markers and/or genetic (e.g., unique sequences) or phenotypic (e.g., expression of fluorescent gene products) tags [33, 58, 61–63]. This approach has the advantage of facilitating the identification of the mutation site associated with a specific mutant phenotype. However, unlike random mutagenesis and adaptive laboratory evolution, it has the disadvantage of producing GMOs via the introduction of foreign DNA.

In regard to reverse genetics methodologies, once the genotype is available, most include the introduction of foreign genetic material into cells, rendering them GMOs, and, consequently, bringing forth several concerns and commercialisation hurdles, as discussed below (Figure 2.1B) [40, 58, 64, 65]. However, genetic engineering allows the generation of tailor-made genotypes with specific mutations in the genes of interest, which may affect their sequence and/or expression, usually leading to a loss-of-function mutation through, for example, gene silencing via RNA interference (RNAi) [42, 43, 51, 52, 66]. Engineered nuclease systems, namely ZFN, transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9, are versatile tools that create double-strand breaks by cleaving DNA, which enable knockin mutations through the insertion of an intervening DNA fragment, or

knockout/knockdown mutations via deletion or changes in the nucleotide sequence. These strategies can either enhance or impair gene expression, and add or delete genes [21, 58, 67–72]. However, applying these gene-editing techniques requires a priori knowledge of the target organism's genome (Figure 2.1B), but not of the gene function; hence, it enables the analysis of the effects of such mutations on the phenotype [43]. Alternatively, unspecific gene disruption can be promoted to generate mutant phenotypes, for example, by targeting-induced local lesions in genomes (TILLING) [42, 43, 51]. TILLING has the advantage of not introducing foreign genetic material, since it consists of finding mutations in target genes with heteroduplex formations through the use of an endonuclease that specifically cleaves ethyl methanesulfonate (EMS)-induced mismatches [51, 73, 74]. Basically, this technique involves EMS-induced chemical mutagenesis, followed by high-throughput screening for point mutations [73]. Despite the potential specificity of the site-directed approaches described earlier, defining a specific genomic target is difficult. A given alteration in the genome often has pleiotropic effects on a wide set of genes, which may not only hinder the isolation of mutants with the desired phenotypes, but may also impair their viability and/or growth patterns (Figure 2.1B). Moreover, molecular biology models and transformation protocols are available for only a few microalgal species (e.g., *Chlamydomonas reinhardtii*). As a result, efficient molecular and transformation tools need to be developed for other species; thus, this is a time-consuming and potentially expensive strain improvement approach (Figure 2B) [21, 52, 62, 70].

Major factors leading to failure in microalgae production, and in the commercialisation of their biomass and bioproducts, have been addressed by each strain improvement strategy described above. One such factor is the lack of robustness and tolerance of environmental abiotic factors and cultivation conditions, in particular temperature, salinity, shear stress, and exposure to toxic compounds and pollutants. In the following sections, we provide examples of the application of strain improvement strategies for improving biomass productivity and enhancing yields of target compounds (e.g., triacylglycerols, PUFAs, and pigments). Each strain improvement approach might be used to achieve different goals, and so their respective benefits, concerns, and restrictions (summarized in Figure 2.1) will also be discussed.

2.3 Random Mutagenesis

2.3.1 Historical Perspective

Plant and animal wildtype strains have seldom been considered suitable for large-scale production [75]. Over the years, mankind has tried to improve the robustness, productivity, and nutritional value of plants, animals, and microorganisms for food and feed production [40]. Early in the 20th century, geneticists and biologists were interested in gene mutations and heredity, acknowledging them as the basis of the evolution of life [76]. However, naturally occurring mutations are rare events, and are difficult to detect and study [76]. The first immediate approach to altering natural organisms for the benefit of humans was carried out by breeders who started to recombine different genetic materials to obtain new

strains that combined the features of both parental organisms, and selecting the best-performing individuals [40, 76]. Nevertheless, scientists were eager to go beyond naturally occurring mutations and breeding. The discovery of random mutagenesis refers back to 1921, when Mavor [77] first demonstrated that X-rays had a mutating effect on *Drosophila melanogaster* chromosomes; this was followed by Little and Bagg [78], whose work corroborated this effect of X-rays on mice.

The discovery of mutagenic agents, such as X-rays, led to an experimental revolution in genetics, since researchers could now partially control mutagenesis to generate mutant progenies [79]. In the 1950s, random mutations in microalgae were also studied in order to understand pigment biosynthesis [80]. Other genetic agents, and their mutagenic properties, began to be studied, namely chemical mutagens [81]. Reports of one of the first microalgal mutants refer back to 1960, when Schwarze and Frandsen [82, 83] obtained a colourless *Chlorella* mutant through exposure to radioactive isotopes. EMS and nitrosoguanidine (MNNG) also began to be studied as mutagens applied to algae [84, 85]. At this time, EMS became, and remains to this day, one of the most frequently used mutagenic agents [81].

Over the past 100 years, random mutagenesis has been used as an easy-to-perform and robust tool to develop mutants based on a phenotype-driven search instead of focusing on specific gene modifications [53, 54, 86]. Random mutagenesis has received increased attention in the microalgae field, as it has been recognized as a very useful approach for creating more productive strains, regarding biomass and target compounds, and for adapting strains to tolerate a wider range of environmental conditions, with the advantage of not requiring extensive knowledge of microalgal genetics [16, 54, 55, 75]. Similar to adaptive laboratory evolution, unspecific mutagenic action targets a set of genes simultaneously, which, with suitable selection methods, can be used to readily isolate strains associated with the intended phenotype, at a much faster rate [75, 86]. Furthermore, since both chemical and physical mutagens are well-characterised, it is a ready-to-use technology that produces rapid results [43, 53, 87]. However, mutations are often deadly or hamper growth, and can revert to the wildtype over time, which hinders the isolation of surviving stable mutant strains [55, 88]. Strategies to prevent phenotypic reversion should be studied to enable the persistence of the improved mutants. In addition, a beneficial phenotype can only be isolated if it is possible to select it [55]; thus, more selection methods are required to enable the isolation of different mutants, namely, by resorting to high-throughput screening methods, as indicated below.

2.3.2 Physical and Chemical Mutagenesis

Random mutagenesis is carried out by treating a cell culture with a mutagen that usually induces single-nucleotide changes or small deletions in the genome that might encompass multiple genes or regulatory sequences [40, 86]. Both chemical and physical mutagenic agents can be used, and will act on the genome in different ways (Figure 2.2). Upon mutagenesis, strain selection is carried out depending on the target phenotype. However, these mutagenic agents are dangerous to work with; contact should be avoided, and precautions must be taken to handle them. For example, chemical toxic agents should be handled in a fume chamber [87, 89, 90].

Physical mutagenesis consists of applying specific dosages of radiation to cells by means of ultraviolet (UV), laser, X-ray, heavy-ion, and gamma irradiation [16, 53, 87, 91–93]. Atmospheric and room temperature plasma (ARTP) mutagenesis is a more recent method that uses room temperature plasma to generate mutagens, but also chemical species that might be mutagenic, being thus a possible physicochemical method [94].

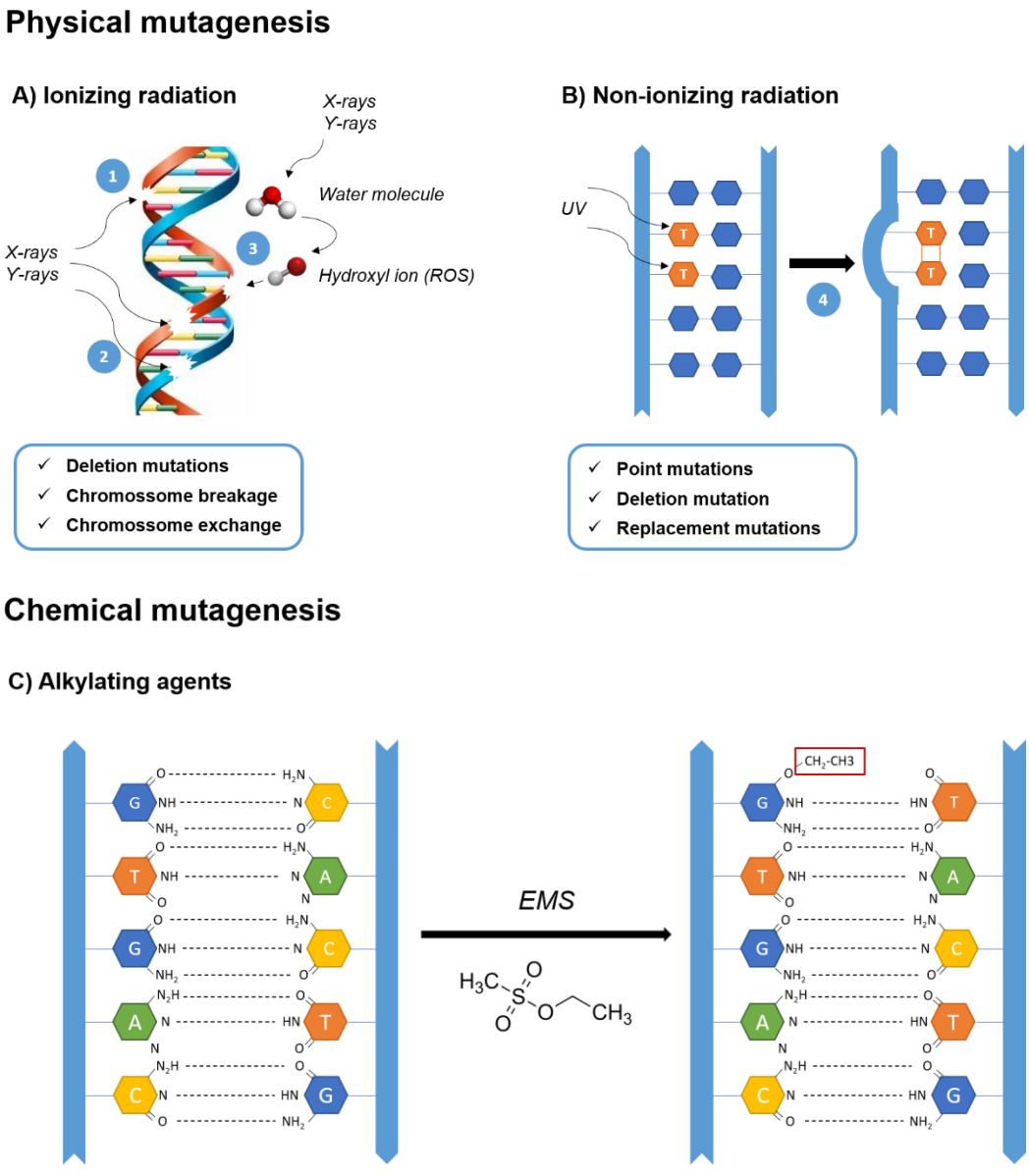


Figure 2.2 - DNA mutation mechanisms by physical and chemical mutagenesis. (**A**) Ionizing radiation may induce the following lesions on DNA: 1—single-strand breakage; 2—double-strand breakage; and 3—reactive oxygen species (ROS) damage. (**B**) Non-ionizing radiation might cause: 4—thymine dimerization (DNA kink). (**C**) Alkylating agents, such as EMS, replace a hydrogen ion with an alkyl group on a DNA base, often guanine (G).

Gamma and heavy-ion beam irradiation are both forms of high-frequency radiation that cause double-stranded DNA breakage by ionization, which often leads to the deletion of nucleotides, as well

as chromosome breaks and exchanges, respectively (Figure 2.2) [92, 95, 96]. Their higher frequency enables stronger cell penetration, which, by interacting with molecules such as water, gives origin to free radicals, which are able to disrupt macromolecules, namely DNA, causing high mutation rates [86], [91]. Both methods, along with others, such as laser mutagenesis, involve the application of electromagnetic fields to mechanically induce changes in the DNA; however, these procedures usually require sophisticated equipment [93]. Therefore, UV radiation-mediated mutagenesis is often more appealing, since it is simpler, less expensive, and easier to apply; it basically consists of exposing cells to UV sterilizing lamps commonly found in flow chambers, a basic piece of equipment available in most laboratories [97]. Moreover, it facilitates the isolation of mutants in sterile conditions, which often prevents the occurrence of biological contaminants [97]. Despite its lower frequency and lower mutation rate, UV radiation usually induces point mutations, deletions, and replacements [98, 99]. The underlying mechanism causing these mutations is based on UV absorption by DNA molecules, which leads to covalent linkage of pyrimidines, forming dimers that prevent normal base pairing and distort the DNA double-helix structure (Figure 2.2) [100, 101]. Likewise, normal base pairing and double helix unwinding for replication and transcription cannot occur, resulting in a wide range of mutations [43, 86, 98]. Nonetheless, UV mutations are more prone to reversion and impermanence, due to the existence of several UV damage repair mechanisms. To cope with the most common lesions induced by UV radiation, the cyclobutane pyrimidine dimers carry out nucleotide excision repair, which replaces lesion sites with newly synthesized oligonucleotides [102–105].

Chemical mutagens have also been widely used, and their mutagenic potential, as well as their mechanisms of action, are well-characterised [87, 106]. The most commonly used chemical mutagens are alkylating agents, i.e., molecules that carry an active alkyl group, which substitutes a hydrogen ion for an alkyl group on a DNA base, often guanine (Figure 2.2) [16, 53, 87, 107, 108]. Upon DNA replication, nucleotide substitutions, insertions, or deletions are introduced into the DNA sequence, often due to the misreading of the nucleotides on the chemically altered template strand by the DNA polymerase. The common alkylating agents are ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), nitrosoguanidine (NTG, MNNG), ethyl-nitrosourea (ENU), and *N*-methyl-*N*-nitrosourea (MNU), whereby EMS and NTG/MNNG are most frequently used in microalgal strain improvement [82,90,93]. These agents trigger a similar chemical mutagenesis mechanism in DNA, which enables high-frequency point mutation and the emergence of novel phenotypes [54, 86, 95, 106]. However, EMS alkylation is specific to guanine, resulting in G/C to A/T transitions, while MNNG induces a wide spectrum of mutations [54, 95, 109, 110].

2.3.3 Mutant Selection Methods

The result of random mutagenesis is the generation of hundreds of mutant colonies. However, only a minute portion of them has the desired phenotype; thus, an efficient screening method is often necessary (Figure 2.3). Mutants can be selected via different properties, such as visual appearance, autofluorescence, or growth performance measured by absorbance. The large sizes and different

colours of the colonies are good indicators for fast-growing mutant strains, as well as changes in pigment contents, respectively. On the other hand, the autofluorescence of pigments, such as chlorophyll and carotenoids, measured by fluorescence imaging provides a good selection tool for differently pigmented mutants, e.g., truncated antenna size mutants [111]. Nevertheless, these screening techniques are very time-consuming, as each colony needs to be inspected individually, and do not necessarily lead to the desired improved strain.

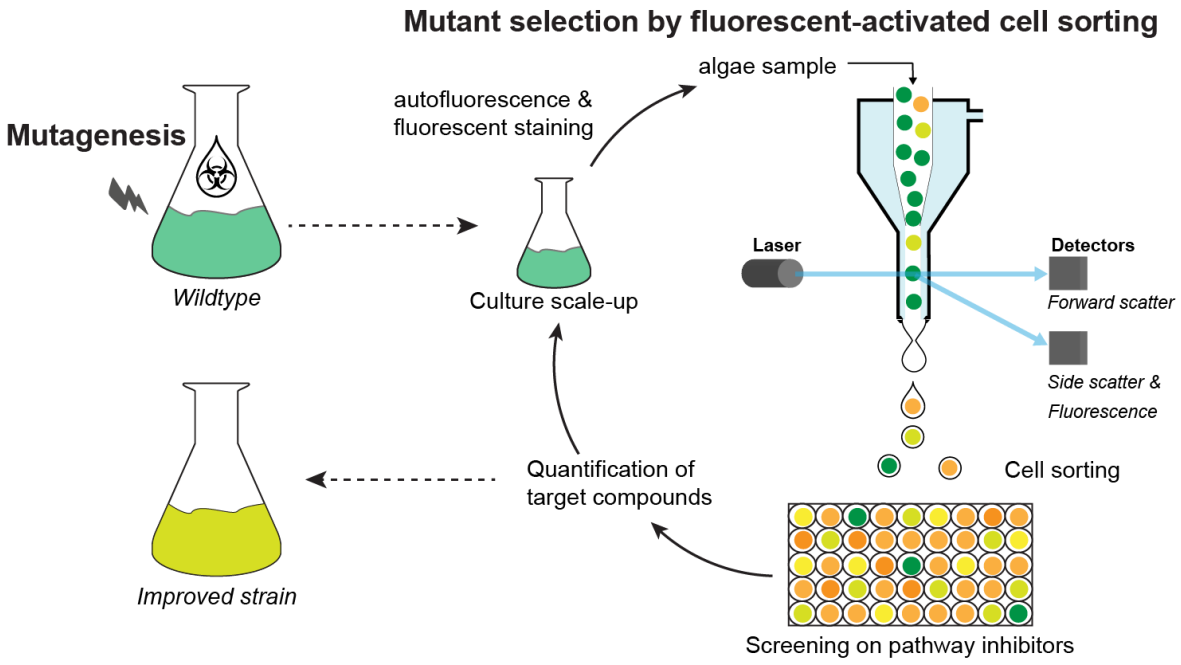


Figure 2.3 - Random mutagenesis and high-throughput mutant selection pipeline using FACS and pathway inhibitor screening.

A more direct approach is the exposure of newly generated mutants to environmental stresses, such as extreme salinities or temperatures, light or dark conditions, CO_2 levels, or nutrient stress. For example, a yellow mutant of *Chlorella vulgaris* was isolated upon mutagenesis, followed by growth in the dark; mutagenesis was crucial to suppress the need for energy supply via photosynthesis, and thus, chlorophyll synthesis (Table 2.1) [19]. Furthermore, the exposure of mutagenized cells to high salinity, high temperature, or high pH, followed by the selection of large colonies, led to the generation of salt-resistant, thermotolerant, and alkali-tolerant strains of *Chlorella* sp., respectively (Table 2.1 and Table S1) [112–116].

Table 2.1 - Relevant examples of recent random mutagenesis reports aiming at different targets, indicating the respective mutagenesis method used, species, screening strategy, and obtained improvement. An extended version of this table can be found in the Supplementary Materials (Table S1).

Species	Method	Target	Screening	Improvement	Reference
Chemical mutagenesis					
Chlorella sp.	EMS 100 mM, 30 min	Lipid content	FACS using BODIPY 505/515 staining	1.4-fold increased lipid content	[117]
Chlorella sp.	EMS 100 mM, 60 min	Thermotolerance	Incubation at 40 °C; size	Increase of 1.8-fold at 25 °C and 6.7-fold at 40 °C for growth rate	[112]
Chlorella sp.	NTG 5 μg mL^{-1} for 60 min	Alkali tolerance	pH 11.5; size	CO_2 utilization efficiency	[114]
Chlorella vulgaris	EMS 300 mM, 60 min	Chlorophyll deficiency	Colour and norflurazon	Up to 99% lower chlorophyll and 60% higher protein content	[19]
Coelastrum sp.	EMS 400 mM, 60 min	Carotenoid content	Glufosinate 25 μM and size	2-fold higher astaxanthin content	[118]
Desmodesmus sp.	EMS 600–800 mM, 30–60 min	Lipid content	Nile red fluorescence	Increased lipid productivity of up to 74%	[106]
Nannochloropsis gaditana	EMS 70 mM, 60 min	Chlorophyll deficiency	In vivo fluorescence imaging	Photosynthetic activity and biomass productivity	[119]
Physical mutagenesis					
Chlamydomonas reinhardtii	UV, 30 min	Sterols	On 0.1–1.0 mM terbinafine	50% overproduction of sterols and squalene, higher resistance to oxidative stress	[120]
Chlorella sp.	Gamma ray, 800 Gy	Lipid content	Nile red fluorescence	Increased lipid content and productivity	[121]
Phaeodactylum tricornutum	Heavy-ion irradiation	Carotenoid content	FACS (chlorophyll autofluorescence)	25% higher fucoxanthin content	[96]
Tetradesmus (*Scenedesmus*) *obliquus*	UV 254 nm (40 000 μJ cm^{-1})	Starchless mutants	Iodine vapor staining to screen for starch	41% increased total fatty acid productivity	[122]
Hybrid mutagenesis					
Chlorella vulgaris	UV 254 nm (0.5–10 min) + EMS 25 mM 60 min	Lipid content	Growth and Nile red staining;	Lipid content and biomass were, respectively, 67% and 35% higher than those of the wildtype	[123]

The most frequently used and selective process of mutant screening for high compound accumulation is the utilization of pathway inhibitors that specifically target rate-limiting enzymes of the biosynthesis of the desired compounds (Figure 2.3). Upon mutagenesis, colonies resistant to these inhibitors often contain mutations in the gene encoding an enzyme or a regulatory factor of the (partially) suppressed metabolic pathway. These mutations often cause higher metabolic flows through enhanced gene expression to overcome the metabolic inhibitor during the selection procedure [124]. In turn, such changes often lead to higher compound content and/or productivities in the respective mutants.

Carotenoid hyperproducing mutants can thus be isolated by the screening of pathway inhibitors, such as norflurazon, fluoridone, nicotine, and diphenylamine, that block carotenoid biosynthesis (Table S1). More specifically, norflurazon and fluoridone inhibit phytoene desaturase, which is responsible for the desaturation of phytoene to phytofluene [125]. However, norflurazon-resistant mutants of *Tetraselmis striata* did not only show higher carotenoid content, but also higher eicosapentaenoic acid (EPA) content, which suggests that norflurazon may also block the fatty acid desaturases of the PUFA pathway [124]. A similar pleiotropic effect has been found for diphenylamine (DPA), which is also widely used as an inhibitor of phytoene desaturase; however, in *Haematococcus pluvialis*, DPA-induced inhibition of *β*-carotene oxygenation and hydroxylation has been described, two key steps for the production of the xanthophyll astaxanthin [124, 126, 127]. To further enhance the levels of carotenoids, nicotine-induced

blockage of lycopene cyclase can be used to isolate mutants able to overcome the inhibition of lycopene cyclization into *β*-carotene [128].

When lipid or fatty acid contents are the target of improvement, inhibitors such as cerulenin, quizalofop, or erythromycin can be applied. Cerulenin is known to inhibit the *β*-ketoacyl-(acyl carrier protein) synthase I [129], while quizalofop inhibits the acetyl-CoA carboxylase (ACCase), both leading to alterations in fatty acid biosynthesis [130]. On the other hand, erythromycin is an antibiotic, targeting the protein synthesis of bacteria, but has been shown to affect chloroplast metabolism in microalgae by inhibiting the photosynthetic electron transport chain, leading to the damage of the photosystems and decreased pigment biosynthesis [131]. Nevertheless, Chaturvedi and Fujita [132] developed an erythromycin-resistant mutant of *Nannochloropsis oculata*, yielding increased contents of EPA (Table S1).

A different pathway of interest is the synthesis of sterols, which can be blocked by the herbicide terbinafine, which inhibits the enzyme squalene epoxidase. Upon mutagenesis, terbinafine-resistant mutants of *C. reinhardtii*, that overproduced sterols and squalene without compromising growth performance, were isolated (Table 2.1) [120].

Instead of targeting pathways specific to the synthesis of the biomolecules chosen for improvement, the focus can be on general metabolic fluxes. For example, ammonia assimilation can be inhibited by glufosinate, which blocks the essential enzyme glutamine synthetase. In this way, a metabolic condition similar to nitrogen starvation is triggered, which is a known inducer of lipids and certain carotenoids. Glufosinate-resistant mutants of *Haematococcus pluvialis* and *Coelastrum* sp., with higher astaxanthin contents than the wildtype, have been isolated (Table 1 and Table S1) [107, 118].

Taken together, the utilization of pathway inhibitor screening has led to promising mutants with improved biochemical profiles in different types of microalgae; however, these inhibitors often have a pleiotropic effect on overall metabolism, and may lead to unexpected or unwanted mutants.

Another approach to mutant screening is the selection of desired traits by high-throughput methods such as FACS (Figure 2.3). A key characteristic of microalgae is the autofluorescence of several pigments, such as the wine-red fluorescence of chlorophyll *a*, or the carotenoid fluorescence in the green range of the electromagnetic spectrum [133, 134]. Furthermore, lipids can be stained by fluorescent dyes such as Nile red or BODIPY505/515. Upon random mutagenesis, lipid- or carotenoid-rich mutants have been isolated by the high-throughput selection of target cells via FACS [16]. Nevertheless, the difficulty in this method is the need for fluorescence, which is only displayed by pigments, solvatochromic dyes, and other signal-specific fluorochromes. Therefore, the correlation of fluorescence to certain compounds needs to be established. For example, in a study on *Phaeodactylum tricornutum*, the correlation between fucoxanthin and chlorophyll autofluorescence was used to isolate high-fucoxanthin-producing mutants (Table 2.1) [96].

2.3.4 Random Mutagenesis Applications

One of the most important targets in strain improvement is growth performance and the resulting biomass volumetric productivity. Since the evolution of microalgae occurred under light-limiting

conditions, microalgae possess increased contents of chlorophyll molecules and large chlorophyll antenna to maximize light utilization [135]. However, under photoautotrophic cultivation, growth performance depends heavily on a sufficient light supply, and the self-shading effects of highly concentrated cultures often limit cell growth at an industrial scale. To improve light distribution in the reactor, mutants with lower chlorophyll contents and/or truncated antenna size are of interest. These often pale-green mutants have been isolated from *Chlorella vulgaris*, *Chlorella saccharophila*, *Chlorella sorokiniana*, *Nannochloropsis gaditana*, *Cyclotella* sp., and *Chlamydomonas reinhardtii* upon EMS- or UV-induced mutagenesis (Table 2.1 and Table S1) [63, 111, 119, 136–139].

In recent years, researchers have also aimed for more appealing biochemical profiles with increased contents of a group of biomolecules (e.g., lipids and protein) or higher yields of added-value target compounds (e.g., pigments and/or PUFAs), depending on the species. Upon UV radiation-mediated mutagenesis, followed by screening using iodine vapor staining, starchless mutants of *Tetradesmus obliquus* (syn. *Scenedesmus obliquus*) with 41% increment in total fatty acid (TFA) productivity were isolated (Table 2.1) [122]. Conversely, Zhang et al. [106] used EMS-induced mutagenesis and Nile red fluorescence-based screening to isolate high-lipid-producing *Desmodesmus* sp. mutants (Table 2.1). A similar approach was also used for other species, such as *Nannochloropsis gaditana, Nannochloropsis oceanica*, and *C. reinhardtii* (Table S1) [140–142]. Furthermore, a FACS-based selection using BODIPY staining showed success in the isolation of high-fatty-acid-producing mutants of *Microchloropsis salina* (syn. *Nannochloropsis salina*) and *Chlorella* sp. upon mutagenesis with EMS (Table 1) [117, 143]. Moreover, Sarayloo et al. [123] used a combination of UV radiation and EMS to mutate *C. vulgaris* and its isolated mutants, which exhibited 67% increased lipid content and 35% increased biomass than those of the wildtype (Table 2.1). In a different study, physically induced random mutagenesis using gamma rays led to *Chlorella* mutants suitable for biodiesel production (Table 2.1) [121].

Tolerance of unfavourable environmental conditions can also be improved through random mutagenesis approaches. For example, Ong et al. [112] and Sachdeva et al. [144] managed to create thermotolerant mutants of *Chlorella* sp. through random mutagenesis with EMS, which allowed them to improve its growth rate by 1.8–6.7-fold at temperatures ranging between 25–40 °C (Table 2.1 and Table S1). On the other hand, NTG-induced mutagenesis combined with a screening of large colonies on pH 11.5 agar plates led to the isolation of alkali-tolerant *Chlorella* strains [114].

Interestingly, out of the 75 articles published in the literature using random mutagenesis to improve microalgae, EMS was the most widely used mutagenic agent, being the chosen method in 43% of the reports. UV treatment came in second place, with 33% of the studies adopting this method (Figure 2.4A). The most frequently targeted genera were *Chlorella* and *Nannochloropsis* with, respectively, 36 and 15% of studies applying these techniques to these microalgae, most probably a consequence of them being of high commercial interest (Figure 2.4B). Concerning metabolism as the target for improvement, most research was carried out to enhance lipid and carotenoid productivity, 25 and 22% of the studies, respectively (Figure 2.4C).

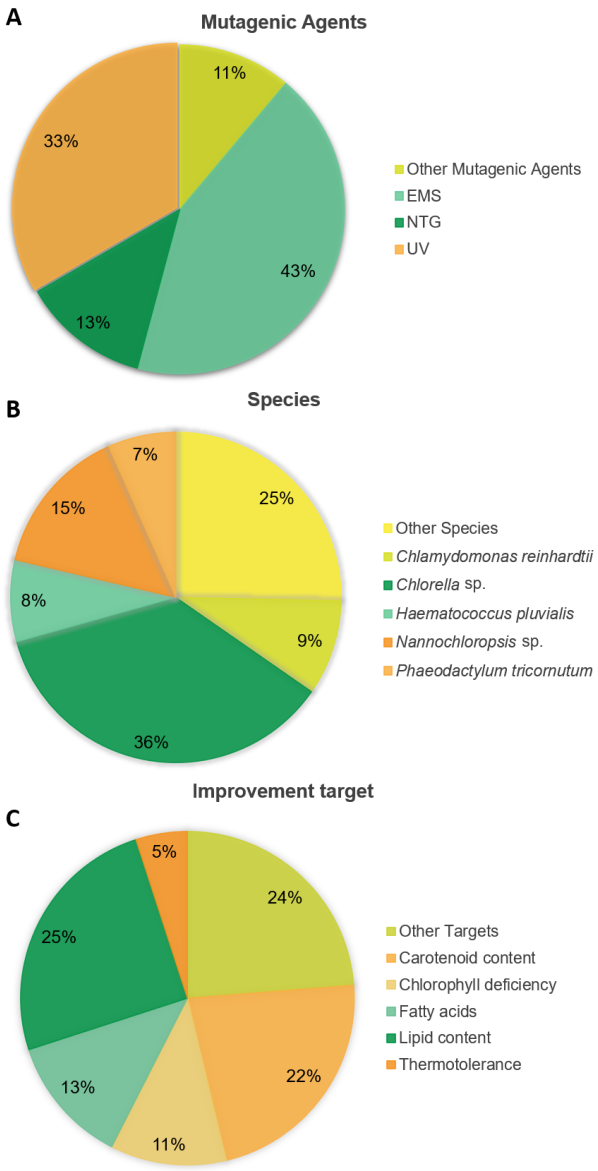


Figure 2.4 - Statistics of random mutagenesis publications (% of reports out of all the examples in this review. **A**—Mutagenic agents; **B**—Genera and species; **C**—Improvement target.

2.4 Adaptive Laboratory Evolution

It is common knowledge among the research community that stressful conditions induce microalgae to produce and accumulate different molecules, generally lipids and pigments, that help them survive and cope with environmental stress [16, 145, 146].

Likewise, adaptive laboratory evolution has been used not only to create hyperproducing strains for industrial cultivation, but also to generate more robust and tolerant strains capable of bioremediating toxic compounds, through growing under high concentrations of CO_2, phosphate, nitrate, or heavy metals, for example [16, 18, 145].

This approach consists of exposing microalgae to specific stress conditions (e.g., high salinity, CO_2, glucose, or flue gas concentration) during prolonged periods (months or years) to promote the selection of spontaneous mutations that confer an adaptive advantage to the growth conditions (Figure 2.5). Usually, in adaptive laboratory evolution experiments, the mutations detected have been mapped to stress-induced genes. Under stressful conditions, the stress-induced genes are activated at the expense of housekeeping genes and growth. If the stressful conditions are withdrawn, the stress-induced genes are repressed, and the cell resumes its normal activity. The conditions of adaptive laboratory evolution keep the stress constant from one generation to the next, and the stress response is kept active, so that any mutation that enables the cell to grow under stressful conditions is likely to be favoured. Likewise, each generational cycle improves the original wildtype strain, selecting cells with higher environmental tolerance, and thus displaying more robust, tailor-made phenotypes [18, 86, 147].

Adaptive laboratory evolution is an effective strategy to isolate improved strains, since it stimulates the accumulation of beneficial mutations in several genes in parallel, acting in a genome-wide manner, which favours the permanence and stability of the intended alterations [18, 86]. Moreover, by inducing stress conditions, the underlying microalgal metabolic mechanisms and responses to environmental stress might be further scrutinized, along with information about genes imparting stress tolerance and the design of novel strains through synthetic biology (experimental evolution) [16]. It is also useful to apply tools, such as FACS, to assist in the selection of the fittest mutants, based on, for example, their cell morphology or pigment content [18]. Adaptive laboratory evolution also allows the study of evolutionary trade-offs, since adaptations that provide better fitness in one environment might lead to maladaptation in another.

However, cells grown in the laboratory might be under evolutionary constraints imposed by lower genetic variation due to the smaller population size, as compared to the genetic diversity found in larger microalgal populations present in nature; this can hamper or delay the isolation of mutants with the desired phenotype [59]. As a result, a significant and uncertain number of generations is usually necessary to complete the evolutionary process, which can take from months to years [18, 59, 86]. This lag in microalgae adaption is also related to their larger genomes and lower growth rates compared to those of bacteria and yeast, and thus, the efficiency of this approach depends on the initial strain chosen for improvement and the stress factors applied [18]. In addition, creating laboratory mutant strains might result in organisms that are unable to thrive on more variable, less predictable environments, such as those of outdoor industrial reactors, since it is hard to mimic such conditions in a laboratorial context [18].

In Figure 2.5, two different experimental designs of adaptive laboratory experiments are represented: serial dilution (or batch) and continuous. Batch experiments are characterised by the sequential passage of the culture to different media (liquid or solid) under increasing levels of the selective stress condition. Photobioreactors in a continuous operation mode can be used in order to impose an uninterrupted selective pressure to the culture over prolonged periods of time.

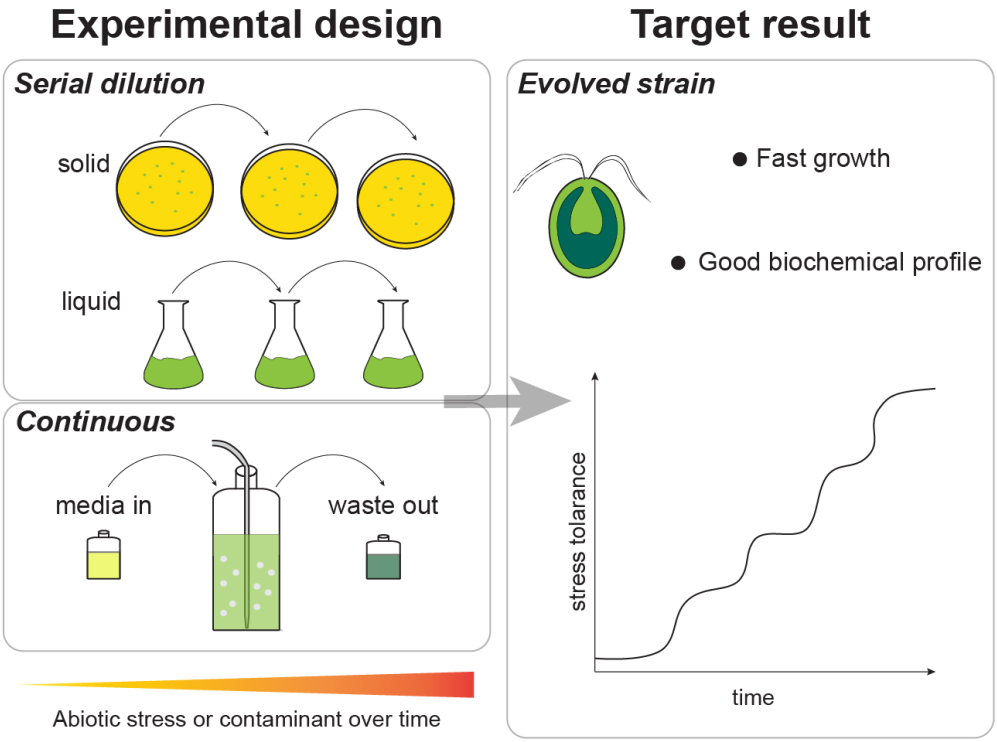


Figure 2.5 - Diagram of adaptive laboratory experiments and expected results. Left—adaptive laboratory evolution experimental designs in batch and continuous mode. The abiotic stress is kept constant or increased, and this leads to the improvement of the culture. Right—after adaptive laboratory evolution, the evolved microalgal strain will be able to tolerate the abiotic stress while maintaining favourable growth parameters and a balanced biochemical profile.

In 1997, Rebound and Bell [148] reported the first experiment of adaptive laboratory evolution with microalgae. In this work, they adapted *Chlamydomonas* cell lines to light or dark environments by submitting the populations to different light/dark stress patterns.

As with random mutagenesis, researchers aimed to improve the biochemical profile of the microalgae, namely carotenoids and fatty acids, while maintaining or improving their growth rate (Table 2.2). Regarding biochemical improvements, Gao et al. [37] have recently reported the isolation of an improved *Tisochrysis lutea* with only two rounds of selection based on fucoxanthin fluorescence-activated cell sorting (FACS), of which fucoxanthin and DHA contents were, respectively, 3.1 and 1.6-fold higher (Table 2.2). Wang et al. [149] managed to increase the EPA content of *Phaeodactylum tricornutum* cells to 139 μg/mg biomass using hyposaline and fulvic acid treatments (Table 2.2).

Scientists are also focusing on the development of strains able to perform bioremediation, which have to be robust and able to grow in media with high amounts of potentially harmful compounds (e.g., phenol, NOx, SOx, and CO_2) in order to remove them from the environment. For example, Cheng et al. [86] improved a *Chlorella* strain through an adaptive laboratory evolution of 46 cycles to flue gas, which developed tolerance and became able to grow exposed to the aforementioned pollutants (Table 2.2). Another example comprising bioremediation is the adaptive evolution reported by Wang et al. [149], in which *Chlorella* sp. was submitted to 31 cycles of exposure to phenol. The improved strain doubled the maximum biomass concentration and removed 100% of phenol from the wastewater (Table 2.2).

Table 2.2 - Examples of adaptive laboratory evolution reports obtained by different methods. An extended version of this table can be found in the Supplementary Materials (Table S2).

Species	Method	Target	Improvement	Reference
Chlorella sp.	31 cycles under 500 mg/L of phenol	Phenol wastewater removal	100% phenol removal in 7 days; maximum biomass concentration increased 2-fold	[149]
Chlorella sp.	46 cycles with flue gas	Tolerance to flue gas	Growth under 10% CO_2, 200 ppm NOx, and 100 ppm SOx	[86]
Phaeodactylum tricornutum	11 cycles, 5 days each, light-induced oxidative stress supplied by LED	Carotenoid content	2-fold higher biomass production and fucoxanthin content	[150]
Phaeodactylum tricornutum	35 cycles, 7 days each, of hyposaline treatment	Fatty acid content	EPA content increased up to 139 μg/mg biomass; improved growth	[151]
Picochlorum sp.	390 days under temperature stress	Thermotolerance	1.5 ˚C increase in the maximum tolerable temperature	[152]
Tisochrysis lutea	2 rounds of direct evolution + FACS	Carotenoid and fatty acid content	3.1-fold fucoxanthin and 1.6-fold DHA higher productivities	[37]

Recently, adaptive laboratory evolution was successfully applied to increase the maximum temperature tolerance of microalgae. Barten et al. [152] applied high temperature as a stress factor, and were able to increase the maximum temperature that *Picochlorum* sp. tolerated by 1.5 ˚C (Table 2.2). This is an important breakthrough, as temperature is one of the factors affecting the production costs of microalgae.

2.5 Genetic Engineering

Genetic engineering has been used as a tool to manipulate microalgal genomes in order to create more productive strains with tailor-made features, and to enhance the biosynthesis of valuable target metabolites [12]. Once the target pathways and respective genes are identified, as in forward genetics, genetic engineering and the available molecular tools allow one to insert (“knockin”), delete (“knockout”), or modify a gene in one or more nucleotides [153]. These modifications can lead to the upregulation of a specific gene. Conversely, depending on the mutation generated, it can lead to partial (“knockdown”) or full abrogation (“silencing”) of the expression of the target gene, which can be either permanent or transient [152, 153]. Transient gene expression can be either a time-dependent or condition-dependent phenotype, or both. As these genetic modifications might result in the overexpression or silencing of the target gene, which is associated with one or more metabolic pathways, it can be used to manipulate the metabolism in order to produce a completely new metabolite through a gain-of-function mutation (usually through a knockin mutation), or to simply improve the production of a pre-existing metabolite [12]. There are several genetic engineering methods that can be employed to modify microalgae, namely via ZFNs [154], transcription activator-like effector (TALE) nucleases (TALENs) [155], RNAi, *Agrobacterium tumefaciens*-facilitated DNA transformation [156], and/or CRISPR-Cas9 [157].

ZFNs, TALENs, and CRISPR-Cas9 technologies have been used to edit genomic DNA in order to generate mutants by using the catalytic domain of a DNA-cleaving enzyme, which is then targeted to specific sequences. While ZFNs use zinc finger DNA binding domains to target the nuclease, TALENs contain TALE repeat arrays that can be engineered to bind to specific DNA sequences [156]. CRISPR-Cas9 is a more recent technology that instead uses a guide RNA to target the Cas9 nuclease to cleave at a specific genomic site [158]. All these techniques depend on the generation of double-strand breaks,

which can be repaired by non-homologous end-joining (NHEJ), generating indel (insertion/deletion) mutations, or by homology-directed repair (HDR) with the help of donor templates, which can generate precise mutations, down to single-nucleotide mutations and indels (Figure 2.6) [155, 159].

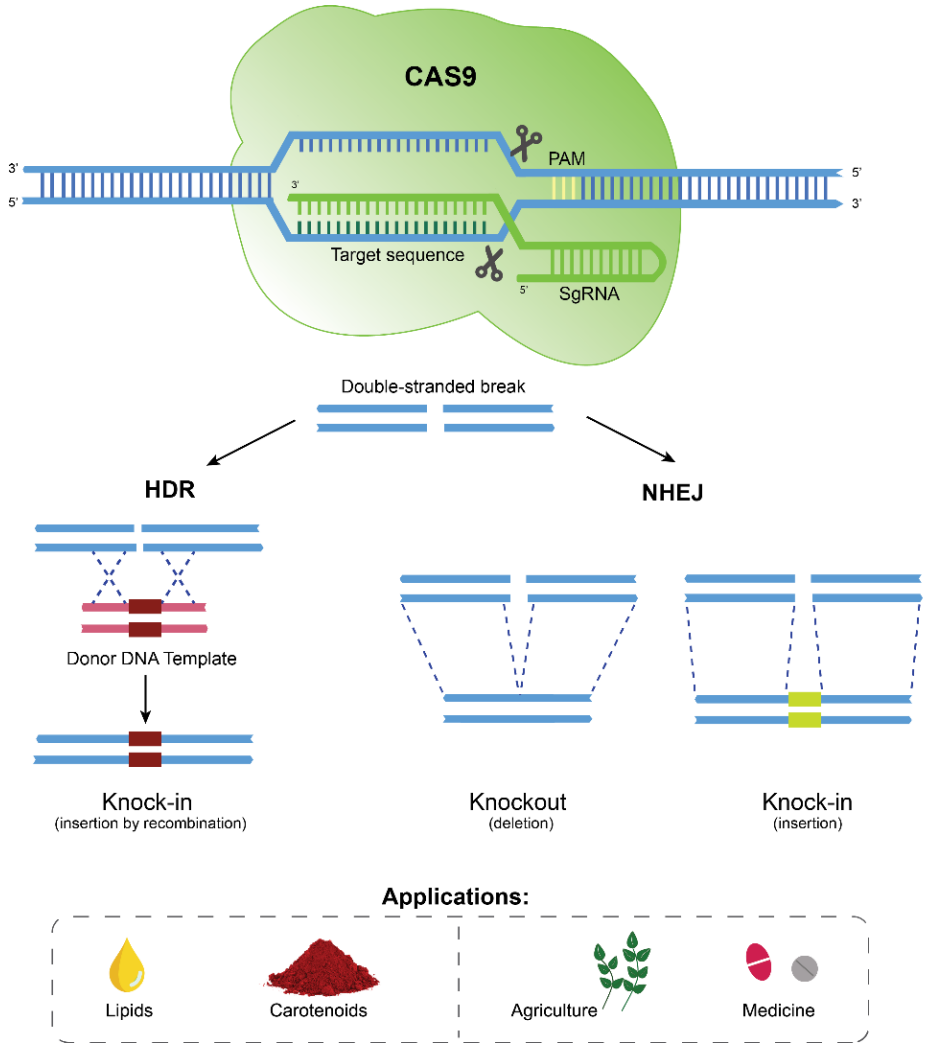


Figure 2.6 - Genome editing using CRISPR-Cas9. The nuclease Cas9, with a custom single-guide RNA (SgRNA), cuts DNA on a specific sequence near a protospacer adjacent motif (PAM), a short sequence recognized by the enzyme downstream of the cleavage site. In the presence of exogenous DNA, homology-directed repair (HDR) can take place, generating a knockin mutant; otherwise, non-homologous end join (NHEJ) repair might occur, so that the ends of the DNA fragments are brought together. The mutant might contain a disrupted target gene (knockout) or an inserted gene or DNA fragment (knockin), which could generate a loss- and/or gain-of-function phenotype. The main applications of this technology are related to improving lipid content and profile, the production of high-value compounds such as carotenoids, the development of tolerance for agroindustrial applications, and the production of recombinant proteins for pharmaceutical and medical applications.

The first microalgae to undergo nuclear transformation was *C. reinhardtii* in 1990, using glass bead agitation and electroporation [160]. Other oleaginous species have been the focus of interest in this area, such as *Nannochloropsis* sp., *Phaeodactylum tricornutum, Dunaliella* sp., and *Tetraselmis* sp., as shown in Table 2.3. Different strategies have been used to enhance lipid production, such as the

overexpression of enzymes involved in TAG assembly, PUFA production (EPA and DHA), or NADH biosynthesis [146]. The overexpression of the enzyme diacyl glycerol acyl transferase (DGAT), involved in the last step of TAG synthesis, led to an increment in 79% of EPA and 69% of the neutral lipid contents in *P. tricornutum* and *N. oceanica,* respectively (Table 2.3) [161, 162]. Glycerol-3-phosphate acyltransferase 2 (GPAT) is the first enzyme involved in TAG synthesis, and led to a 2.9-fold increase in TAG content when overexpressed in *P. tricornutum* (Table 2.3) [163]. Moreover, a combination of genetic transformation of *C. reinhardtii* to overexpress acetyl-CoA synthetase or type-2 diacylglycerol acyl-CoA acyltransferase and nitrogen or phosphorus starvation resulted in a 2.4-fold and 2.5-fold increase in TAG content, respectively (Table 2.3) [164, 165]. Gene edition has also been applied to microalgae to obtain higher lipid contents. For example, a CRISPR-Cas9-mediated knockout of NO06G3670 transcription factor was used by Südfeld et al. [166] to enhance lipid accumulation in *N. oceanica* by 40% (Table 3). In addition, Xue et al. [167] obtained a 2.5-fold increase in total lipid concentration in *P. tricornutum* through the overexpression of the malic enzyme (ME), a biocatalyst able to convert malate to pyruvate, with the production of NADPH, which plays an important role in lipid biosynthesis (Table 2.3) [168].

There are several methods used to transfer exogenous DNA into cells, such as electroporation, glass beads, and mediation using biological vectors, such as *Agrobacterium tumefaciens.* The use of the latter vector has been demonstrated to be effective in plants and fungi [169]. When CRISPR-Cas9 technology was applied for the first time in *Chlorella vulgaris,* transformants from the *A. tumefaciens*-mediated method displayed a 46% (w/w) higher lipid accumulation (Table 2.3) [170].

For the purpose of increasing biomass productivity, RuBisCo activase has often been targeted in microalgae to improve the limiting rate of CO_2 assimilation in photosynthesis. Wei et al. [171] overexpressed this enzyme in *Nannochloropsis oceanica*, and obtained mutants with a growth rate 32% higher compared to the wildtype (Table 2.3).

In addition, the overexpression of enzymes involved in carotenogenesis is also a strategy to increase high-value compounds, such as pigments. The first steps of carotenoid biosynthesis are catalysed by phytoene desaturase (PDS) and phytoene synthase (PSY). For example, Cordero et al. [172] enhanced violaxanthin and lutein content 2-fold and 2.2-fold, respectively, in *C. reinhardtii*, through the heterologous overexpression of PSY, while Galarza et al. [173] achieved an increase of 67% in astaxanthin content in *H. pluvialis* through PDS overexpression (Table 2.3).

Gene editing techniques have great potential to create hyperproducing and more productive microalgal strains, since specific genes of interest can be targeted to tailor the genome to attain the desired traits. Unlike adaptive laboratory evolution and random mutagenesis, genetic engineering can be directed to modify a specific gene or regulatory sequence, whose phenotype can be tested under laboratory conditions [12, 86].

Nonetheless, there are several limitations to genome editing, which explains why there are few reports on the successful genetic engineering of microalgae. Firstly, the phenotypes resulting from the mutation of specific genomic sequences must be identified for each species, and the development of consistent genome editing techniques for microalgae are far from a ready-to-use technology [68, 86]. Secondly, obtaining genetically stable mutant strains able to thrive under industrial settings and

overcome the resistance of transformants to Cas9 toxicity remains a challenge [72]. Finally, some desirable features, such as stress tolerance, are often complex processes that encompass a wide range of genes, making it difficult to achieve significant strain improvement by targeting specific genes via genetic and metabolic engineering [18]. Hence, researchers have resorted to omics (e.g., metabolomics, proteomics, lipidomics, and transcriptomics) technologies to predict complex interactions among gene products. This strategy might enhance the outcome of genome editing and overcome the difficulty of improving multigenic traits [12, 86].

Table 2.3 - Examples of genetic engineering methods in microalgae and the results obtained. An extended version of this table can be found on the Supplementary Materials (Table S3).

Species	Method	Target	Improvement	Reference
Chlamydomonas reinhardtii	Heterologous overexpression of phytoene synthase (PSY)	Carotenoid content	2.0- and 2.2-fold higher in violaxanthin and lutein content	[172]
Chlamydomonas reinhardtii	Overexpression of acetyl-CoA synthetase (ACS)	Lipid content	2.4-fold more TAG in N depletion media	[165]
Chlamydomonas reinhardtii	Overexpression of type-2 diacylglycerol acyl-CoA acyltransferase (DGTT4)	Lipid content	2.5-fold increased TAG content in P depletion media	[164]
Chlorella vulgaris	Heterologous overexpression of mGFP	Lipid content	46% (w/w) higher lipid accumulation	[170]
Haematococcus pluvialis	Overexpression of phytoene desaturase (PDS) gene	Carotenoid content	67% increase in astaxanthin accumulation	[173]
Nannochloropsis oceanica	Knockout of NO06G03670	Lipid content	Increase in neutral lipids content by 40 %	[166]
Nannochloropsis oceanica	Overexpression of RuBisCO activase	Growth productivity	Growth rate and photosynthesis increase by 32 and 28%, respectively, induced under low level of CO_2	[171]
Nannochloropsis oceanica	Overexpression of type 2 diacylglycerol acyltransferase (DGAT)	Lipid content	69% increase in neutral lipid content	[162]
Phaeodactylum tricornutum	Overexpression of glycerol-3-phosphate acyltransferase 2 (GPAT2)	Lipid content	2.9-fold increase in TAG content	[163]
Phaeodactylum tricornutum	Overexpression of malic enzyme	Lipid content	2.5-fold increase in total lipid content	[167]
Phaeodactylum tricornutum	Overexpression of type 2 DGAT	Lipid content	76% increase in EPA content	[161]

2.6 Regulatory Frameworks on Genetically Modified Organisms (GMOs) Applied to Microalgae

There is a general concern about generating genetically modified organisms by means of the techniques discussed above. In the European Union (EU), a GMO is defined as “an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” [40, 174]. Using this definition, most microalgal mutants that have not been generated by spontaneous mutations would fall under this definition of a GMO. However, because of their extensive safety track record, and the fact that no foreign genetic material is introduced into the mutant genome, microalgal strains improved by random mutagenesis or adaptive laboratory evolution are exempt from the requirements for those obtained by heterologous DNA transformation/transfection using genetic engineering [16, 40, 55, 174, 175]. In the United States, three agencies, U.S. Food and Drug Administration (FDA), U.S. Environmental Protection Agency (EPA), and U.S. Department of Agriculture (USDA), work together to regulate and ensure that GMOs are safe for

humans, animals, plants, and the environment on a case-by-case basis [176, 177]. These safety measures have been put in place, since the interactions of new strains with natural environments are unknown and there is the possibility of gene flow between species with unpredictable consequences that might unbalance ecosystems, particularly in primary producers at the food web base [12, 64]. Higher risks are assigned to herbicide and antibiotic resistance transgenes or genomes with enhanced growth performances that might outcompete microalgal strains in natural environments [40, 174]. Accordingly, careful risk assessment and close monitoring should be carried out before providing GMO products to the microalgal market [40, 64]. However, as randomly generated mutants or, even better, spontaneous mutants selected by adaptive laboratory evolution, are exempt from the requirements of genetically engineered GMOs, the commercialisation of the first two types of improved strains appear to be more promising in terms of market demand, as well as existing regulatory frameworks.

2.7 Conclusions

Microalgal strain improvement is essential to provide more productive and robust strains, and to address the current challenges in industrial production. The decision of investing in one strain improvement approach over another should be made in accordance with the improvement target and the intended application. As such, random mutagenesis is a cost- and time-effective strategy to deliver more competent strains for microalgae industry. However, there is still a long way to go concerning the screening and selection of mutants with the desired phenotype. It is important to test and study new metabolic inhibitors and selective pressures to develop selection methods that enable a more effective identification and isolation of different phenotypes. In addition, the potential of high-throughput methods, such as FACS, is still underexploited, since it is limited to a few markers (e.g., pigments autofluorescence and lipid dyes) and, subsequently, few metabolic targets (e.g., lipid and carotenoids contents). These technologies should be further studied to shed light on how the different cell characteristics and fluorescent dyes are related, and the information they can provide about a cell and its compounds. Furthermore, the study of microalgal omics, such as genomics and metabolomics, has an important role in elucidating the regulation of the pathways responsible for the biosynthesis and catabolism of target compounds. This interconnected knowledge will enable the identification, selection, and isolation of different factors (e.g., gene products and conditions) that are crucial for the improvement of a specific microalgal strain with a given target phenotype.

2.8 Supplementary Materials

The following are available online at https://www.mdpi.com/1660-3397/20/7/440, Table S1: Extended version of Table 1: examples of random mutagenesis reports, where the respective mutagenesis method used, target, species, screening strategy and obtained improvement is referred.; Table S2: Extended version of Table S2: examples of adaptive laboratory evolution reports aiming at different targets,

where the respective method used, target, species, screening strategy and obtained improvement is referred.; Table S3: Extended version of Table 3: examples of genetic engineering reports, where the respective method used, target, species and obtained improvement is referred.

3

RANDOM MUTAGENESIS AND SELECTION WITH METABOLIC INHIBITORS TO ISOLATE MICROALGAL CHLOROPHYLL-DEFICIENT MUTANT STRAINS FOR NUTRITIONAL APPLICATIONS

3.1 Oxyfluorfen: a novel metabolic inhibitor to select microalgal chlorophyll-deficient mutant strains for nutritional applications

This chapter was adapted from the following published research paper:
M. Trovão, L. Cardoso, L. M. Schüler, A. Machado, G. Espírito Santo, H. Pedroso, A. Reis, A. Barros, N. Correia, M. Costa, S. Ferreira, H. Cardoso, M. Mateus, J. Silva, H. Pereira, F. Freitas and J. Varela, "Oxyfluorfen: a novel metabolic inhibitor to select microalgal chlorophyll-deficient mutant strains for nutritional applications," *Algal Res.*, vol. 81, no. 103572, 2024, doi: 10.1016/j.algal.2024.103572

ABSTRACT

Nowadays, there is an increasing demand for novel feedstocks and alternative protein sources to meet global needs. Because of their rich nutritional profiles and high protein contents, microalgae-based food products and supplements are being developed. Nonetheless, these products present organoleptic characteristics such as taste, smell and colour that are often considered unpleasant by human and animal consumers. To address this constraint, strain improvement approaches such as random mutagenesis have been used, which combined with the right selection strategy, lead to more appealing microalgal biomass. In this work, a novel selection strategy using oxyfluorfen, an inhibitor of the chlorophyll synthesis pathway, was applied for the first time to isolate chlorophyll-deficient strains of

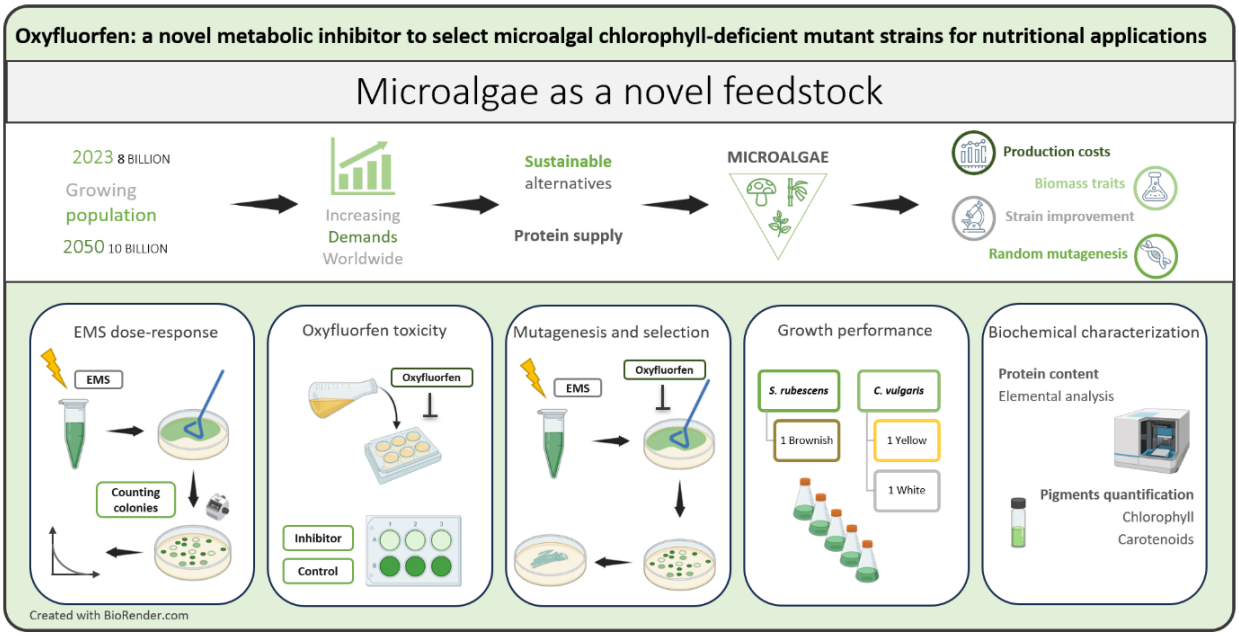
Scenedesmus rubescens and *Chlorella vulgaris* upon treatment with ethyl methanesulfonate (EMS). With this approach, one *S. rubescens* brownish (37Y01) mutant strain, as well as two *C. vulgaris* mutant strains, one yellow (31Y15) and one white (31W62), were obtained. *S. rubescens* 37Y01 displayed a reduced protein content of 19.1% dry weight (DW) compared to that of the wildtype, which presented a protein content of 25.0% DW. *C. vulgaris* wildtype and mutants exhibited higher protein contents, in the 42.8-44.3% DW range, compared to *Scenedesmus rubescens* (p<0.05). The selective pressure of this inhibitor allowed the selection of *S. rubescens* and *C. vulgaris* mutants displaying 55% and 95% decrease in chlorophyll content, respectively. The reduced chlorophyll content greatly improves the sensory properties and consumer acceptance of established mutants, increasing the potential of both strains as feedstocks to develop novel food products.

Keywords: Chlorophyll; Heterotrophic; Microalgae; Protein; Random mutagenesis; Selection method; Strain improvement.

Highlights

- Strain improvement enabled the development of appealing microalgal products;
- A novel selection method to screen randomly-generated mutants is proposed;
- Oxyfluorfen was used to select mutants with up to 55-95% less chlorophyll;
- A brownish mutant of *S. rubescens* was isolated with 19% DW of protein content;
- Yellow and white mutants of *C. vulgaris* with protein of 43-44% DW were isolated;

GRAPHICAL ABSTRACT



3.1.1 Introduction

Global demand for food and feed has been increasing steadily, along with the world population (www.un.org/en/global-issues/population), resulting in a progressively compromised food security worldwide, particularly regarding protein supply [13, 14]. Thus, it is urgent to explore alternatives to conventional feedstocks and adapt the food system towards more sustainable, healthy and affordable diets [178]. In this context, microalgae have been a subject of greater interest as one of the promising alternatives for food and feed and other biotechnological applications [179].

Microalgae are natural sources of vitamins, pigments, bioactive compounds and high-quality vegan protein with all the essential amino acids [180].

However, microalgae-based industries have been facing multiple challenges. Despite all the investment in R&D, microalgal products struggle to reach competitive market prices, mainly due to low biomass and target compounds productivities as well as costly inputs [23–25]. The organoleptic characteristics of the biomass, namely the green colour and grassy taste originated by chlorophyll, also hinder consumers' and even animals' acceptance [7, 9, 19, 181, 182]. Finally, only a limited number of microalgal strains, such as *Chlorella* spp., are currently approved as novel foods in Europe (www.algae-novel-food.com) (EU, 2017/2470; EU, 2015/2283).

Overcoming the current bottlenecks of microalgae production requires a multistage optimisation approach in the whole cultivation and processing pipeline, starting with strain selection and improvement [16, 21, 22, 33]. Several strategies have been applied to generate and select mutants suitable for large-scale cultivation, such as adaptive laboratory evolution, random mutagenesis and gene editing tools [12, 33, 183]. A more detailed discussion of these methodologies is available in a recent review by Trovão et al. [183].

Random mutagenesis is a well-established and cost-effective technology that enables the isolation of more productive and tolerant microalgal strains for different biotechnological applications [55, 183]. However, there are two limiting steps concerning random mutagenesis: lack of effective selection methods for different characteristics and phenotype instability [55, 88, 183].

Several selection strategies have been developed to select a target phenotype out of numerous mutant colonies. Methods based on the characterisation of each colony, such as visual appearance and autofluorescence, are often used to select fast-growing and differently coloured mutants. Nevertheless, these techniques are heavily time-consuming and hamper the reduction of the number of colonies to test. High-throughput technologies, such as fluorescence-activated cell sorting (FACS), are promising tools to address this lack of efficiency but still require further innovation and study to take full advantage of it [21, 53, 55, 133]. Exposure to stressful conditions is also a commonly used approach to select mutants with tolerant phenotypes [183]. Finally, a more direct approach might be applied to select mutants producers of specific biocompounds by resorting to pathway inhibitors that target enzymes or regulatory factors involved in the biosynthetic pathway of the compounds of interest, such as carotenoids [126, 130, 183]. Although the latter enables the isolation of mutants with improved biochemical profiles, these mutations often hamper or are detrimental to growth due to a loss of function. In addition, mutants

might revert their phenotype, either through DNA damage repair mechanisms or secondary mutations [103, 104]. Thus, new selection strategies, namely using new inhibitors that directly target specific pathways, should be studied to select improved phenotypes and prevent their reversion.

In this context, oxyfluorfen, an inhibitor that specifically targets the chlorophyll biosynthetic pathway [184, 185], holds high potential to isolate stable chlorophyll-deficient mutants with different pigmentation profiles. Oxyfluorfen is a diphenyl ether that has been used as an herbicide in agriculture since 1976 [186, 187]. This fluorinated chemical was reported to inhibit protoporphyrinogen oxidase (PPO) by inducing the formation of reactive oxygen species (ROS) through the interaction of oxygen with protoporphyrin IX, which leads to the inhibition of the synthesis of photosynthetic pigments as chlorophyll (Figure 3.1) [184–188].

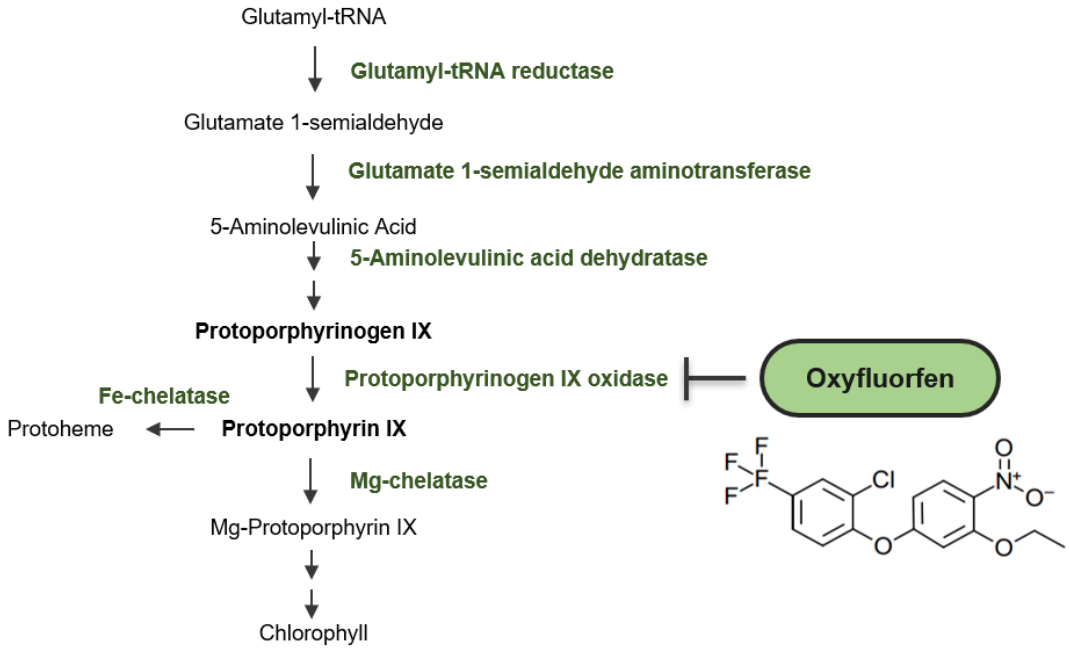


Figure 3.1 - Simplified chlorophyll synthesis pathway and inhibition target of oxyfluorfen. Adapted from [185, 189, 190].

Therefore, this work focused on the optimisation of a novel strain improvement pipeline using oxyfluorfen to establish chlorophyll-deficient mutants from *Chlorella vulgaris* and *Scenedesmus rubescens* with protein contents similar to the wildtype strains but with improved organoleptic characteristics. To the best of our knowledge, this is the first report of using oxyfluorfen as a selective inhibitor upon random mutagenesis with ethyl methanesulfonate (EMS) to isolate improved mutants of *C. vulgaris* and *S. rubescens*. Moreover, the mutagenesis for altered pigment profiles with EMS is here described for the first time for the genus *Scenedesmus*.

3.1.2 Materials and Methods

3.1.2.1 Wildtype inocula and culture media

Scenedesmus rubescens and *Chlorella vulgaris* axenic cultures were obtained from Allmicroalgae Natural Products S.A. culture collection. The wildtype seeds were obtained from cryopreserved aliquots stored in liquid nitrogen (–196 °C).

The optimised medium reported by Espírito Santo et al. [191] was used to cultivate *S. rubescens* with a modification of the nitrogen source (Sr-HM): sodium nitrate (10 mM) and urea (30 mM) were used instead of ammonia. The heterotrophic medium (HM) described by Barros et al. [192] was used to cultivate *C. vulgaris* (Cv-HM), with 20 mM of ammonium sulphate. For both species, glucose at 20 g L^{-1} was used as the carbon source.

3.1.2.2 EMS dose-response experiments

According to the protocol described by Schüler et al. [19], WT inocula in the early exponential phase were concentrated 10-fold in the respective Sr-HM or Cv-HM medium by centrifugation. Concentrated *S. rubescens* culture (1.54×10^6 cells mL^{-1}) was treated with 0-600 mM of EMS (Sigma-Aldrich, St. Louis, USA) and incubated under constant agitation (100 rpm) at 28 °C for 1 h in the dark. Similarly, *C. vulgaris* culture (2.16×10^5 cells mL^{-1}) was exposed to 0-300 mM of EMS. The cells were incubated at 30 °C and 100 rpm for 1 h in the dark. The EMS reaction was stopped by adding sodium thiosulfate (5%, w/w) and cells were resuspended in the respective diluted HM medium (1:2) after washing thrice. The mutagenized cells were incubated for 24 h in the dark at 28 °C and 30 °C, respectively, for *S. rubescens* and *C. vulgaris*. In order to determine the survival rate, cultures were plated in triplicates onto Plate Count Agar (PCA; HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated in the dark at the corresponding growth temperatures for 15-21 days.

3.1.2.3 Oxyfluorfen toxicity

Scenedesmus rubescens (1.01×10^6 cells mL^{-1}) and *Chlorella vulgaris* (2.16×10^5 cells mL^{-1}) in early exponential phase were concentrated 10-fold in the respective diluted HM medium (1:2) by centrifugation. Then, 0.1 mL of concentrated culture was spread onto 6-well plates containing PCA supplemented with oxyfluorfen. All conditions were tested in triplicates. Oxyfluorfen solution in methanol was prepared at 500 mg L^{-1}, which was filter-sterilised with 0.22 µm PTFE filters (Labbox Labware, SL, Barcelona, Spain). Oxyfluorfen was tested at 0-700 µg L^{-1} for *S. rubescens* and at 0-5000 µg L^{-1} for *C. vulgaris*.

Plates were incubated in the dark at the respective growth temperatures for 14 days, after which the growth in each well was observed and compared to the control. In addition, a sample from each condition was replated onto PCA without the inhibitor. After 3-5 days, the growth was visually analysed to determine the optimal concentration range of oxyfluorfen to be used in mutant selection.

3.1.2.4 Random mutagenesis and mutants' selection

According to the dose-response curves previously established (Section 3.1.2.2.), random mutagenesis was performed on *S. rubescens* (1.54×10^6 cells mL^{-1}) with 150, 200 and 250 mM of EMS, while for *C. vulgaris* (2.16×10^5 cells mL^{-1}) the EMS concentrations used were 100, 125 and 150 mM. The mutagenesis protocol was as described above (Section 3.1.2.2.).

Upon mutagenesis, a selection step was applied by resorting to oxyfluorfen. *S. rubescens* mutagenized cells were plated onto PCA with 100, 200 or 300 µg L^{-1} of oxyfluorfen and incubated in the dark at 28 °C for 28 days. For selecting *C. vulgaris* mutants, the cultures were plated onto PCA with 300, 400 and 500 µg L^{-1} of oxyfluorfen and incubated in the dark at 30 °C for 28 days. For each plate, 0.1 mL of concentrated culture was spread. Differently coloured colonies were picked and streaked onto PCA with the same concentration of oxyfluorfen and re-streaked for 10 generations to ensure phenotype stability. A pre-selection step was carried out, in which 9 yellow and 11 white mutants of *C. vulgaris* and 4 brownish mutants of *S. rubescens* were isolated. These mutants were streaked several times in plate, as explained, and only one of each species was selected, with stable phenotype and the most appealing colour.

3.1.2.5 Growth performance of wildtype vs mutants

Mutants with a stable phenotype on solid medium were transferred to liquid medium (without inhibitor) to compare their growth performance with the respective wildtype strains. Growth assays were conducted in 250-mL Erlenmeyer flasks in triplicate with a final working volume of 50 mL.

Scenedesmus rubescens WT (37WT) and its mutants were grown at 28 °C, in Sr-HM medium, at pH 6.5, with PIPES buffer at 60 mM, in an orbital incubator set at 200 rpm (ArgoLab® shaker SKI 4, Carpi, Italy). Likewise, *C. vulgaris* WT (31WT) and its mutants were cultivated at 30 °C, 200 rpm, in Cv-HM medium, at pH 6.5, with PIPES buffer at 50 mM.

Cultures were sampled and analysed daily by measuring the optical density at 600 nm (OD_{600}) (Genesys 10S UV-Vis®; Thermo Fisher Scientific, Massachusetts, EUA) and medium pH (Metria universal pH test paper strips; Labbox Labware, SL, Barcelona, Spain), as well as through optical microscopy (Axio Scope A1®, Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Dry weight (DW) was determined by filtering microalgal suspensions using pre-weighed 0.7 µm glass microfibre filters (VWR International, Pennsylvania, USA) and washed with demineralized water.

Finally, the samples were dried at 120 °C and weighed using a moisture analyser (MA 50.R Moisture Analyser, Radwag®, Radom, Poland). The *DW* was calculated as follows:

$$DW\ (\mathrm{g\ L^{-1}}) = \left(m_f - m_i\right) \div V \quad (1)$$

where m_f - m_i represent the algal mass collected by filtration and *V* corresponds to the volume of filtrated cell suspension.

A *DW* vs. *OD* correlation was established for *S. rubescens* (Equation 2; R^2 = 0.8478) and *C. vulgaris* (Equation 3; R^2 = 0.9136):

$$OD_{600} = (0.5566 \times DW) + 1.416 \quad (2)$$

$$OD_{600} = 0.5934 \times DW \quad (3)$$

The growth rate was obtained by equation 4 and biomass productivity by equation 5, where DW_f and DW_i correspond to the final and initial dry weights measured at the time points t_f and t_i, respectively.

$$\mu\ (\mathrm{d}^{-1}) = \ln\left(DW_f \div DW_i\right) \div (t_f - t_i) \quad (4)$$

$$r_p(\mathrm{g\ L^{-1}d^{-1}}) = \left(DW_f - DW_i\right) \div (t_f - t_i) \quad (5)$$

At the end of each assay, samples were centrifuged at 4500 *g* for 15 min (Hermle® Z300 centrifuge, Gosheim, Germany), and pellets were frozen at −20 °C, freeze-dried in a Coolvacuum, Lyomicron (Barcelona, Spain) and stored in a desiccator for posterior biochemical analysis.

3.1.2.6 Biochemical characterisation

3.1.2.6.1 Protein content

The protein content was estimated by performing an elemental analysis (Vario EL III®, Elementar Analyser System; GmbH, Hanau, Germany) according to the manufacturer's instructions and by multiplying the nitrogen content by a factor of 6.25 [193].

3.1.2.6.2 Pigments extraction and quantification

Chlorophyll extraction and quantification were performed according to Ritchie's method [194]. Briefly, 10 mg of biomass were weighed into a tube to which 2 g of glass beads (d_p = 1 mm) and 6 mL of acetone (99%) were added. Milling was performed by vortexing for 10 min, followed by centrifugation for 10 min at 2547 *g* (Hermle® Z 300 centrifuge, Wehingen, Germany). The supernatant of the samples was collected and kept in the dark, while the extraction step was repeated until the pellet became colourless. Quantification of chlorophyll *a* and *b* was performed by measuring the absorbance (*Abs*) of the supernatant at 630, 647, 664 and 691 nm, and applying Equations 6 and 7.

$$Chl_a = -0.3319Abs_{630} - 1.7485Abs_{647} + 11.9442Abs_{664} - 1.4306Abs_{691} \quad (6)$$

$$Chl_b = -1.2825Abs_{630} + 19.8839Abs_{647} - 4.8860Abs_{664} - 2.3416Abs_{691} \quad (7)$$

Carotenoid extraction was carried out with 1 mL of methanol containing 0.03% butylhydroxytoluene for 5-10 mg of dry biomass, followed by bead milling with 0.6 g of glass beads (d_p ~ 425-600 µm) using a mixer mill (Retsch MM 400) at 30 Hz for 3 min. Then, samples were centrifuged at 24000 *g* for 3 min, and the supernatant was collected. The extraction procedure was repeated until both the pellet and the supernatant became colourless. Afterwards, the extracts were evaporated under continuous nitrogen flow. Finally, the dried samples were dissolved in 1 mL HPLC grade methanol and filtered through a 0.22 µm PTFE filter.

Carotenoids quantification was performed using a Chromaster HPLC System (Hitachi, VWR, Portugal), equipped with a diode array detector (5430 DAD, Hitachi, VWR, Portugal) and a Purospher® STAR RP-18 endcapped (Merck, Portugal) (250 × 2.1 mm, 5 µm) chromatographic column set at 20 °C. A mobile phase composed of solvent A (acetonitrile:water 9:1, v/v) and solvent B (ethyl acetate) were applied according to the following gradient: 0-16 min, 0-60% B; 16-30 min, 60% B; 30-32 min 100% B and 32-35 min 100% A [195]. A Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, US) was used for carotenoid identification. Quantification was carried out using calibration curves of neoxanthin, violaxanthin, lutein and β-carotene standards (Sigma-Aldrich, Portugal). The injection volume of both extracts and standards was 100 µL.

3.1.2.7 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 as well as graphical representation (GraphPad Software, San Diego, USA, http://www.graphpad/.com). For each test, the mean and standard deviation were determined among biological triplicates.

3.1.3. Results and Discussion

3.1.3.1. EMS dose-response experiments

The first step of this work was to determine the survival rate of *S. rubescens* and *C. vulgaris* exposed to EMS (Figure 3.2).

In *S. rubescens*, EMS concentrations of 100, 200, 300 and 400 mM led to the respective survival rates of 58%, 9%, 6% and 0.1%, while at higher concentrations of EMS the survival rate was 0% (Figure 3.2). On the other hand, at 100 mM of EMS, the survival rate of *C. vulgaris* was 9%, which reduced to 2% at 150 mM and 0% for higher concentrations.

The optimal mutagen concentration to apply to a mutagenesis protocol should correspond to a survival rate between 5-10%, since lower survival rates tend to favour colonies with multiple mutations, and these should be avoided as they might compromise genome integrity and generate a more complex, pleiotropic phenotype that might revert more readily. On the other hand, survival rates should not be too high to improve the probability of finding cells with at least one mutation, decreasing the number of

mutants to screen [19, 196]. Accordingly, the optimal EMS concentrations to use in the conditions tested were 200-300 mM and 100-150 mM for mutagenesis of *S. rubescens* and *C. vulgaris*, respectively

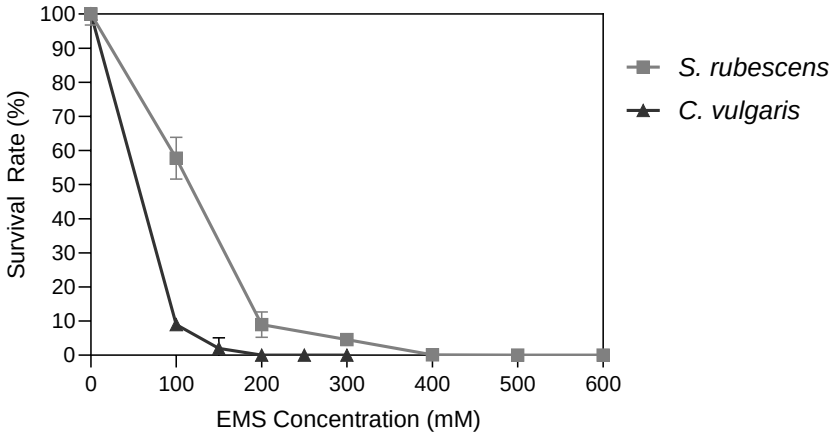


Figure 3.2 - Survival rate (%) of *S. rubescens* and *C. vulgaris* exposed to different ethyl methanesulfonate (EMS) concentrations. Results are shown as mean ± SD, n=3.

Zhang et al. [197] studied the effect of EMS on *Scenedesmus* sp. (FACHB-489) and reported a survival rate of 46% at 200 mM EMS, 5-fold higher than the results obtained for *S. rubescens* strain in this experiment. On the other hand, varying susceptibilities of *Chlorella* sp. to EMS were reported by several authors [19, 112, 117, 123, 138, 198–201]. Shin et al. [198] obtained a 10% survival rate with 150 mM EMS, while Guardini et al. [199] determined that 161 mM EMS resulted in a similar survival rate (5-10%). In contrast, Schüler et al. [19] described a 5-10% survival rate with 300 mM EMS.

The observed differing susceptibilities of *C. vulgaris* and *S. rubescens* to EMS are probably due to strain-specific differences in sensitivity to this agent and/or differences in the experimental conditions used. More specifically, it is noteworthy that, for example, in this work, 2.16×10^5 cells of *C. vulgaris* were subjected to EMS, which accounts for 14 times less cells than, for example the 3.20×10^6 cells reported in Schüler et al. [19], which resulted in a 50% lower EMS concentration needed to kill 90% of the cells under similar experimental conditions. Similarly, in this experiment, only 5-10% out of the 1.54×10^6 cells of *S. rubescens* survived 200 mM EMS, while Zhang et al. [197] reported 46% cell viability at the same EMS concentration, with 2.20×10^7 cells of *Scenedesmus* sp. In addition, these authors used different species, exposed the culture to light and used a different culture medium. Therefore, to decrease discrepancies in the results, reported protocols should be standardized, as the survival rate upon an EMS treatment can be influenced by factors such as species, strain, cell concentration, growth stage, and culture medium used [202, 203]. Incubation conditions such as time, temperature and the presence or absence of light also significantly impact the results. In particular, exposure to light might increase the susceptibility of cells to the mutagenic agent and/or induce light-dependent DNA-repair mechanisms [202, 203].

3.1.3.2. Oxyfluorfen toxicity

To determine the optimal range of oxyfluorfen for mutants' selection, several concentrations of this inhibitor were tested on *S. rubescens* (Figure 3.3) and *C. vulgaris* (Figure 3.4).

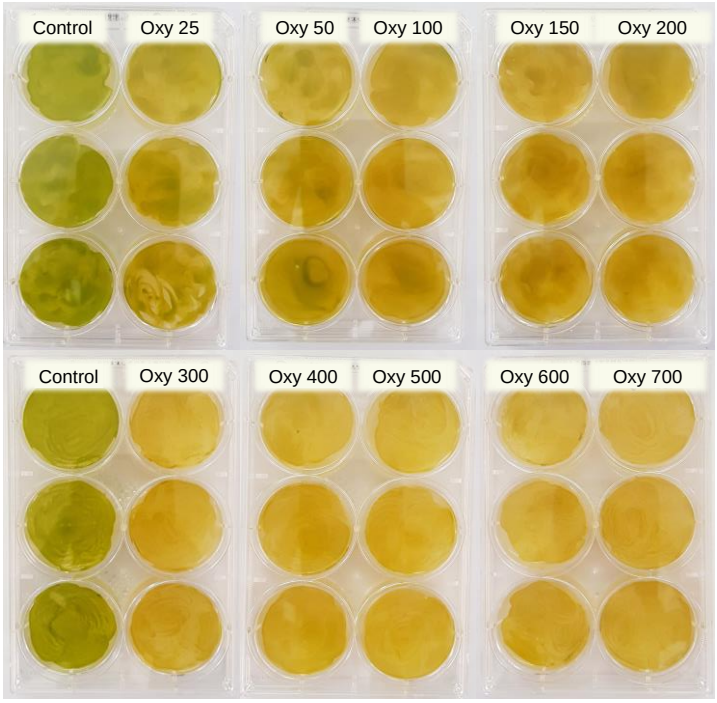


Figure 3.3 - *Scenedesmus rubescens* oxyfluorfen (Oxy) toxicity test. The culture in early exponential phase was plated onto PCA with Oxy and incubated in the dark. Oxyfluorfen concentrations are represented in µg L^{-1}.

In 6-well plates, *S. rubescens* growth (Figure 3.3) was fully inhibited by concentrations equal to or higher than 300 µg L^{-1} of oxyfluorfen, while only concentrations higher than 500 µg L^{-1} inhibited growth of *C. vulgaris* completely (Figure 3.4).

As the optimal inhibitor concentration to use for mutant selection should be between the sublethal and lethal concentrations [138, 183], the oxyfluorfen concentration ranges to test for *S. rubescens* and *C. vulgaris* strains after mutagenesis were established between 200-300 µg L^{-1} and 400-500 µg L^{-1}, respectively (Table S1 – Supplementary Material). This strategy ensures that the surviving colonies that gained a suitable mutation that imparts their resistance to this chemical are more likely to be selected, avoiding non-mutant false positives and false negatives that have acquired a stable mutant phenotype.

In the presence of this inhibitor, both cultures acquired a brownish colour that can be associated with decreased chlorophyll contents and possibly with the toxic effect of increased oxidative stress that has been linked to diphenylethers, such as oxyfluorfen [185, 204].

Geoffroy et al. [184] studied the toxicity of this inhibitor on the growth of an autotrophic culture of *Tetradesmus obliquus* (synonym of *Scenedesmus acutus*), in microplates with liquid medium. To inhibit the growth of 10% of the population (IC_{10}), 3 µg L^{-1} of oxyfluorfen was required, 15 µg L^{-1} for 50% (IC_{50}), while 22 µg L^{-1} were sufficient to inhibit the growth of 90% of the cells (IC_{90}). These concentrations are

significantly lower than the ones reported in the present work. The difference in these values can be attributed to different experimental conditions that significantly impact the toxicity of oxyfluorfen such as the presence or absence of light, culture medium, cell concentration, well volume and respective altered partial pressure of atmospheric gases, temperature and agitation.

Figure 3.4 - *Chlorella vulgaris* oxyfluorfen (Oxy) toxicity test. The culture in early exponential phase was plated onto PCA with Oxy and incubated in the dark. Oxyfluorfen concentrations are represented in µg L^{-1}

On the other hand, Cheng et al. [205] studied the toxicity of a similar diphenyl ether, acifluorfen, in *T. obliquus* when grown photoautotrophically in liquid medium at 25 °C, under continuous illumination on a 16:8 light:dark cycle. The effective concentration of acifluorfen resulting in a 50% decrease in *T. obliquus* growth (after 96 h) was 341 µg L^{-1}. Although this concentration is closer to the concentration of 300 µg L^{-1} of oxyfluorfen that inhibited growth of *S. rubescens*, it is noteworthy that the experimental conditions were also completely different.

Regarding *Chlorella* sp., Zhao et al. [206] studied the toxicity of a polybrominated diphenyl ether (BDE-47), a molecule with a chemical structure related to oxyfluorfen, and reported a 97% growth inhibition at a concentration of 120 µg L^{-1}. Once more, it is difficult to compare the inhibitory concentrations not only because different species and different inhibitors were used (although chemically related), but also due to several differences in the experimental conditions. However, this inhibitor seems to resemble oxyfluorfen with respect to the toxic effects caused on *Chlorella* sp. and *Scenedesmus* sp. [184, 207].

Oxyfluorfen is a fluorinated chemical that induces a bleaching effect, *i.e*., decrease in pigments content, either by inhibiting the synthesis of these molecules and/or by inducing the loss of pigments. While inhibitors like norflurazon have been linked only to the first hypothesis, causing the accumulation of phytoene and other carotenoids precursors, oxyfluorfen also causes a loss of pigments in algae as aimed for this work [204]. As mentioned previously, oxyfluorfen has been reported to inhibit chlorophyll biosynthesis by inhibiting the enzyme PPO (Figure 3.1), which leads to the accumulation of protoporphyrinogen IX [187, 188, 208]. This molecule goes through a non-enzymatic oxidation, causing the formation of ROS, which triggers lipid peroxidation. Since the precursor protoporphyrin IX is not produced, chlorophyll synthesis does not occur either. Moreover, phytotoxicity of these herbicides has been linked to the peroxidative degradation of cellular components, mainly membrane lipids [187]. In 1987, Lambert et al. [209] investigated the toxicity of this inhibitor to microalgae, namely the binding and peroxidative action of oxyfluorfen in *Scenedesmus acutus*. Geoffroy et al. [188] indicated antenna size and chlorophyll content as the most sensitive biomarkers to evaluate the toxic effect of oxyfluorfen on *T. obliquus*. More recently, the biochemical effects of oxyfluorfen on other species has also been reported [208, 210].

Overall, oxyfluorfen affects the transcription levels and activities of enzymes of both carotenoids and chlorophyll biosynthetic pathways, which affect pigment biosynthesis, which in this study would lead to a loss of pigments and subsequent improved sensory properties of the microalgal biomass [185].

3.1.3.3. Isolation of mutants with different pigmentation

Based on the previous results, cells were mutagenized with EMS and exposed to oxyfluorfen. Mutagenized cultures of *S. rubescens* and *C. vulgaris* isolated on oxyfluorfen resulted in the appearance of yellow, brown and green colonies and also white in the case of *C. vulgaris* (Figure 3.5).

A brownish *S. rubescens* colony isolated with 100 µg L^{-1} of oxyfluorfen and generated with 200 mM of EMS, designated 37Y01, was picked and streaked for 10 generations to ensure phenotype stability (Table 3.1). Regarding *C. vulgaris*, 9 yellow and 11 white mutants were selected with this inhibitor (data not shown). Although the growth of all *C. vulgaris* mutants was compared among each other, only the most significant data for the continuity of this work are shown below, namely the characterisation of the yellow and white mutants with growth performances comparable to the WT, that were selected for future work, 31Y15 and 31W62, both generated with 150 mM EMS and selected with 500 µg L^{-1} of oxyfluorfen (Table 3.1).

These results demonstrate the feasibility of using a metabolic inhibitor of the chlorophyll biosynthesis pathway, like oxyfluorfen, to select EMS-mutagenized cells with impaired pigmentation.

Random mutagenesis has been extensively used previously in several microalgal species, namely as a strategy to generate mutants with altered chlorophyll and carotenoid contents (Table S2 – Supplementary Material) [19, 53, 96, 111, 133, 138, 139, 172, 198–200, 211, 212]. Regarding Scenedesmaceae species, there are a few papers describing the application of EMS-based random mutagenesis. For example, Zhang et al. [197] used both EMS and UV-radiation to generate mutants of

Scenedesmus sp. with a high biomass productivity, while Zhang et al. [106] isolated *Desmodesmus* sp. mutants not only with improved biomass yield but also lipid productivity. Conversely, *Chlorella* has been one of the most frequently targeted genera for strain improvement, accounting for 36% of the reports concerning microalgal random mutagenesis in 2022 [183].

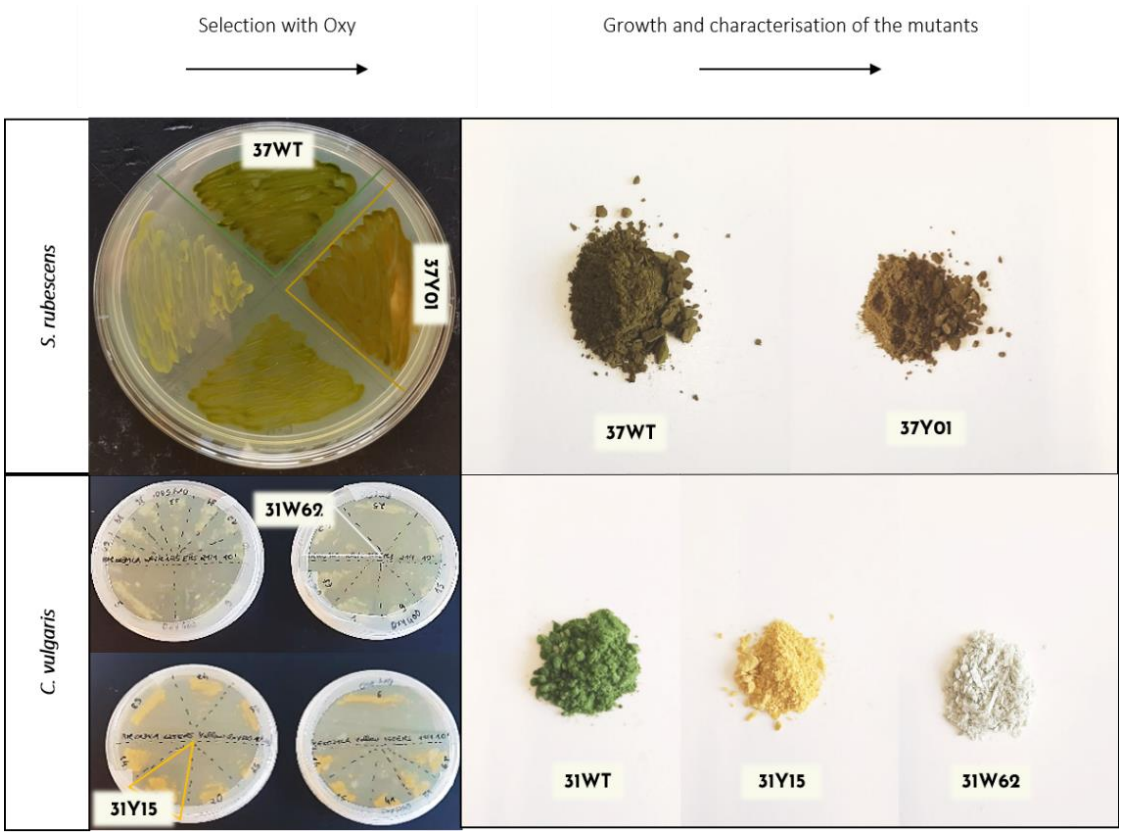


Figure 3.5 - Selection of mutants of *S. rubescens* and *C. vulgaris* with oxyfluorfen (oxy) and streaking on plate (left). Lyophilized biomass obtained after growth trials (right).

Table 3.1 - Conditions applied in the isolation of *S. rubescens* and *C. vulgaris* mutants chosen for future characterisation.

Species	Mutant	EMS concentration (mM)	Oxy concentration (µg/L)
Scenedesmus rubescens	37Y01	200	100
Chlorella vulgaris	31Y15	150	500
Chlorella vulgaris	31W62	150	500

Although with different goals, EMS-random mutagenesis has allowed to generate mutants with different pigmentation profiles, such as a pale-green phenotype, white and yellow strains and carotenoids hyper-producers [19, 138, 139, 198–200, 211]. The limiting step of this approach has always been keeping the number of mutants to be screened to a minimum and having a suitable screening strategy to identify and select cells exhibiting phenotypes of interest. Several screening strategies have been applied, such as the selection of colonies by visual appearance, more specifically by their colour [139, 198, 211]. To select randomly generated and differentially pigmented mutants more efficiently, high-

throughput technologies as, for example, FACS, have been used [96, 111, 200]. On the other hand, metabolic inhibitors can be used to speed up the selection process. In particular, for the selection of mutants with different pigmentation, two inhibitors have been often reported: nicotine and norflurazon, which target the carotenoid pathway, leading to the generation of mutants with enhanced carotenoids content [133, 172, 183, 199]. However, the isolation of mutants with norflurazon with decreased carotenoid and chlorophyll contents has also been reported [19, 185]. Nevertheless, the application of a direct inhibitor of the chlorophyll biosynthetic pathway has not been reported, either to enhance chlorophyll content or to obtain chlorophyll-deficient mutants. This study describes for the first time the use of a chlorophyll biosynthetic pathway inhibitor, oxyfluorfen, that enabled the selection of chlorophyll-deficient mutants of *S. rubescens* and *C. vulgaris* with improved colour, odour and taste. Moreover, the combined use of different selection strategies simultaneously might improve the success rate of random mutagenesis experiments [33, 183, 213]. For example, Yi et al. [53] combined the use of diphenylamine, another inhibitor of the carotenoid biosynthetic pathway, with the selection of colonies by visual appearance (size and colour), with a subsequent FACS selection step. This strategy led to a 69% increment of fucoxanthin content in a *Phaeodactylum tricornutum* mutant compared to the levels found in the WT.

3.1.3.4. Wildtype vs mutants' growth performances

Oxyfluorfen-derived *S. rubescens* and *C. vulgaris* mutants and respective wildtype strains were compared at lab-scale regarding their growth performance, namely biomass productivity (r_p) and growth rate (μ) (Figure 3.6).

Strain	r_p (g L^{-1} d^{-1})	μ (d^{-1})
37WT	2.13 ± 0.08^{b}	1.17 ± 0.03^{b}
37Y01	1.66 ± 0.10^{c}	1.05 ± 0.02^{c}
31WT	2.39 ± 0.01^{a}	1.31 ± 0.01^{a}
31Y15	2.19 ± 0.15^{ab}	0.99 ± 0.02^{d}
31W62	1.84 ± 0.08^{c}	0.93 ± 0.01^{e}

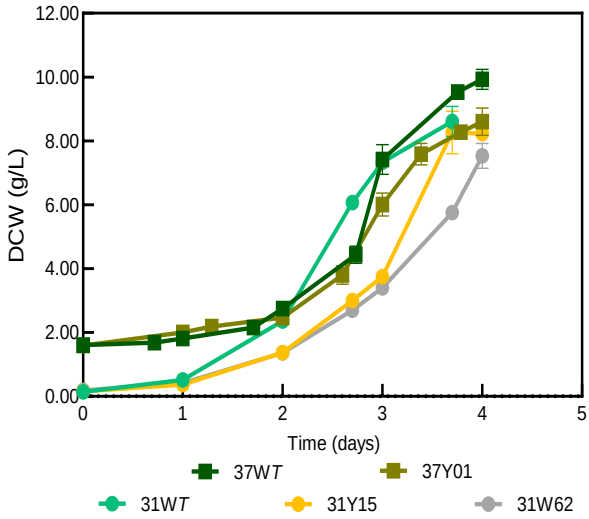


Figure 3.6 - Biomass productivies (r_p) and growth rates (μ) (on the left) and growth curves (on the right) of *S. rubescens* and *C. vulgaris* WT and mutant strains, grown heterotrophically in 250-mL Erlenmeyer flasks for 4 days (in the absence of inhibitor). Results are shown as mean ± SD, n=3. Different letters indicate significant differences ($p < 0.05$) between strains.

As for *S. rubescens*, the WT productivity and growth rate (2.13 ± 0.08 g L^{-1} day^{-1} and 1.17 ± 0.03 day^{-1}) were significantly higher than those of 37Y01 (1.66 ± 0.10 g L^{-1} day^{-1} and 1.05 ± 0.02 day^{-1}).

Chlorella vulgaris WT strain showed significantly higher biomass productivity and growth rate values than those of 37WT, 2.39 ± 0.01 g L^{-1} day^{-1} and 1.31 ± 0.01 day^{-1}, respectively ($p < 0.05$). The yellow mutant 31Y15 displayed a biomass productivity of 2.19 ± 0.15 g L^{-1} day^{-1}), with no significant differences compared to the WT ($p > 0.05$). However, the growth rate of 31Y15 (0.99 ± 0.02 day^{-1}) was significantly lower than that of the WT ($p < 0.05$). The white mutant 31W62 displayed a significantly lower biomass productivity (1.84 ± 0.08 g L^{-1} day^{-1}) and growth rate (0.93 ± 0.01 g L^{-1} day^{-1}) compared to the WT and 31Y15 (Figure 3.6) ($p < 0.05$)

Chlorella and *Scenedesmus* have been reported to achieve some of the highest biomass and protein productivities [19, 191, 192, 214], which might be related to the fact that these are two of the few genera of microalgae with the ability to grow heterotrophically, which allows to achieve much higher cell concentrations than with photoautotrophic cultivation [23, 24, 191, 192].

Recently, Espírito Santo et al. [191] studied the heterotrophic growth of the same wildtype strain of *S. rubescens* in similar conditions, reaching a biomass productivity of 2.86 g L^{-1} day^{-1} and a specific growth rate of 1.18 day^{-1}. Although the productivity was higher than the one obtained in the present work, the growth rate was similar. Other studies on the heterotrophic growth of *Scenedesmus* spp. have, however, attained lower values, demonstrating the variability of growth within microalgae of the same genus and under different cultivation conditions. For example, Ren et al. [215] studied the heterotrophic growth of *Scenedesmus* sp. and reached a lower growth rate of 0.82 day^{-1}, while for *T. obliquus* a biomass productivity of 0.46 g L^{-1} day^{-1} was reported [216].

Regarding *C. vulgaris* growth performance, there is a wide range of values that can be found in literature, which is always dependent on cultivation conditions, medium used, type of reactor and scale. Under lab-scale heterotrophic conditions, most studies report growth rates and biomass productivities lower than the ones reported in the present work, between 0.55-0.79 day^{-1} and 1.65-1.99 g L^{-1} day^{-1} [217–220].

Among several reports in literature, different outcomes in terms of biomass productivity and growth rate have been reported upon mutagenesis. Similarly to the results obtained for the WT and the 31Y15 yellow mutant strain, which displayed similar growth performances, Schüler et al. [19] reported a statistically equivalent growth for the WT and a yellow mutant in an Erlenmeyer assay under similar conditions. Moreover, the same authors also generated a white mutant that propagated slower than the WT and the yellow mutant, similarly to what is described in the present study. Concerning the improvement of biomass productivity through random mutagenesis, there are several reports on *C. vulgaris* [123, 200], *Chlorella* spp. [112, 221] and other species [222]. Aiming at improving photosynthetic efficiency, Patil et al. [139] developed chlorophyll-deficient mutants of *C. vulgaris* using EMS-dependent mutagenesis, which allowed the biomass productivity to increase by 27% as compared to that of the WT, while Shin et al. [198] reported a 45% increment under autotrophic conditions.

Regarding Scenedesmeceae genus, growth has also been previously improved by resorting to random mutagenesis. For example, Xi et al. [223] isolated a *T. obliquus* mutant through $^{12}C^{6+}$ ion beam mutagenesis, which resulted in the generation of a mutant with a 57% and 25% improvement in biomass productivity and growth rate, respectively.

Overall, many of these reports in which biomass productivities and growth rates were improved, described decreased chlorophyll levels along with smaller antenna sizes, which under autotrophic cultivation conditions will likely increase the efficiency of the process by preventing self-shading effects and limited growth [183]. The same seems inapplicable to heterotrophic cultivation, so that mutagenesis along with impaired pigmentation likely leads to slower or similar growth performances comparing to the wildtype strains. Moreover, in this work the growth parameters obtained are the same or close to the maximum reported for these species at this scale. Finally, higher biomass productivities and growth rates have been reported upon scale-up and process optimisation, both for *S. rubescens* [191] and *C. vulgaris* [192], which points out the potential of further improving the mutants selected in this work.

3.1.3.5. Biochemical characterisation

3.1.3.5.1. Protein content and productivity

The protein content and productivity of both wildtype strains of *S. rubescens* and *C. vulgaris* and respective mutants are presented in the figure below (Figure 3.7).

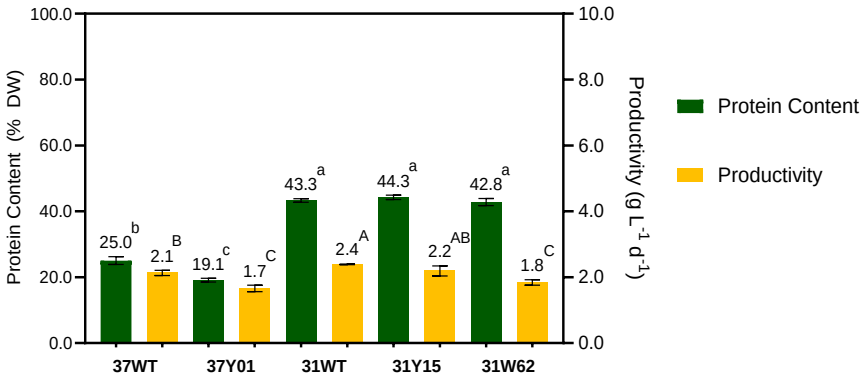


Figure 3.7 - Protein contents (% of DW) and protein productivities (g L^{-1} d^{-1}) of *S. rubescens* and *C. vulgaris* wildtype and mutant strains. Protein contents were determined by measuring total N in an elemental analyser. Results are shown as mean ± SD, n=3. Different letters indicate significant differences (p < 0.05) between strains.

S. rubescens WT displayed a significantly higher protein content, 25.0% of DW, compared to that of mutant 37Y01, 19.1% (p<0.05), with protein productivities of 2.1 and 1.7 g L^{-1} d^{-1}, respectively (Figure 3.7). Concerning *C. vulgaris*, the ranges of protein contents and productivities obtained were between 42.8-44.3% and 1.8-2.4 g L^{-1} d^{-1}, respectively (Figure 3.7), whose contents were considerably higher than the values of *S. rubescens* (p<0.05). The yellow (31Y15) and white (31W62) mutants selected exhibited protein contents of 44.25% and 42.80%, which were similar to the one of the WT strain containing 43.28% (Figure 3.7).

A recent study reported a higher protein content of 31-33% [191], for the same *S. rubescens* strain used, grown in the same culture medium, but in a fed-batch 7-L bench top fermenter (Table 3.2).

Furthermore, when grown photoautotrophically, this strain can reach protein contents as high as 60% (Table 3.2) [214]. Other studies carried out with Scenedesmeceae report a wider range, 13-54%, when grown autotrophically (Table 3.2). The species *T. obliquus* was also reported to have a protein content of 44%, when grown autotrophically [224]. Under heterotrophic conditions, it has been suggested that *T. obliquus* can similarly achieve a protein content of 55% [225].

Table 3.2 - Reports of protein content of *C. vulgaris*, *S. rubescens* and *Scenedesmus* spp. under different trophic modes.

Species	Protein content (% DW)	Trophic mode	Reference
Scenedesmus bajacalifornicus	32.9	Autotrophic	[228]
Scenedesmus rubescens	31.0-33.0	**Heterotrophic**	[191]
Scenedesmus rubescens	60.0	Autotrophic	[214]
Scenedesmus sp.	56.0	Autotrophic	[229]
Scenedesmus sp.	12.5-28.2	Autotrophic	[230]
Tetradesmus obliquus	28.5-44.4	Autotrophic	[224]
Tetradesmus obliquus	55.0	**Heterotrophic**	[225]
Scenedesmus rubescens			
37WT	25.0 ± 1.2	**Heterotrophic**	This study
37Y01	19.1 ± 0.6		
Chlorella vulgaris			
WT	30.5		
MT01	39.5	**Heterotrophic**	[19]
MT02	48.7		
Chlorella vulgaris	52.2	Two-stage	[192]
Chlorella vulgaris	20.0	**Heterotrophic**	[218]
Chlorella vulgaris	64.1	**Heterotrophic**	[220]
Chlorella vulgaris	52.3	Autotrophic	[226]
Chlorella vulgaris	24.9	Autotrophic	[32]
Chlorella vulgaris	44.3	**Heterotrophic**	[227]
Chlorella vulgaris			
31WT	43.3 ± 0.5		
31Y15	44.3 ± 0.7	**Heterotrophic**	This study
31W62	42.8 ± 1.1		

As for the trophic mode, there is a wide range of values that can be found for *C. vulgaris* protein content (Table 3.2). Under autotrophic cultivation conditions, Chen et al. [226] achieved a protein content of 52.3% DW, while Lai et al. [32] reported half of that, reaching only 24.9% DW. However, under heterotrophic conditions and with microalgae of the same species, both Lau et al. [218] and Barros et al. [192] attained lower protein levels, 20.0%, even though more recently Xie et al. [227] and Cai et al. [220] reported values 2- and 3-fold higher, reaching 44.30% and 64.14% DW, respectively. Interestingly, Barros et al. [192] reported the use of a combination of the two trophic modes as a strategy to achieve

the highest biomass and protein productivities possible within the shortest time period, which enabled a protein content of 52.2% DW.

Although protein content improvement was not the goal of this study neither a strain selection criterion, there are almost no reports regarding the improvement of protein contents on microalgae through random mutagenesis, since most studies found in literature are more directed towards lipids and carbohydrates. Nonetheless, Schüler et al. [19] reported increased protein content in a WT *C. vulgaris* strain (30.5%) in its yellow (39.5%) and white (48.7%) mutants. In the present study, as mentioned above, there were no significant differences among the protein contents of the WT and the yellow and white mutants isolated. However, the protein content reported for the WT is already very similar to the highest value reported in Schüler et al. [19] and close to the highest values found in literature (40-60%) (Table 3.2). It becomes clear that there is a significant heterogeneity of values regarding protein content of these species (Table 3.2), which might be explained by diverse reasons, namely trophic mode, cultivation conditions, strain used, culture medium, the method used to quantify protein content, among others. Moreover, the nitrogen concentration during growth is a crucial factor for protein synthesis. In fact, it has been shown that the protein content of *T. obliquus* can range from 11.4 to 55.6% if the nitrogen levels in the medium are increased [225].

Taken together, these results confirm that *C. vulgaris* is one of the most promising microalgal species as a protein source [226] and the contents of the strains generated can probably be further enhanced by optimisation of cultivation conditions. Although several biochemical characterisations of both *Chlorella* and *Scenedesmus* biomasses have been reported, namely regarding proximal composition [19, 191] and amino acids profiles [231, 232], it would be of great interest to analyse and compare the nutritional profiles of wildtype strains and mutants, namely mutants selected with different inhibitors. Few studies have been carried out regarding this matter. For example, Maurício et al. [233] compared the lipid profile of *Chlorella vulgaris* wildtype and yellow and white mutants, while Cabrol et al. [234] compared the amino acids profiles obtained in pork frankfurters with and without enrichment with the same yellow and white mutants.

As for future perspectives concerning the improvement of protein productivity, it is key to study the mechanisms and metabolic pathways underlying the production and accumulation of protein in the microalgal cell. In addition, screening methods must be developed to allow the selection of mutants with even higher protein contents, if possible.

3.1.3.5.2. Chlorophyll content and carotenoids profile

Carotenoids and chlorophyll concentrations of both WTs and respective mutants were analysed. The profiles of pigments are shown in Table 3.3.

Macroscopically, 37WT colonies were dark-green, while 37Y01 presented a brownish colour. This difference was apparently due to their pigmentation profiles. Accordingly, mutant 37Y01 displayed a 55% and a 18% decrease in total chlorophyll and carotenoid contents, respectively, compared to the WT. Despite the decrease in the carotenoid content, neoxanthin was detected in 37Y01 biomass at a

concentration of 0.31 mg g^{-1}, whereas this carotenoid was not present in the WT profile (Table 3.3). On the other hand, the *β*-carotene content in the mutant strain decreased by 68%, from 0.88 mg g^{-1} DW, to 0.28 mg g^{-1} DW (Table 3.3).

Table 3.3 - Carotenoids concentrations (mg g^{-1} of DW) of wildtypes and mutants of *S. rubescens* and *C. vulgaris*, determined by HPLC, and chlorophyll contents (mg g^{-1} of DW) determined by Ritchie method. n.d. – not detected.

Species	Strain	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Neoxanthin	Lutein	*β*-Carotene
S. rubescens	**37WT**	16.45 ± 1.12	6.58 ± 0.94	23.07 ± 2.06	n.d.	0.53 ± 0.02	0.88 ± 0.04
S. rubescens	**37Y01**	6.23 ± 0.25	4.09 ± 0.39	10.32 ± 0.63	0.31 ± 0.05	0.50 ± 0.02	0.28 ± 0.03
C. vulgaris	**31WT**	4.79 ± 0.29	1.89 ± 0.29	6.68 ± 0.57	0.27 ± 0.02	0.52 ± 0.01	1.29 ± 0.10
C. vulgaris	**31Y15**	0.14 ± 0.03	0.18 ± 0.03	0.32 ± 0.05	n.d.	n.d.	n.d.
C. vulgaris	**31W62**	0.12 ± 0.03	0.14 ± 0.03	0.26 ± 0.06	n.d.	n.d.	n.d.

C. vulgaris WT biomass also presented a dark-green colour, while mutant 31Y15 and 31W62 exhibited a yellow and white colour, respectively. Pigments profile of the three strains reflected these descriptions (Table 3.3). As expected, the total chlorophyll content of the WT was the highest, 6.7 mg g^{-1} of DW, and was practically null for both mutants (~95% reduction), with about 0.3 mg g^{-1} of DW for both the yellow and white mutant. The WT displayed high concentrations of carotenoids, namely 0.27, 0.52 and 1.29 mg g^{-1} of DW for neoxanthin, lutein and *β*-carotene, respectively. However, none of the carotenoids analysed were identified in the biomass of the yellow and white mutants.

Mutants with different pigments' profile have been selected after mutagenesis whether aiming at higher chlorophyll contents or lower, depending on the authors' intent (Table 3.4 and Table S2 - Supplementary Materials). A chlorophyll-deficient mutant of *Desmodesmus armatus* has been previously isolated by resorting to UV-mutagenesis, although the objective was to isolate mutants resistant to fluazinam, which resulted in increased autotrophic growth rate and biomass productivity [235]. On the contrary, the mutant of *T. obliquus* isolated by Xi et al. [223], with the objective of obtaining increased photosynthetic efficiency and lipid content, displayed a 33% increment in chlorophyll content and a 48% increase in total carotenoids content. There are also some reports of pale-green and/or chlorophyll-deficient phenotypes of *C. vulgaris*, selected with the purpose of having decreased antenna sizes and improved photosynthetic efficiencies (Table 3.4). For example, Shin et al. [198] and Dall'Osto et al. [138] obtained a 50% chlorophyll reduction through EMS-mutagenesis and selection of mutants with lighter green colour, while Cazzaniga et al. [111] achieved the same 50% reduction but with UV-mutagenesis and selection through chlorophyll fluorescence. Moreover, Shin et al. [198] also reported a reduction of 75% of the carotenoids content, while Cazzaniga et al. [111] reported lower levels of neoxanthin and lutein for the mutants, while *β*-carotene, violaxanthin and zeaxanthin were more abundant, comparing to the WT.

Table 3.4 - Examples of random mutagenesis reports that generated *C. vulgaris* and *S. rubescens* mutants with altered chlorophyll and carotenoid contents. An extended version of this table can be found in the Supplementary Materials (Table S2).

Species	Mutagenic agent	Target	Screening	Improvement	Reference
C. vulgaris	EMS	Chlorophyll deficiency;	Colour and Norflurazon;	Up to 99% decreased chlorophyll and 60% increased protein content.	[19]
C. vulgaris	EMS	Chlorophyll deficiency;	Colour;	Reduction by 57% in chlorophyll *a*, 76% in chlorophyll *b* and 45% increase in biomass productivity.	[198]
C. vulgaris	EMS	Chlorophyll deficiency; oxidative stress resistance;	Colour; Red Bengal 12 µM;	50% chlorophyll reduction; 68% higher biomass yield.	[138]
D. armatus	UV	Fluazinam tolerance;	Fluazinam;	33-38% decrease in chlorophyll fluorescence; improved productivity and quantum efficiency.	[235]
S. vacuolatus	UV	Improved biodegradative performance;	Chlorophyll content; Substrate affinity; Sugar utilization; Growth rate;	89% increase in chlorophyll *a* content, 24% carotenoid content and 44% in protein content.	[236]
T. obliquus	$^{12}C^{6+}$ ion beam	Increased photosynthetic efficiency; Increased lipid content;	Chlorophyll fluorescence;	Up to 48% increase in carotenoid content; up to 33% in chlorophyll *a* content; improved photosynthetic efficiency and lipid productivity; decreased protein content.	[223]
S. rubescens and *C. vulgaris*	EMS	Chlorophyll deficiency;	Oxyfluorfen;	55% and 95% chlorophyll reduction.	This study

According to several published data, differently coloured chlorophyll-deficient mutants (*e.g.,* yellow and white) in heterotrophic conditions, as in the present work, generally exhibit lower chlorophyll and carotenoids contents. Schüler et al. [19] isolated a yellow and white heterotrophic mutants of *C. vulgaris*, selected with norflurazon and by colour, that contained less 82% and 95% chlorophyll than the WT, respectively. Additionally, the carotenoids content of both mutants was much lower than the WT, being below the limit of quantification (LOQ) for the white one, while the most abundant carotenoid in the yellow mutant was lutein with 0.86 mg g^{-1} of DW. The lutein content of the WT in the present work was lower and not even detectable for the yellow and white mutants. However, for example *β*-carotene was much higher for this WT (1.29 mg g^{-1}), while in Schüler et al. [19] the WT had only 0.28 mg g^{-1} as well as neoxanthin and violaxanthin. These differences can be related to the use of different strains, different cultivation conditions, namely culture medium, and also to different extraction and quantification methods, such as instrumental techniques used (spectrophotometric or chromatographic).

In contrast to the objective of the present study, there are also several works published targeting the increment of carotenoids content, through mutagenesis and different selection strategies (Table 3.4 and Table S2 - Supplementary Materials). Recently, Eregie et al. [236] selected larger colonies after UV-mutagenesis, which led to a 1.2-fold increase of the total carotenoid content of *S. vacuolatus*.

Concerning lutein, one of the most abundant carotenoids in *Chlorella* sp., Cordero et al. [172] enhanced this pigment by 2-fold in an autotrophic *Chlorella sorokiniana*, after mutagenesis with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and selection with nicotine, while Chen et al. [133] attained a lutein productivity 1.5-fold higher than the WT, but in mixotrophic conditions. On the other hand, Huang et al. [212] attained some of the highest carotenoid concentrations reported for this genus by inducing the accumulation of lutein, *β*-carotene and zeaxanthin on *Chlorella zofingiensis*, after mutagenesis with MNNG, followed by selection under dim light and diphenylamine, attaining 13.8 mg g^{-1}, 7.18 mg g^{-1} and 7 mg g^{-1}, an increment of 2-, 5- and 7-fold of lutein, β-carotene and zeaxanthin, respectively.

Oxyfluorfen affects the transcription levels and activities of enzymes of both carotenoids and chlorophyll biosynthetic pathways, which affect pigment biosynthesis. Altered metabolite levels on one pathway impact the gene expression in the other pathway, which also varies with the level of oxidative stress that cells are exposed to and often cause unpredictable effects on pigment biosynthesis. As discussed above, carotenoid inhibitors, as norflurazon or nicotine, sometimes lead to the accumulation of some carotenoids [172, 199], but it has also been reported that it might lead to decreased carotenoid concentrations [19, 185]. Accordingly, the exposure to these inhibitors can either induce the accumulation of chlorophyll or cause its depletion. Likewise, it is not straightforward whether oxyfluorfen will cause chlorophyll depletion or accumulation as well as its effect on the biosynthesis of carotenoids, which will also be influenced by the cultivation conditions. In the future it would be of great interest to study which genes were mutated through the usage of this inhibitor to understand the impact on the pigments' metabolic pathways of these mutated cells.

3.1.4. Conclusions

The metabolic inhibitor oxyfluorfen, tested for the first time as a selection strategy upon mutagenesis, allowed the isolation of chlorophyll-deficient mutants of *S. rubescens* and *C. vulgaris* with a 55% and 95% decrease in total chlorophyll content, respectively. *S. rubescens* WT and mutant 37Y01 exhibited protein contents between 19.1-25.0% DW, while *C. vulgaris* WT, yellow 31Y15, and white 31W62 mutants reached higher contents, 42.8-44.3% DW. Therefore, *C. vulgaris* mutant strains are more promising protein sources for developing novel food and feed applications with more appealing organoleptic characteristics due to the high protein and almost null chlorophyll contents. Nevertheless, their nutritional profile should be further characterised since it might present other interesting features, such as amino acid and lipids profile, vitamins, minerals and other bioactive compounds.

Overall, the oxyfluorfen-based random mutagenesis pipeline reported here established stable chlorophyll-deficient mutants of *C. vulgaris* and *S. rubescens* in an efficient, straightforward manner, expanding the mutant generation / selection pipeline as well as the portfolio of strains with high potential for nutritional applications.

3.1.5. Supplementary Materials

The following are available online at https://www.sciencedirect.com/science/article/pii/S221192642400184X?via%3Dihub#s0115, Table S1: Results of the oxyfluorfen resistance tests on *S. rubescens* and *C. vulgaris*. Table S2: Examples of random mutagenesis reports that generated microalgal mutants with altered chlorophyll and carotenoid contents.

3.2 Isolation and characterisation of chlorophyll-deficient mutants of *Chlorella vulgaris*

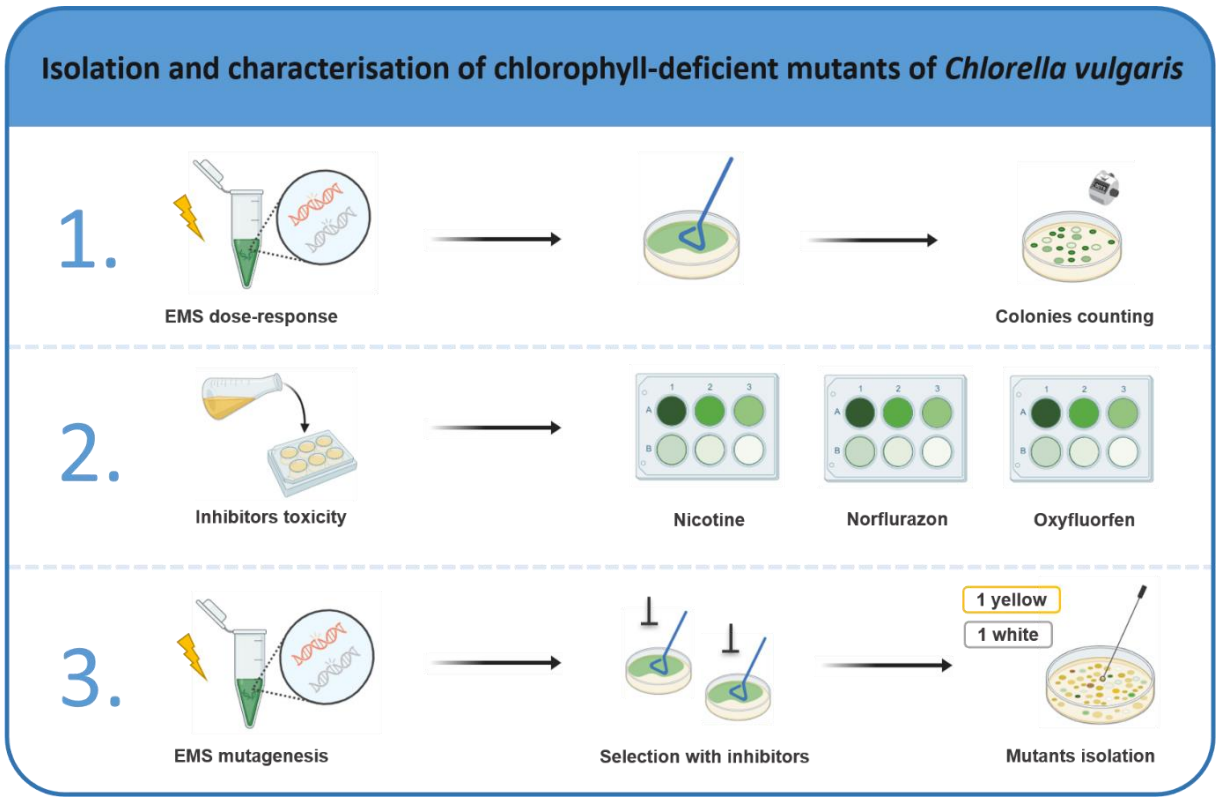
ABSTRACT

The development and commercialisation of more appealing microalgal products relies on strain improvement to generate strains with improved sensory properties, such as colour, smell and taste. Random mutagenesis combined with selection with metabolic inhibitors is a powerful methodology to isolate novel strains with target phenotypes, such as chlorophyll-deficient mutants. Nicotine and norflurazon are two well-reported inhibitors of the carotenoids' biosynthetic pathway, while oxyfluorfen is a known inhibitor of the chlorophyll metabolic pathway. Thus, in this work, chemical mutagenesis was applied to *Chlorella vulgaris*, to generate novel chlorophyll-deficient mutants, whose selection was carried out with the aforementioned metabolic inhibitors.

Nicotine allowed the selection of a yellow mutant, 7Y. Additionally, to obtain a white mutant of *C. vulgaris*, 31W, a two-round mutagenesis was performed, and a hybrid selection methodology was applied. The combined use of norflurazon in the first round, with oxyfluorfen in the second round, allowed the isolation of a whiter mutant than the ones generated in the first round. These methodologies enabled the reduction of chlorophyll by up to 95%. Moreover, the isolated mutants displayed high protein contents, 45% and 41% of DW in the case of mutant 7Y and 31W, respectively. These novel mutants presented more attractive organoleptic features, with great potential to be used in food applications.

Keywords: Metabolic inhibitor; Heterotrophic; Nicotine; Norflurazon; Random mutagenesis; Selection method; Strain improvement.

GRAPHICAL ABSTRACT



3.2.1 Introduction

The background introduced in subchapter 3.1. applies to this subchapter as well. In addition, the methodology applied is the same, with some differences, as it will be explained in the dedicated section.

As explained previously, oxyfluorfen inhibits the enzyme protoporphyrinogen oxidase (PPO) of the chlorophyll pathway. In contrast, nicotine blocks lycopene cyclase (that converts lycopene into *β*-carotene), and norflurazon inhibits phytoene desaturase of the carotenoids' biosynthetic pathway (Figure 3.8) [125, 128, 185].

This work used the same pipeline described in subchapter 3.1. to isolate chlorophyll-deficient mutants of *C. vulgaris* with high protein contents and improved organoleptic characteristics by resorting to three metabolic inhibitors: nicotine, norflurazon and oxyfluorfen. Additionally, the combined use of inhibitors of both chlorophyll and carotenoids biosynthetic pathways was also tested.

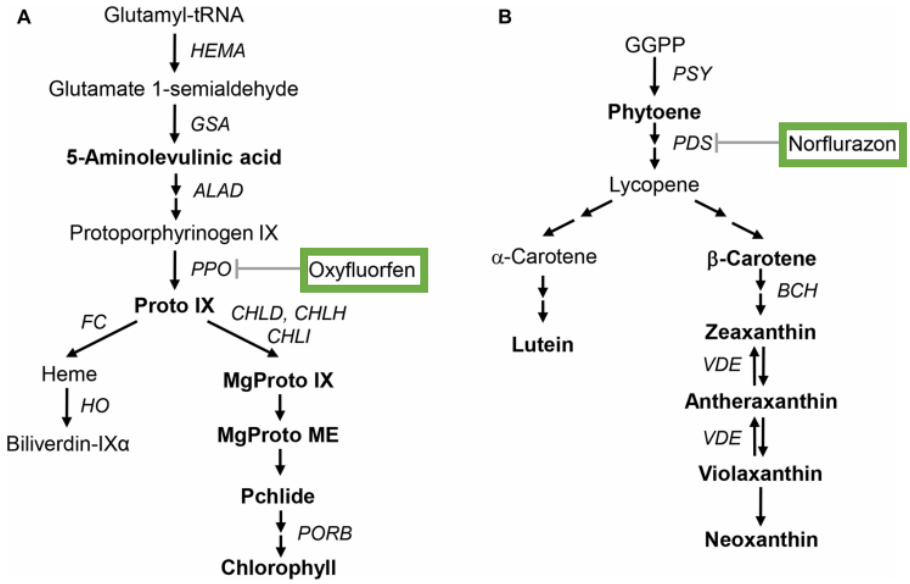


Figure 3.8 - Schematic biosynthetic pathways of chlorophyll and carotenoid in plants. Adapted from Park et al. [185].

3.2.2 Materials and Methods

3.2.2.1. Wildtype inocula and culture media

Chlorella vulgaris axenic cultures were obtained from Allmicroalgae Natural Products S.A. culture collection. The wildtype seeds, 0007CA, 0008CA and 0031CA, were obtained from cryopreserved aliquots stored in liquid nitrogen (–196 °C). These strains' codes are internal codes from Allmicroalgae culture collection.

3.2.2.2. EMS dose-response experiments

According to the protocol described in section 3.1.2.2. for *C. vulgaris* strain 0031CA, two other strains of *C. vulgaris*, 0007CA (2.88×10^8 cells mL^{-1}) and 0008CA (1.90×10^8 cells mL^{-1}), were treated with 0-300 mM of EMS to establish the dose-response correlation.

3.2.2.3. Nicotine and norflurazon toxicity

The same protocol described in section 3.1.2.3. was applied with nicotine and norflurazon instead. Nicotine stock solution was at 6.23 M and norflurazon solution was prepared in acetone at 5.26 mM, which were filter-sterilised with 0.22 µm PES and PTFE filters, respectively. Nicotine was tested at 0-7 mM and norflurazon at 0-20 µM.

3.2.2.4. Random mutagenesis and mutants' selection

According to the dose-response curves previously established (Section 3.2.2.2.), random mutagenesis was performed on *C. vulgaris* 0007CA (1.96×10^8 cells mL^{-1}) with 100, 125, 150 and 200 mM of EMS and on 0008CA (2.86×10^8 cells mL^{-1}) with 150 and 200 mM of EMS, according to the same

protocol described in section 3.1.2.4. Upon 0007CA mutagenesis, selection was carried out either under 2.5, 4, 5 or 7.5 mM of nicotine, or 2.5, 5, 10, 15 or 20 µM of norflurazon, while upon 0008CA mutagenesis, a concentration of 4 or 5 mM of nicotine and 10 or 15 µM of norflurazon were used. Several mutagenesis rounds were performed. However, only the most relevant results in the context of this project are shown.

Differently coloured colonies were picked and streaked for 10 generations to ensure phenotype stability. Strains that kept a stable phenotype were subsequently compared regarding their growth performance, protein content and pigment profile.

Aiming at isolating a whiter mutant, after a pre-selection step based on growth performance, protein content, pigments' profile and biomass colour, as it will be further explained, a second-round mutagenesis was carried out with the white mutant generated upon mutagenesis of 0031CA with 150 mM of EMS, selected under 500 µg L^{-1} of oxyfluorfen (colony number 62 – mutant named 31W150.500.62; also mentioned under the name 31W62 in the subchapter 3.1). This second round was performed with 125 and 150 mM of EMS, and mutants were plated onto PCA with: i) 500 and 600 µg L^{-1} of oxyfluorfen, or, ii) 3 and 4 mM of nicotine, or, iii) 15 and 20 µM of norflurazon. The whitest colonies were picked and streaked for further characterisation.

3.2.2.5. Growth performance of wildtype vs mutants

Mutants with a stable phenotype on solid medium were transferred to liquid medium (without inhibitor) to compare their growth performance with the respective wildtype strains. Growth assays, sampling and daily analysis were conducted as described in section 3.1.2.5.

3.2.2.6. Biochemical characterisation

To estimate protein content and extract and quantify pigments, the same protocols that were described in section 3.1.2.6. were used.

3.2.2.7. Statistical analysis

All assays were carried out in biological triplicates, and results were analysed as described in section 3.1.2.7.

3.2.3 Results and discussion

3.2.3.1 EMS dose-response experiments

The survival rate of the three strains of *C. vulgaris* used throughout this work to EMS is shown in Figure 3.9.

The optimal EMS concentration to apply to mutate these three strains of *C. vulgaris* is within 100-200 mM range. However, strain 0008CA presents a higher tolerance, so this range should be narrowed

to 150-200 mM, while for strains 0007CA and 0031CA, concentrations between 100-150 mM of EMS should be used.

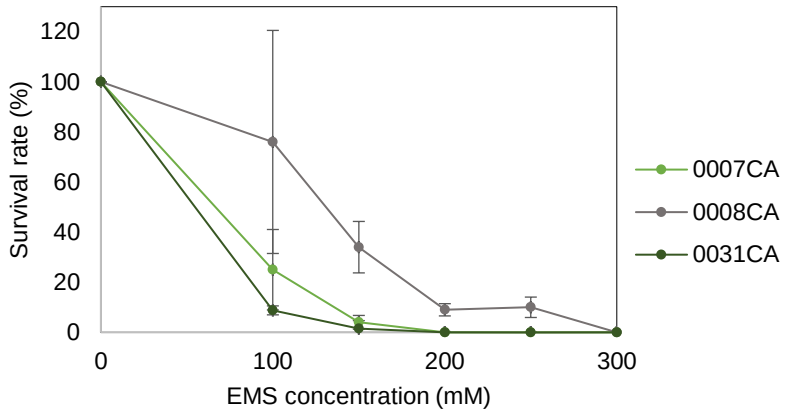


Figure 3.9 - Survival rate (%) of *C. vulgaris* 0007CA, 0008CA and 0031CA strains, exposed to different ethyl methanesulfonate (EMS) concentrations. Results are shown as mean ± SD, n=3.

3.2.3.2. Nicotine and norflurazon toxicity

To determine the optimal range of nicotine and norflurazon for mutants' selection, several concentrations of these inhibitors were tested on the three strains of *C. vulgaris* (Table 3.5).

The concentrations of nicotine and norflurazon that fully inhibited *C. vulgaris* were similar among the three strains. To assure an effective selection methodology, 3-5 mM of nicotine should be used, while with norflurazon concentrations should be between 10 and 20 µM.

Table 3.5 - Results of the nicotine (Ni) and norflurazon (NF) resistance tests on *C. vulgaris* 0007CA, 0008CA and 0031CA strains. G – Growth; NG – No Growth; - not tested.

Ni (mM)	**0**	**2**	**2.5**	**3**	**4**	**5**	**5.5**	**6**	**7**
0007CA	G	-	G	-	G	-	NG	-	NG
0008CA	G	G	-	G	NG	NG	-	NG	NG
0031CA	G	G	-	G	G	NG	-	NG	NG
NF (µM)	**0**	**5**	**7.5**	**10**	**12.5**	**15**	**17.5**	**20**	
0007CA	G	G	-	G	-	NG	-	NG	
0008CA	G	G	G	G	G	G	G	NG	
0031CA	G	G	G	G	G	G	G	NG	

Regarding the use of norflurazon as a pathway inhibitor upon mutagenesis with EMS on *C. vulgaris*, a concentration of 10 µM has also been reported previously [19]. However, in the case of nicotine, only

two reports were found, both using 400 µM to improve carotenoids' content in *Chlorella sorokiniana* upon mutagenesis with methylnitronitrosoguanidine (MNNG) [133, 172]. This concentration is much lower than the range obtained in this work, which might be related to mutagenesis and cultivation conditions, as previously discussed extensively.

3.2.3.3. Isolation of mutants with different pigmentation

Based on the previous results, cells were mutagenized with EMS and exposed to nicotine, norflurazon or oxyfluorfen. Mutagenized cultures isolated under these inhibitors resulted in the appearance of yellow, white, orange, brown and green colonies (Table 3.6, Figure 3.10).

Table 3.6 - Conditions applied in the isolation of *C. vulgaris* mutants selected for future characterisation (bold). WT strains 0007CA, 0008CA and 0031CA were subjected to mutagenesis with EMS (concentrations in mM) and selection was performed under nicotine (Ni, in mM), or norflurazon (NF, in µM) or oxyfluorfen (Oxy, in µg L^{-1}). In the "Results" column, only the mutants with stable phenotype after 10 times streaking on solid medium were accounted.

Round	Strain	EMS	Ni	NF	Oxy	Results
1	0007CA	100, 150, 200	5, 7.5	10, 15, 20	-	No growth
2	0007CA	100, 125, 150	5, 7.5	10, 15, 20	-	No growth or turned green
3	0007CA	100, 125, 150	2.5, 4	2.5, 5, 10	-	**1 yellow** and **1 white**
1	0008CA	150, 200	4, 5	10, 15	-	Only green strains
1	0031CA	100, 125, 150	2.5, 5	10, 15, 20	-	**1 yellow**
2	0031CA	100, 125, 150	4	8, 10, 15	300, 400, 500	**9 yellow** and **11 white**

To illustrate the colourful colonies obtained on the solid media, as an example, plates after mutagenesis with 150 mM of EMS and 500 µg L^{-1} of oxyfluorfen are shown in Figure 3.10.

A yellow colony of *C. vulgaris* 0007CA was generated upon mutagenesis with 125 mM of EMS and selected under 2.5 mM of nicotine, while 10 other of 0031CA were isolated after treatment with either 125 or 150 mM of EMS and 500 µg L^{-1} of oxyfluorfen (Table 3.6). Moreover, 1 white mutant from 0007CA mutagenesis was generated in the same conditions as the yellow one, 125 mM of EMS and 2.5 mM of nicotine, and 11 white mutants from 0031CA were picked after incubation with 125 or 150 mM of EMS and 400 or 500 µg L^{-1} of oxyfluorfen (Table 3.6). The mutants that originated from the WT strain 0031CA are the same as described in subchapter 3.1.

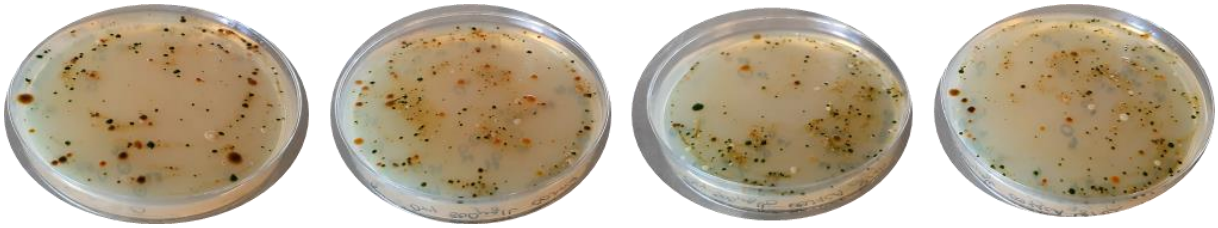


Figure 3.10 - Differently coloured colonies obtained on plates after mutagenesis of wildtype strain 0031CA *C. vulgaris*, with 150 mM of EMS and 500 µg L-1 of oxyfluorfen.

These results demonstrate not only the feasibility of using a metabolic inhibitor of the chlorophyll biosynthesis pathway, like oxyfluorfen, but also validate the previously reported feasibility of using nicotine and norflurazon as inhibitors of the carotenoids' pathway, to select chemically-mutagenized cells with different pigmentation, namely chlorophyll-deficient phenotypes [19, 133, 172, 199, 237].

3.2.3.4. Wildtype vs mutants: growth performances

Chlorella vulgaris mutants and respective wildtype strains were compared at lab-scale regarding their growth performance (Figure 3.11, Figure 3.12).

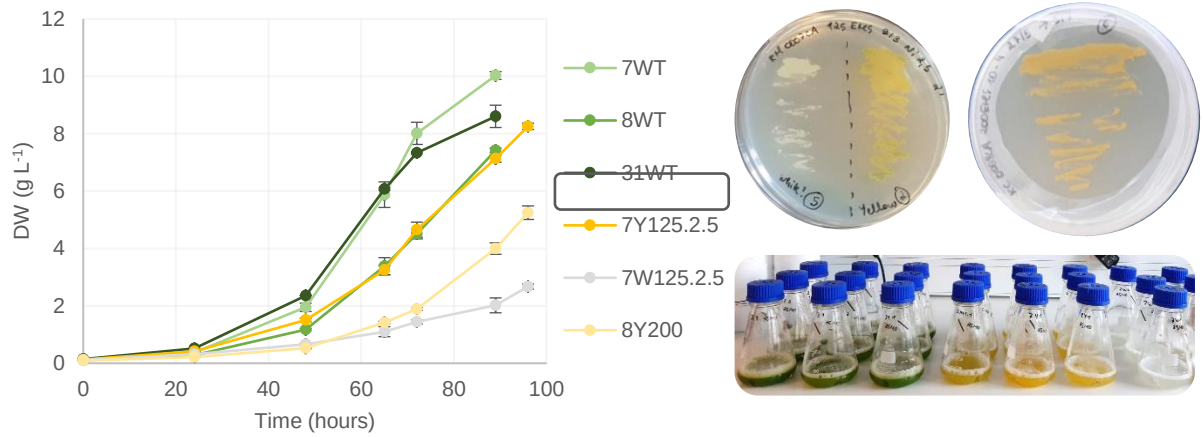


Figure 3.11 - Growth curves of *C. vulgaris* wildtype (WT) strains 0007CA (7WT), 0008CA (8WT) and 0031CA (31WT), yellow (Y) mutants derived from 0007CA (7Y) and 0008CA (8Y) and a white (W) mutant originated from 0007CA (7W), grown heterotrophically in 250-mL Erlenmeyer flasks (on the left). Pictures of yellow and white mutans on solid medium and of the growth trial of these strains in liquid medium (on the right). Results are shown as mean ± SD, n=3.

From the 11 yellow mutants selected, mutant 7Y125.2.5, henceforth named 7Y, and mutant 31Y150.500.15, named 31Y15, as mentioned in the subchapter 3.1., or simply 31Y displayed faster growth performance and were selected for further analysis. The white mutant 7W125.2.5 was excluded for having a significantly slower growth. Regarding the other 11 white mutants obtained from strain 31, growth performance was similar among them, and all acquired a green-mint tone in liquid medium. However, mutant 31W150.500.62, named 31W62 (mentioned in the subchapter 3.1.), was selected for further work and analysis for having a slightly whiter tone. For this reason, this mutant was also

subjected to a second-round mutagenesis, which generated 5 potential whiter mutants, whose growth performance was also compared (Figure 3.13).

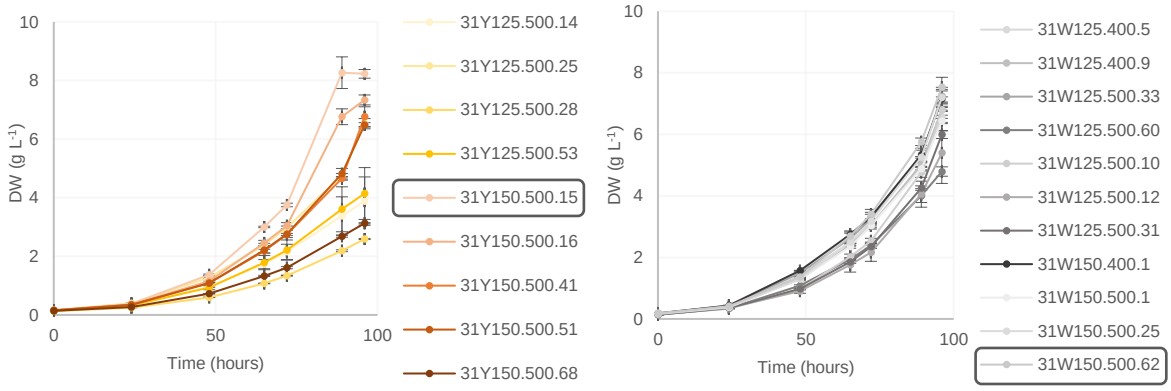


Figure 3.12 - Growth curves of *C. vulgaris* mutants of 0031CA (31): yellow (Y) (on the left) and white (W) mutants (on the right), grown heterotrophically in 250-mL Erlenmeyer flasks (on the left). In the caption, in front of yellow (31Y) and white mutants (31W), appears the concentration of EMS used in the mutagenesis (in mM), followed by the concentration of oxyfluorfen (in µg L^{-1}), which ends with the number of the colony isolated. Results are shown as mean ± SD, n=3.

In this second-round mutagenesis, 4 out of the 5 mutants were selected again under 500 or 600 µg L^{-1} of oxyfluorfen and 1 under 15 µM of norflurazon. These mutants exhibited a similar growth performance. Nevertheless, mutant 31W125.15.5, henceforth named 31W, displayed a whiter tone (Figure 3.**13**).

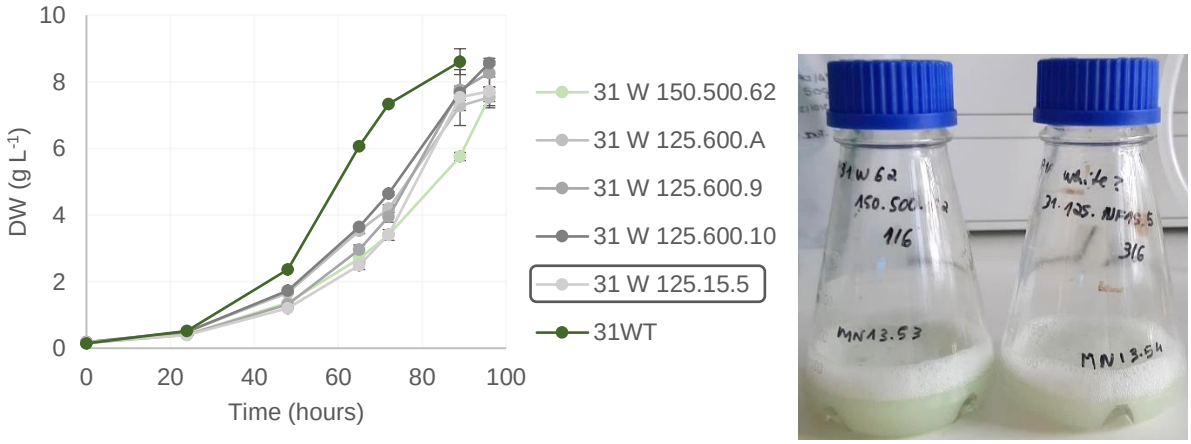


Figure 3.13 - Growth curves of *C. vulgaris* wildtype strain 0031CA (31WT), white mutant of the first-round mutagenesis (31W150.500.62) and of white mutants generated on the second-round mutagenesis of the latter, grown heterotrophically in 250-mL Erlenmeyer flasks (on the left). In the caption, in front of white mutants (31W), appears the concentration of EMS used in the mutagenesis (in mM), followed by the concentration of oxyfluorfen (in µg L^{-1}) or norflurazon (in µM), which ends with the number of the colony isolated. On the right, there is a picture displaying the colour improvement in liquid medium from mutant 31W150.500.62 to mutant 31W125.15.5. Results are shown as mean ± SD, n=3.

3.2.3.5. Wildtype vs mutants: biochemical profiles

Besides growth performance, higher protein content was another characteristic of interest that was searched in the screening of these mutants, so that the contents of the wildtype strains and yellow and white mutants were analysed (Table 3.7).

In addition, since colour is important not only for developing products with more appealing sensory properties, but also for the bioactivity of such products, namely provided by the antioxidant pigments, carotenoids and chlorophyll contents of the most promising mutants were quantified (Table 3.8).

Table 3.7 - Protein contents (% of DW) of *C. vulgaris* wildtype (WT) strains 0007CA (7WT), 0008CA (8WT) and 0031CA (31WT), yellow (Y) mutants derived from 0007CA (7Y), 0008CA (8Y) and 0031CA (31Y) and white (W) mutants originated from 0007CA (7W) and from 0031CA (31W), determined by elemental analysis (factor 6.25). In the caption, in front of yellow (31Y) and white mutants (31W), appears the concentration of EMS used in the mutagenesis (in mM), followed by the concentration of oxyfluorfen (in µg L^{-1}) or norflurazon (in µM) or nicotine (in mM), which ends with the number (or letter if number > 100) of the colony isolated. The prefix "2nd" refers to the mutants obtained in the second-round mutagenesis of mutant 31W150.500.62. Results are shown as mean ± SD, n=3.

Strain	Protein (%)	Strain	Protein (%)
7WT	41.34 ± 2.17	**31W125.400.5**	41.78 ± 0.56
8WT	45.52 ± 1.53	**31W125.400.9**	43.59 ± 3.35
31WT	43.28 ± 0.54	**31W125.500.10**	36.06 ± 3.02
7Y125.2.5	45.36 ± 1.08	**31W125.500.12**	39.20 ± 1.12
8Y200	35.54 ± 0.65	**31W125.500.31**	37.39 ± 0.86
31Y125.500.14	42.35 ± 1.26	**31W125.500.33**	43.78 ± 1.60
31Y125.500.25	43.37 ± 2.78	**31W125.500.60**	41.50 ± 1.42
31Y125.500.28	31.48 ± 1.47	**31W150.400.1**	42.73 ± 0.88
31Y125.500.53	41.33 ± 0.30	**31W150.500.1**	41.65 ± 1.76
31Y150.500.15	44.25 ± 0.68	**31W150.500.25**	38.62 ± 5.48
31Y150.500.16	42.22 ± 1.84	**31W150.500.62**	42.80 ± 1.09
31Y150.500.41	41.74 ± 0.98	**2nd 31W125.600.A**	33.82 ± 2.04
31Y150.500.51	41.84 ± 1.15	**2nd 31W125.600.9**	34.15 ± 0.60
31Y150.500.68	38.07 ± 0.48	**2nd 31W125.600.10**	32.62 ± 1.03
7W125.2.5	32.35 ± 0.67	**2nd 31W125.15.5**	41.12 ± 1.45

Chlorella vulgaris wildtype strains presented a protein content between 41-45% of DW. In addition, WT strains also displayed the highest contents of carotenoids and chlorophyll, as expected, since the inhibitors used in this selection method target these biosynthetic pathways. As supported by the growth trials and colour of these cultures in liquid medium, mutant 7Y (7Y125.2.5) and mutant 31W (31W125.15.5) were selected for further work. As highlighted in Table 3.7, the protein contents were similar to the WT strains, 45% and 41%, respectively and, in Table 3.8, 73% and 91% lower chlorophyll contents, compared to the WT, were exhibited by mutant 7Y and 31W, respectively.

Table 3.8 - Carotenoids concentrations (µg mg^{-1} of biomass), determined by HPLC, and chlorophyll contents (g 100 g^{-1} of biomass), determined by Ritchie method, of *C. vulgaris* wildtype strains 0007CA (7WT), 0008CA (8WT) and 0031CA (31WT), yellow (Y) mutants derived from 0007CA (7Y), 0008CA (8Y) and 0031CA (31Y) and white (W) mutants derived from 0007CA (7W) and 0031CA (31W). The prefix "1st" refers to the first-round mutagenesis of 0031CA that gave origin to mutant 31W150.500.62, and "2nd" refers to the second-round mutagenesis of the latter, which originated mutant 31W125.15.5. n.d. - not detected.

	Neoxanthin	Violaxanthin	Lutein	*β*-Carotene	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll
7WT	0.373 ± 0.084	0.170 ± 0.018	0.572 ± 0.110	0.836 ± 0.202	0.602 ± 0.071	0.293 ± 0.016	0.895 ± 0.078
8WT	0.360 ± 0.010	0.166 ± 0.005	0.533 ± 0.008	0.738 ± 0.017	0.354 ± 0.036	0.212 ± 0.027	0.568 ± 0.060
31WT	0.274 ± 0.015	0.182 ± 0.013	0.516 ± 0.010	1.292 ± 0.096	0.479 ± 0.029	0.189 ± 0.029	0.668 ± 0.057
7Y	0.149 ± 0.005	0.155 ± 0.010	0.380 ± 0.025	0.315 ± 0.045	0.105 ± 0.018	0.138 ± 0.024	0.244 ± 0.042
8Y	0.180 ± 0.005	0.160 ± 0.006	0.477 ± 0.014	0.583 ± 0.003	0.094 ± 0.008	0.118 ± 0.009	0.212 ± 0.018
31Y	n.d.	n.d.	n.d.	n.d.	0.014 ± 0.003	0.018 ± 0.003	0.032 ± 0.005
7W	0.114 ± 0.004	0.154 ± 0.005	0.205 ± 0.007	0.236 ± 0.008	0.073 ± 0.019	0.069 ± 0.028	0.143 ± 0.047
1st31W	n.d.	n.d.	n.d.	n.d.	0.012 ± 0.003	0.014 ± 0.003	0.026 ± 0.006
2nd31W	n.d.	n.d.	n.d.	n.d.	0.031 ± 0.007	0.029 ± 0.009	0.061 ± 0.014

Although mutant 8Y exhibited higher carotenoids' contents, this strain displayed a slower growth rate and a 21% lower protein content, compared to mutant 7Y. For this reason, mutant 7Y was selected for the upcoming tasks. Additionally, despite mutant 31W (2nd) presented a higher chlorophyll content than 31W15 (1st), the colour of the culture in liquid medium was clearly whiter after the second-round mutagenesis. This phenomenon might be explained by such low concentrations of chlorophyll to be quantified, so that the colour is not translated in the concentrations, either by lack of sensitivity of the method and/or all the chlorophyll might not have been extracted from the biomass. Furthermore, these mutants presented similar protein contents, also equivalent to the WT, while mutant 31W (2nd) displayed a faster growth rate.

3.2.4 Conclusions

Several promising mutants were established, including a yellow (7Y) and a white (31W) mutant of *C. vulgaris,* which were selected out of hundreds of colonies generated upon treatment with EMS. The yellow mutant 7Y was generated with 125 mM of EMS and selected under 2.5 mM of nicotine, while the white mutant 31W was isolated after 2 rounds of mutagenesis and selection under two inhibitors, the first with 150 mM of EMS and 500 µg L^{-1} of oxyfluorfen, inhibitor of the chlorophyll pathway, and the second with 125 mM of EMS and 15 µM of norflurazon, inhibitor of the carotenoids' pathway. Thus, the combination of more than one selection strategy, namely several pathway inhibitors, might facilitate the isolation of mutants with the desired target phenotype.

3.3 Isolation of novel *Scenedesmus rubescens* mutants with high-quality protein and improved sensory properties

This chapter was adapted from the following submitted research paper (to the journal Applied Microbiology and Biotechnology):
M. Trovão, S. Navalho, G. Espírito Santo, A. Reis, H. Pedroso, A. Barros, C. Lourenço, M. Costa, S. Ferreira, G. Bombo, H. Cardoso, F. Freitas, J. Silva, H. Pereira, J. Varela and L. M. Schüler, "Isolation of novel *Scenedesmus rubescens* mutants with high-quality protein and improved sensory properties," *Appl. Microbiol. Biotechnol*, 2024

ABSTRACT

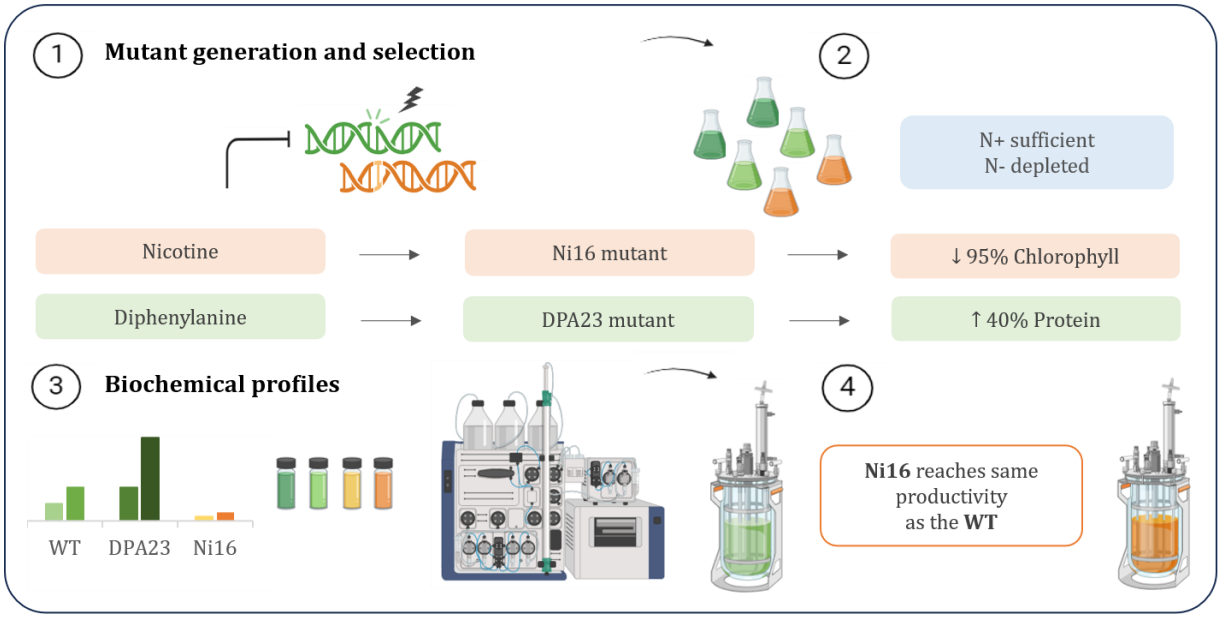
Microalgae are sustainable sources of protein and pigments for feed, food and cosmetics, which can be produced in fermenters. Random mutagenesis has gained increasing attention to improve the phenotype of microalgae for different applications. Chemical mutagenesis and selection with metabolic inhibitors were carried to generate novel mutants of *Scenedesmus rubescens* with improved organoleptic characteristics to develop new products for food and feed applications. In this context, two mutants of *S. rubescens* were established: i) green strain resistant to diphenylamine (DPA23), and ii) orange strain resistant to nicotine (Ni16). Both mutants displayed the same biomass productivity as the wildtype (WT). The highest protein productivity was achieved by strain DPA23, representing a 40% increase compared to the WT. In contrast, strain Ni16 showed significantly lower productivity of pigments (up to 95% decreased), resulting in an orange biomass rich in lutein, *β*-carotene and astaxanthin. The phenotypic stability of Ni16 was further validated in a 7-L fermenter scale-up trial, reaching a final biomass concentration of 66.0 g L^{-1}. Overall, two mutants with optimised phenotypes with high potential for different biotechnological applications were generated.

Keywords: random mutagenesis, microalgae, fermentation, heterotrophic, pigments, amino acids.

Highlights

- Two novel mutants of *Scenedesmus rubescens* were generated by random mutagenesis;
- An orange nicotine-resistant mutant displayed 95% less chlorophyll, 1.23 mg g^{-1} DW;
- A dark green diphenylamine-resistant mutant presented 40% more protein (65.8% DW).

GRAPHICAL ABSTRACT



3.3.1 Introduction

Microalgae have emerged as promising sources of high-quality protein and other bioactive compounds, such as carotenoids. Most microalgal strains are photosynthetic microscopic organisms. However, some microalgal species have evolved under light-limiting conditions and, thus, acquired metabolic mechanisms to grow under heterotrophic conditions, whose large-scale production occurs in fermenters and comes with several trade-offs when compared to photoautotrophic productions. Heterotrophic cultivation is a well-established technology with a lower areal footprint than photoautotrophic cultivation that does not require light and is independent of weather conditions [238–240]. Overall, it is a highly controllable process leading to a reliable and efficient production of high-quality biomass. In addition, productivity under heterotrophic conditions increases by 10-100 times, reducing thereby the downstream costs significantly [192]. Another great advantage of fermentation is the production of biomass with lower or absent chlorophyll contents, which facilitates the incorporation into several feed, food and cosmetic applications due to the reduction of unwanted pigmentation and flavour [19, 241]. The major disadvantage is the supply of an external carbon source such as glucose, which raises concerns about the sustainability of the process unless alternative feedstocks are used [242].

Scenedesmus is an interesting genus of microalgae due to its typically high protein content of up to 60% of dry weight (DW), high levels of lutein, canthaxanthin and, in some species, astaxanthin, as well as the ability to grow under heterotrophic conditions [191, 243, 244]. However, different approaches can be used to further improve productivity and biomass applications by directly manipulating the strain's genetics or adjusting culture conditions. Random mutagenesis represents an interesting tool to generate

tailormade strains, namely with improved pigmentation. Since random mutagenesis leads to the generation of thousands of different strains, one important screening method is the use of pathway inhibitors, as reviewed by Trovão *et al*. [183]. Strains of *Chlorella* sp. rich in carotenoids have been isolated by screening for resistant colonies on nicotine (Ni) or diphenylamine (DPA) [172, 212]. Chlorophyll-deficient and protein-rich strains of *Chlorella vulgaris* have been also isolated by a similar technique [19].

Scenedesmus rubescens has been mostly studied for biofuel and wastewater treatment applications, mainly in autotrophic conditions, due to its robustness, rapid growth rate and rich lipid content [245–247]. Despite this, the potential of this species has not been fully harnessed, namely for different applications, by combining its ability to grow heterotrophically and produce high-quality protein and other bioactive compounds, such as pigments, and essential amino acids.

This study aimed to generate improved strains of *S. rubescens* with increased protein content and altered pigmentation by random mutagenesis. The productivities of these strains were compared to that of the wildtype (WT) strain under heterotrophic conditions using different nitrogen concentrations, which were further validated at pilot scale.

3.3.2 Materials and Methods

3.3.2.1 Wildtype inoculum and growth conditions

Inoculum of the WT microalgal strain *Scenedesmus rubescens* (SAG 5.95 Göttingen) was provided by Allmicroalgae Natural Products S.A. (Pataias, Portugal). The starter culture was grown in a 250-mL Erlenmeyer using 50 mL of plate count broth (PCB) composed of 5 g L^{-1} tryptone, 2.5 g L^{-1} yeast extract and 10 g L^{-1} glucose and incubated at 25 ± 1 °C under shaking at 130 rpm, in the dark.

3.3.2.2 Random mutagenesis and mutant screening

To determine survival rate, microalgal cells (10^6 cells mL^{-1}) in exponential phase were treated with ethyl methanesulfonate (EMS) following a recent protocol [19], as also described in the subchapter 3.1.2.2. The cells were plated in serial dilutions onto plate count agar (PCA) and incubated at 25 ± 1 °C in the dark to determine colony-forming units.

In order to isolate mutants with different expressions in the target pathway, a growth inhibition assay was performed using the minimal lethal concentration of the selected metabolic inhibitor for the WT strain. To establish this concentration, 1 mL of cell suspension in the exponential phase was plated onto 24-well PCA plates containing nicotine (Ni) at concentrations of 0.25, 0.5, 1, 2 and 3 mM or diphenylamine (DPA) at concentrations of 10, 15, 25, 50, 75, 100 and 150 µM and incubated at 25 ± 1 °C in the dark. The minimal concentration inhibiting growth was confirmed on larger agar plates and was finally used to plate mutagenized cells. Mutant selection was carried out by visual observation of differently coloured colonies resistant to either Ni or DPA that were streaked 10 times before scaling up in liquid medium.

3.3.2.3 Growth comparison of mutant strains to the wildtype in Erlenmeyer

WT strain and mutants were grown in a previously optimised medium [191] containing either 70 mM or 1.75 mM of N, using urea and nitrate as nitrogen sources, to compare the induction of different compounds under N+ and N- conditions, respectively. The cultures were incubated in the dark at 28 ± 1 °C under shaking at 200 rpm. The carbon source was glucose at a concentration of 20 g L^{-1}.

Growth assays, sampling and daily analysis were conducted as described in section 3.1.2.5. as well as biomass productivity and growth rate calculations.

DW was calculated by the following correlation (Equation 8) established previously [191]:

$$OD_{600} = DW \times 1.2036 \quad (8)$$

3.3.2.4 Growth comparison in a 7-L fermenter

Strains were sequentially scaled up to reach a final working volume of 5 L in a benchtop fermenter (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany). The temperature was maintained at 28 °C, and the pH was controlled at 6.5 by the addition of phosphoric acid (25%). As in the Erlenmeyer flasks tests, an optimised medium was used [191], but glucose was added in fed-batch so that a non-limiting concentration of 5 to 20 g L^{-1} was kept. The air inlet flowrate was adjusted throughout the growing period to maintain ~1 vvm. Accordingly, the agitation rate ranged from 200 to 1200 rpm, so that the dissolved oxygen in the medium was not a limiting factor for culture growth. Samples were collected aseptically for biomass concentration and supernatant analysis. Freestyle Precision Neo kit (Abbott, Witney, Oxon, UK) was used to determine glucose concentration in g L^{-1}.

3.3.2.5 Pigment analysis

Freeze dried biomass of the different strains was extracted with methanol under dark and cold conditions. Cells were disrupted with a combination of glass beads and one tungsten bead in an MM400 mixer mill (Retsch, Germany) at 30 Hz for 3 min. The supernatant was recovered by centrifugation at 3000 *g* for 3 min, and the biomass was re-extracted until both biomass and supernatant became colourless. After evaporation of the pooled supernatants under a constant nitrogen flow, the extract was resuspended in a known volume of HPLC-grade methanol and filtered.

Chlorophyll *a* and *b* contents were determined spectrophotometrically using the following formulae mentioned previously in section 3.1.2.6.

Carotenoid profiles were analysed by the same protocol as described in section 3.1.2.6.2.

3.3.2.6 Total amino acids analysis

Total amino acids (AA) were extracted from freeze-dried biomass by hydrolysis with 6 N HCl at 121 °C for 72 h. After cooling down, all extracts were concentrated to dryness in a speed vacuum system (Concentrator plus, Eppendorf), resuspended in 0.02 N HCl, and derivatized following Waters AccQ-Tag™ for hydrolysate amino acids procedure for HPLC. AA determination was performed by HPLC

(Chromaster, Hitachi, VWR) equipped with a fluorescence detector (5440 FL detector, Hitachi, VWR). Chromatographic conditions were set according to the certified Waters AccQ-Tag™ for AAs' hydrolysate.

The essential amino acids index (EAAI) was calculated by the following equation, as described previously [180] and based on the WHO/FAO recommendations [248]:

$$EAAI = \sqrt[n]{(aa1 \div AA1) \times (aa2 \div AA2) \times \dots \times (aan \div AAn)} \quad (9)$$

where *aan* is the content of the specific essential amino acid (EAA) in the sample and *AAn* is the AAn content of a standard egg protein in g 100 g^{-1} of protein; *EAAI*; values ≥1, 0.95–1, 0.86–0.95, 0.75–0.86, and ≤0.75 indicate superior quality, high quality, good quality, useful and inadequate, respectively.

3.3.2.7 Statistical analysis

Data were tested for normality using the Shapiro-Wilk test (XLStat software, Vers. 2016.02.27444, Addinsoft, USA). ANOVA and Tukey's HSD post hoc test were performed for the comparison of means of treatments with a confidence interval of 95%.

3.3.3 Results and Discussion

3.3.3.1 Generation and selection of mutants

Random mutagenesis with the alkylating agent ethyl methanesulfonate (EMS) was performed to obtain strains with improved colours and nutritional profile, followed by a strain selection on different pathway inhibitors. The EMS concentration of 600 mM led to a survival rate of 11%, which was chosen in the following experiments to screen for improved strains on different pathway inhibitors (Figure 3.14). This survival rate s is close to the standard choice between 5-10% for microalgal random mutagenesis [19, 196, 249].

For the inhibitors nicotine (Ni) and diphenylamine (DPA), the minimal lethal concentrations to the WT were 0.5 mM and 50 µM, respectively. Therefore, random mutagenesis with 600 mM of EMS combined with this selection method gave rise to two mutants that stood out, one with dark green coloration and increased carotenoid content (hereafter referred to as strain DPA23), isolated under 50 µM DPA; and the other selected under 0.5 mM of Ni nicotine with orange coloration and improved productivity comparing to the WT (data not shown) (hereafter referred to as strain Ni16).

The inhibitors nicotine (Ni) and diphenylamine (DPA) of the carotenoid biosynthetic pathway block the enzymes lycopene cyclase and *β*-carotene hydroxylase, respectively [250]. In this way, the strains isolated in this work, DPA23 and Ni16, resistant to these pathway inhibitors, might represent changes in these enzymes towards higher activities to overcome this inhibition; thus, enhanced contents of carotenoids were expected in these mutants, as it has been shown for other microalgae [172, 212, 251].

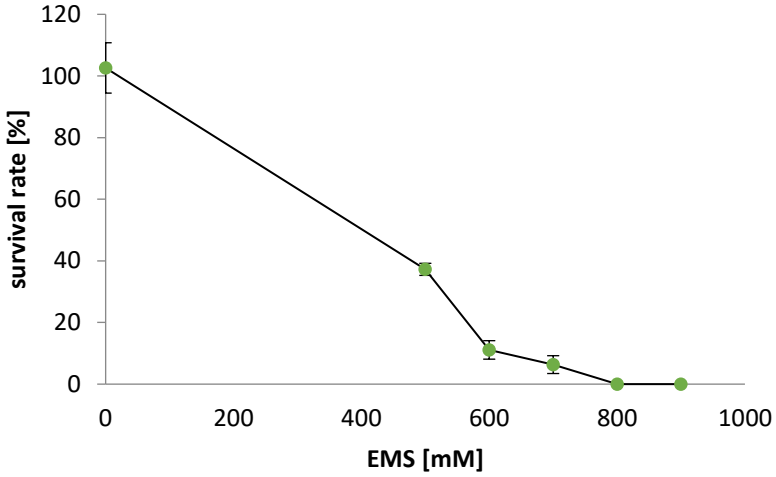


Figure 3.14 - Survival rate of *S. rubescens* using different concentrations of ethyl methanesulfonate (EMS). Data points at each concentration are displayed as mean ± SD (*n*=3).

3.3.3.2 Comparison of WT and mutants under different nitrate concentrations

All strains grown in either 70 mM N (N+) or 1.75 mM N (N-) media showed no lag phase and directly entered the exponential growth phase (Figure 3.15). After 48 h, the growth of the strains in N- medium slowed down, reaching their maximum biomass concentration (on average 6.4 g L^{-1}) on day 3. On the other hand, strains cultivated in the N+ medium reached significantly enhanced maximum biomass concentrations as high as 10.7 g L^{-1} between 65-72 h and specific growth rates as high as 0.95 d^{-1}, without significant differences among strains (Table 3.9). Upon 4 days of growth, the WT cultures and strain DPA23 presented dark green colours in both media. Strain Ni16, on the other hand, showed a brown colour in N+ medium, while it presented a very intense orange colour after growth in N- medium (Figure 3.15).

The growth performance of DAP23 and Ni16 under different nitrogen conditions (70 mM N (N+) or 1.75 mM N (N-)), are in accordance with a study on *S. obliquus*, in which decreased growth rates were shown in nitrogen-limited media under photoautotrophic conditions both for the wildtype and the respective mutant strain [223]. Under nitrogen sufficient conditions, these authors reported growth rates that were increased by up to 57%, achieving up to 0.35 d^{-1}. Despite these improvements, these rates are still much lower than the ones reported in the present study (0.94-0.95 d^{-1}).

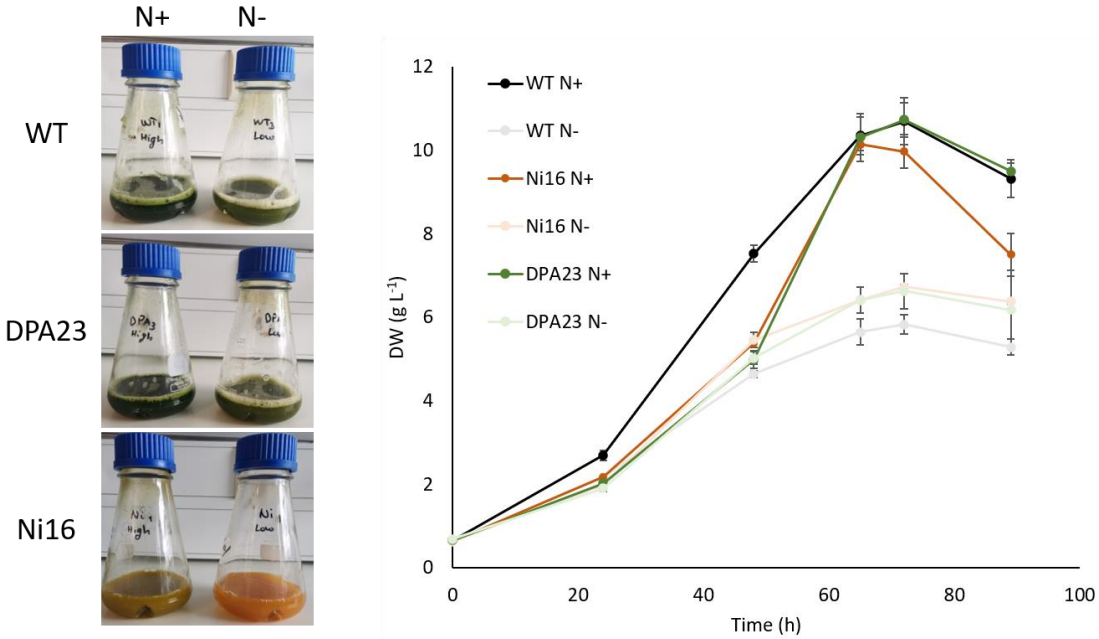


Figure 3.15 - Growth curves of *S. rubescens* strains WT, DPA23 and Ni16 in media supplemented with 70 mM N (N+) or 1.75 mM N (N-). Data points on each day are displayed as mean ± SD (*n*=3).

Table 3.9 - Specific growth data of *S. rubescens* strains WT, DPA23 and Ni16 cultivated in two different types of media using 70 mM N (N+) or 1.75 mM N (N-). Significant differences are indicated by small letters (Tukey test, $p < 0.05$). Values are shown as means ± SD ($n = 3$).

Strain	Medium	r_p (g L^{-1} d^{-1})	X_{max} (g L^{-1})	μ (d^{-1})	μ_{max} (d^{-1})
WT	**N+**	3.35 ± 0.24 ^{a}	10.7 ± 0.70 ^{a}	0.94 ± 0.04 ^{a}	1.03 ± 0.08 ^{a}
Ni16		3.12 ± 0.16 ^{a}	9.97 ± 0.50 ^{a}	0.94 ± 0.01 ^{a}	0.93 ± 0.01 ^{a}
DPA23		3.36 ± 0.16 ^{a}	10.7 ± 0.50 ^{a}	0.95 ± 0.03 ^{a}	1.03 ± 0.05 ^{a}
WT	**N-**	1.73 ± 0.10 ^{b}	5.83 ± 0.28 ^{b}	0.73 ± 0.02 ^{c}	0.76 ± 0.05 ^{b}
Ni16		2.03 ± 0.03 ^{b}	6.73 ± 0.07 ^{b}	0.80 ± 0.01 ^{b}	1.01 ± 0.07 ^{a}
DPA23		1.98 ± 0.18 ^{b}	6.62 ± 0.52 ^{b}	0.77 ± 0.03 ^{b,c}	0.97 ± 0.05 ^{a}

3.3.3.3 Pigment profile

Regarding pigments, the WT grown under N+ conditions represented the highest chlorophyll *a* and *b* contents of 19.6 ± 1.9 and 7.99 ± 0.91 mg g^{-1} DW (Figure 3.16a), resulting in productivities of 45.5 ± 3.3 and 18.6 ± 1.5 mg L^{-1} d^{-1} (Figure 3.16c), respectively. Strain DPA23 displayed significantly lower productivities of 36.1 ± 1.7 and 14.7 ± 1.6 mg L^{-1} d^{-1} for chlorophyll *a* and *b*, respectively. However, chlorophyll productivity decreased by up to 74% under N- conditions in both WT and DPA23. Nevertheless, strain Ni16 had the lowest chlorophyll *a* and *b* productivities independent of the initial nitrogen concentration in the medium, reaching on average 1.68 and 1.04 mg L^{-1} d^{-1}, respectively.

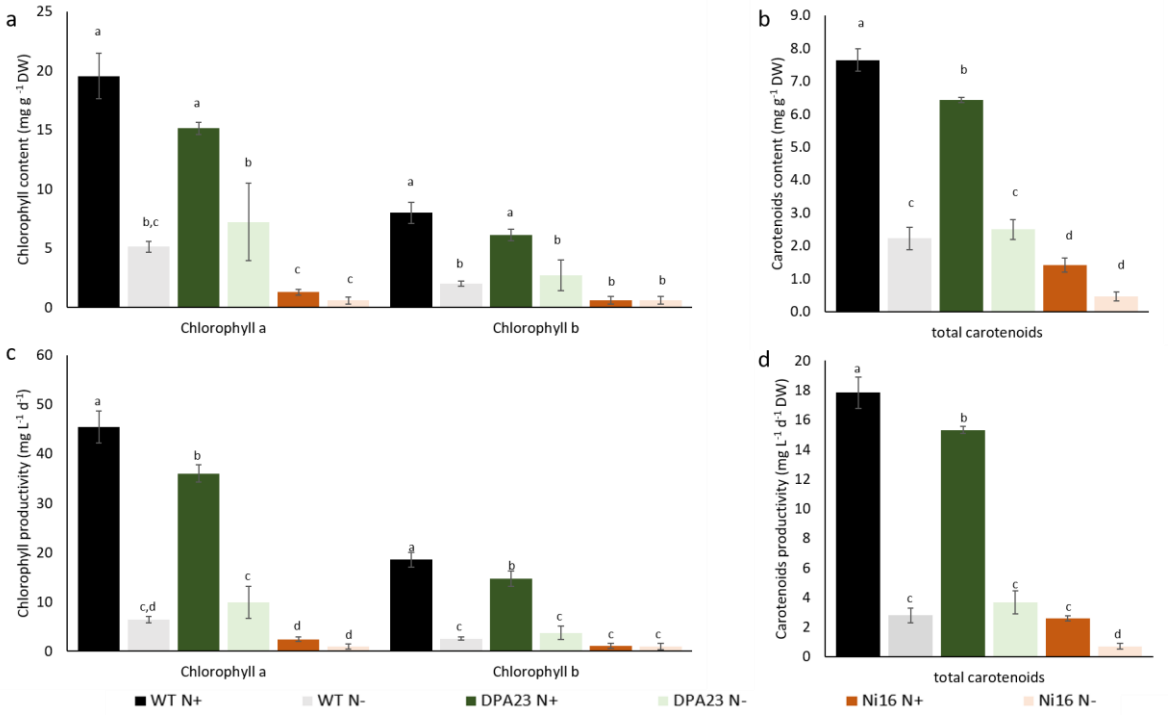


Figure 3.16 - Chlorophyll and carotenoid contents (a, b) and productivities (c, d) of *S. rubescens* WT, DPA23 and Ni16 grown under 70 mM (N+) and 1.75 mM (N-) nitrogen concentrations. Significant differences are indicated by small letters (Tukey test, $p < 0.05$). Values for each compound are displayed as mean ± SD (n=3).

Concerning total carotenoids, the highest content and productivity were obtained in the WT under N+ conditions, reaching 7.64 ± 0.34 mg g^{-1} DW and 17.8 ± 1.1 mg L^{-1} d^{-1}, respectively (Figure 3.16b and d). For strain DPA23, significantly lower values of 6.43 ± 0.07 mg g^{-1} DW and 15.3 ± 0.2 mg L^{-1} d^{-1} were found for total carotenoid content and productivity, respectively (Figure 3.16b and d). However, under N- conditions, carotenoid contents and productivities decreased significantly, by up to 84%, in both WT and DPA23, reaching similar values to those of Ni16 under N+ conditions (1.42 ± 0.21 mg g^{-1} DW and 2.60 ± 0.16 mg L^{-1} d^{-1}, respectively). Nevertheless, the lowest carotenoid contents and productivities were found for strain Ni16 grown under N- conditions, reaching 0.46 ± 0.13 mg g^{-1} DW and 0.71 ± 0.21 mg L^{-1} d^{-1}, respectively.

When looking at the carotenoid profiles, the most abundant carotenoids were *β*-carotene and lutein, reaching the respective contents of 3.26 ± 0.14 and 2.58 ± 0.11 mg g^{-1} DW as well as productivities of 7.60 ± 0.46 and 6.02 ± 0.33 mg L^{-1} d^{-1} in the WT under N+ conditions (Figure 3.17). The content and productivity of lutein in strain DAP23 are similar to those of the WT, while *β*-carotene contents and productivity decreased (2.77 ± 0.15 mg g^{-1} DW and 6.60 ± 0.24 mg L^{-1} d^{-1}, respectively). For strain Ni16, the carotenoid with the highest content of 0.70 ± 0.11 mg g^{-1} DW and productivity of 1.28 ± 0.10 mg L^{-1} d^{-1} was lutein, followed by *β*-carotene (0.21 ± 0.03 mg g^{-1} DW and 0.39 ± 0.02 mg L^{-1} d^{-1}, respectively) under N+ conditions, which were about 6 and 15 times lower than the WT, following the same decreasing trend as chlorophyll, although less pronounced. Interestingly, astaxanthin and canthaxanthin were detected in all strains, although in small amounts, between 0.08 - 0.26 mg g^{-1} DW and 0.03 – 0.07 mg g^{-1}

DW, respectively (Figure 3.17). The highest productivities of astaxanthin and canthaxanthin were reached in N+ conditions by the WT, 0.62 ± 0.06 and 0.15 ± 0.01 mg L^{-1} d^{-1}, respectively.

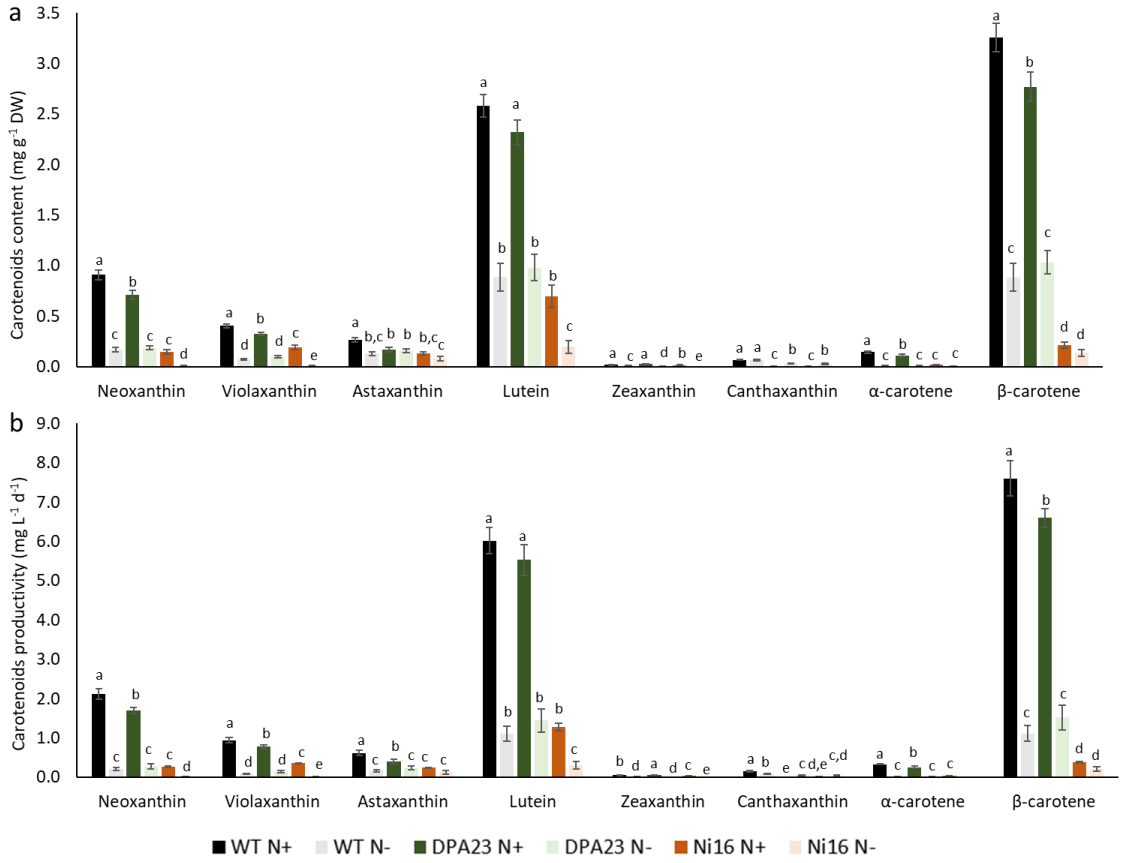


Figure 3.17 - Content (a) and productivity (b) of individual carotenoids of *S. rubescens* WT, DPA23 and Ni16 grown under 70 mM (N+) and 1.75 mM (N-) nitrogen concentrations. Significant differences are indicated by small letters (Tukey test, $p < 0.05$). Values for each compound are displayed as mean ± SD (n=3).

Regarding pigments profiles, chlorophyll *a* contents between 15-20 mg g^{-1} DW were attained by the WT and strain DPA23 under N+ conditions, which is similar to the 16 mg g^{-1} DW that has been reported before for *S. rubescens* under photoautotrophic conditions, whose content decreased to about 1-4 mg g^{-1} DW under stress conditions [252]. Carotenoid contents of the WT and strain DPA23 under N+ conditions, around 7 mg g^{-1} DW, are also in agreement with previous publications on *Scenedesmus* sp. [252, 253]. However, under photoautotrophic conditions, increased carotenoid contents have been found in *Scenedesmus* sp. when abiotic stress such as nitrogen deficiency, high light or salinity were applied [223, 252, 254]. Under heterotrophic conditions, though, photosynthetic pigments decreased significantly in *Scenedesmus* sp., so that other triggers, such as oxidative stress or a shift towards photoinduction might be required to induce their biosynthesis in the dark [255–257]. These results demonstrate that chlorophyll and carotenoids are interdependent since chlorophyll-deficient strains also showed deficient carotenoid accumulation, which has also been observed in chlorophyll-deficient *Chlorella vulgaris* mutants grown under heterotrophic conditions [19]. Therefore, the more intense orange

colour of strain Ni16 is probably not related to increased carotenoid levels but is rather due to decreased chlorophyll contents.

Particular interest has been paid to the genus of *Scenedesmus* due to its high lutein contents reaching up to 8 mg g^{-1} DW under photoautotrophic conditions [253]. Nevertheless, the highest lutein productivity reported of 3.8 mg L^{-1} d^{-1} was found in *S. almeriensis* under photoautotrophic conditions and continuous mode [258]. However, the lower pigment contents attained under heterotrophic conditions, as the lutein content reported in the present study (2.6 mg g^{-1} DW), are compensated by higher final biomass concentrations. Thus, this cultivation strategy leads to higher pigment productivities after all, as it has been demonstrated in this study (lutein productivity of up to 6.02 ± 0.33 mg L^{-1} d^{-1}). In addition, the production of specific compounds can be fine-tuned by controlling growth conditions, such as nutrient supply, pH and temperature. For example, in the present study, N- conditions triggered increased canthaxanthin contents and productivities in Ni16 as compared to N+ conditions, which is in agreement with the study on *S. rubescens*, though in photoautotrophic conditions, that reported contents of up to 2.5, 1.2 and 1.6 mg g^{-1} DW of lutein, astaxanthin and canthaxanthin, respectively, when different abiotic stressors were applied, namely nitrogen deficiency [252]. On the other hand, lutein and protein contents have been increased under nitrate-supplemented conditions in *Scenedesmus* sp. [254, 259].

3.3.3.4 Total amino acid profile

The highest total AA content and productivity were achieved for strain DPA23 when grown under N+ conditions, reaching 65.8 ± 1.4% DW and 1567.1 ± 14.5 mg L^{-1} d^{-1}, respectively (Figure 3.18). Under the same growth conditions, the WT strain reached significantly lower values of 48.1 ± 0.9% DW and 1125.1 ± 91.1 mg L^{-1} d^{-1} for the respective total AA content and productivity. Strain Ni16 reached the lowest protein content and productivity of 34.6 ± 1.1% DW and 640.1 ± 55.8 mg L^{-1} d^{-1}, respectively.

Regarding AA profile, strain DPA23 was particularly rich in arginine, histidine, and phenylalanine (Table 3.10). However, the highest methionine contents were found in strain Ni16, regardless of the growth conditions.

When looking at the essential amino acids (EAA), the WT grown under N+ conditions presented the highest content of 58.5 g 100 g^{-1} protein and superior quality as indicated by the essential amino acids index (EAAI) of 2, followed by that of strain Ni16, which reached an EAA content of 54.9 g 100 g^{-1} (EAAI of 1.88) (Table 3.11).

The protein contents of 30-66% DW attained in this study are higher or similar to what has been reported previously for *S. rubescens* (31% DW [191]) under heterotrophic conditions as well as for *Scenedesmus dimorphus* (34% DW [260]) and *Scenedesmus* sp. grown outdoors under photoautotrophic conditions (52.4% DW [261]).

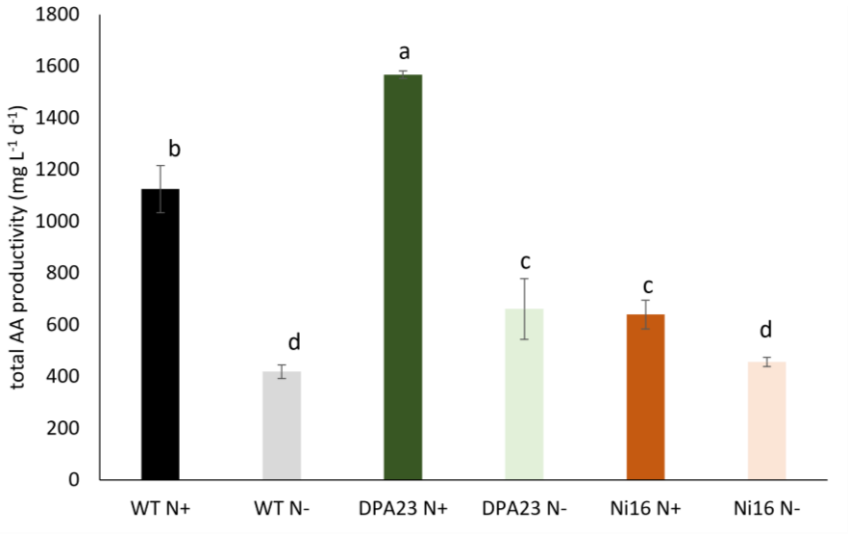


Figure 3.18 - Total amino acid productivity of *S. rubescens* WT, DPA23 and Ni16 grown under 70 mM (N+) and 1.75 mM (N-) nitrogen concentrations. Significant differences are indicated by small letters (Tukey test, $p < 0.05$). Values for each strain are displayed as mean ± SD (n=3).

On the other hand, when the algae were grown under N- conditions, significant decreases by up to 70% of total AA were recorded in all 3 strains. Although in autotrophic conditions, this decreasing trend of protein content under N- depleted conditions was also reported in *S. obliquus*, with decreases of 20-23%, both in the wildtype and mutant [223]. Since N is one of the major components of protein, low nitrogen concentration cannot sufficiently support the biosynthesis of proteins, thus resulting in lower protein content [262–264]. Additionally, in spite of inhibiting the carotenoids pathway, pleiotropic effects have been reported when using DPA and other inhibitors after mutagenesis, namely increased PUFA content [124]. Thus, it is possible that the higher protein and AA contents achieved by strain DPA23 are related to the use of this inhibitor. Therefore, it would be interesting to study whether the genes involved in protein synthesis and amino acids biosynthetic pathways were mutated in this strain.

Furthermore, the AA profiles are similar to other *Scenedesmus* sp., except for the essential amino acid histidine, which presented 10-fold increased contents. However, under N- conditions, both the EAA content and the EAAI decreased significantly in all 3 strains. Moreover, the EAA contents and EAAI of *S. rubescens* WT, DPA23 and Ni16 compare favourably to those of other microalgae such as *Scenedesmus* sp., Spirulina and *Chlorella vulgaris* as well as soy protein, rendering them as promising novel sources of those AA.

Table 3.10 - Amino acid profile (g 100 g^{-1} DW) of *S. rubescens* WT, DPA23 and Ni16 grown under 70 mM (N+) and 1.75 mM (N-) nitrogen concentrations and an example from literature of *Scenedesmus* sp., E (Olsen *et al.* [261]). Values for each AA are given as mean ± SD (*n*=3). n.d. – not determined.

	WT		**DPA23**		**Ni16**		**E** [261]
	N+	N-	N+	N-	N+	N-	
Ala	2.69 ± 0.06^{b}	2.60 ± 0.04^{b}	4.76 ± 0.11^{a}	2.60 ± 0.08^{b}	1.95 ± 0.05^{c}	1.91 ± 0.05^{c}	4.80
Arg	3.11 ± 0.12^{c}	2.05 ± 0.03^{d}	9.76 ± 0.20^{a}	5.24 ± 0.09^{b}	2.01 ± 0.10^{d}	1.79 ± 0.05^{d}	3.10
Asx	2.41 ± 0.03^{b}	2.21 ± 0.07^{b,c}	3.30 ± 0.04^{a}	2.18 ± 0.06^{c}	2.22 ± 0.12^{b,c}	1.33 ± 0.08^{d}	5.20
Cys	0.52 ± 0.00^{a}	0.16 ± 0.01^{d}	0.18 ± 0.01^{c,d}	0.23 ± 0.01^{b}	0.19 ± 0.01^{c}	0.23 ± 0.01^{b}	0.40
Glx	4.27 ± 0.08^{c}	3.18 ± 0.01^{d}	7.07 ± 0.16^{a}	4.13 ± 0.14^{c}	4.20 ± 0.09^{c}	6.22 ± 0.21^{b}	6.10
Gly	1.95 ± 0.06^{b}	1.53 ± 0.02^{c}	2.84 ± 0.07^{a}	1.90 ± 0.05^{b}	1.38 ± 0.05^{d}	1.29 ± 0.04^{d}	3.30
His*	9.79 ± 0.12^{a}	4.83 ± 0.10^{c}	10.1 ± 0.46^{a}	8.86 ± 0.60^{b}	5.39 ± 0.17^{c}	3.94 ± 0.08^{d}	1.00
Ile*	3.07 ± 0.10^{a}	1.29 ± 0.02^{c}	1.78 ± 0.03^{b}	1.29 ± 0.05^{c}	0.90 ± 0.04^{d}	0.96 ± 0.02^{d}	2.10
Leu*	2.34 ± 0.04^{b}	1.95 ± 0.09^{d}	2.78 ± 0.07^{a}	0.28 ± 0.00^{e}	2.17 ± 0.07^{c}	0.19 ± 0.00^{e}	4.90
Lys*	3.03 ± 0.10^{b}	2.03 ± 0.08^{d}	3.47 ± 0.07^{a}	2.93 ± 0.13^{b,c}	2.78 ± 0.06^{c}	1.14 ± 0.03^{e}	2.90
Met*	0.52 ± 0.02^{b}	0.66 ± 0.01^{a}	0.36 ± 0.01^{c}	0.37 ± 0.01^{c}	0.62 ± 0.02^{a}	0.63 ± 0.02^{a}	1.30
Phe*	4.73 ± 0.02^{c}	3.57 ± 0.11^{d}	6.57 ± 0.02^{a}	5.89 ± 0.20^{b}	3.89 ± 0.18^{d}	3.64 ± 0.15^{d}	3.00
Pro	1.92 ± 0.05^{b}	1.87 ± 0.05^{b}	2.71 ± 0.09^{a}	1.91 ± 0.07^{b}	1.45 ± 0.05^{c}	1.42 ± 0.05^{c}	2.50
Ser	2.01 ± 0.04^{b}	1.50 ± 0.05^{c}	2.80 ± 0.05^{a}	1.60 ± 0.08^{c}	1.46 ± 0.02^{c,d}	1.35 ± 0.02^{d}	2.40
Thr*	2.56 ± 0.07^{b}	1.58 ± 0.03^{c}	3.25 ± 0.06^{a}	2.53 ± 0.07^{b}	1.74 ± 0.07^{c}	1.36 ± 0.05^{d}	2.90
Trp*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.20
Tyr	1.11 ± 0.02^{b}	0.84 ± 0.01^{c}	1.71 ± 0.03^{a}	1.10 ± 0.04^{b}	0.75 ± 0.03^{d}	0.62 ± 0.02^{e}	2.10
Val*	2.10 ± 0.05^{b}	1.57 ± 0.03^{d}	2.34 ± 0.07^{a}	1.93 ± 0.05^{c}	1.47 ± 0.03^{d}	1.49 ± 0.06^{d}	3.20
total	48.1 ± 0.9^{b}	33.4 ± 0.7^{d}	65.8 ± 1.4^{a}	45.0 ± 1.7^{c}	34.6 ± 1.1^{d}	29.5 ± 0.8^{e}	

Table 3.11 - Essential amino acids (g 100 g^{-1} protein) of *S. rubescens* WT, DPA23 and Ni16 under 70 mM (N+) and 1.75 mM (N-) nitrogen concentrations compared with other studies, soy protein and recommendations by the World Health Organization (WHO).

Strain	**WT**		**DPA23**		**Ni16**		***Scenedesmus* sp.**	***S. obliquus***	**Spir-ulina**	***C. vulgaris***	**Soy protein**	**WHO**
Media	N+	N-	N+	N-	N+	N-						
His	20.4	14.5	15.3	19.7	15.6	13.3	2.02	2.10	1.90	2.00	2.60	1.50
Ile	6.39	3.86	2.71	2.86	2.61	3.25	4.17	3.60	5.76	3.80	5.30	3.00
Leu	4.86	5.84	4.23	0.63	6.29	0.65	9.31	7.30	9.27	8.80	7.70	5.90
Lys	6.29	6.06	5.28	6.51	8.04	3.87	5.56	5.60	4.85	8.40	6.40	4.50
Met + Cys	2.17	2.44	0.82	1.33	2.35	2.90	2.64	2.10	3.38	3.60	1.30	2.20
Phe + Tyr	12.1	13.2	12.6	15.5	13.4	14.5	5.86	8.00	9.55	8.40	5.00	3.80
Thr	5.31	4.72	4.95	5.62	5.03	4.62	5.34	5.10	5.29	4.80	4.00	2.30
Trp	0.00	0.00	0.00	0.00	0.00	0.00	2.37	0.30			1.40	0.00
Val	4.37	4.71	3.55	4.29	4.25	5.06	6.06	6.00	6.39	5.50	5.30	3.90
EAA	58.5	52.3	46.6	53.5	54.9	45.3	42.4	36.3	39.5	38.0	39.0	
EAAI[a)]	2.01	1.88	1.44	1.38	1.88	1.36	1.56	1.43	1.67	1.63	1.61	
Ref.			This study				[261]	[243]	[124]	[243]		

a) EAAI values ≥1, 0.95–1, 0.86–0.95, 0.75–0.86, and ≤0.75 indicate superior quality, high quality, good quality, useful and inadequate, respectively.

3.3.3.5 Scale up trial in a 7-L fermenter

The growth performance of the orange strain Ni16 was validated by cultivation in a 7-L benchtop fermenter and compared to that of the WT (Figure 3.19). During 7 days of cultivation, strain Ni16 achieved a final biomass concentration, average productivity and growth rate of 66.0 ± 2.1 g L^{-1} and 8.89 ± 0.53 g L^{-1} d^{-1} and 0.54 ± 0.07 d^{-1}, respectively (Table 3.12), without significant differences compared to the WT.

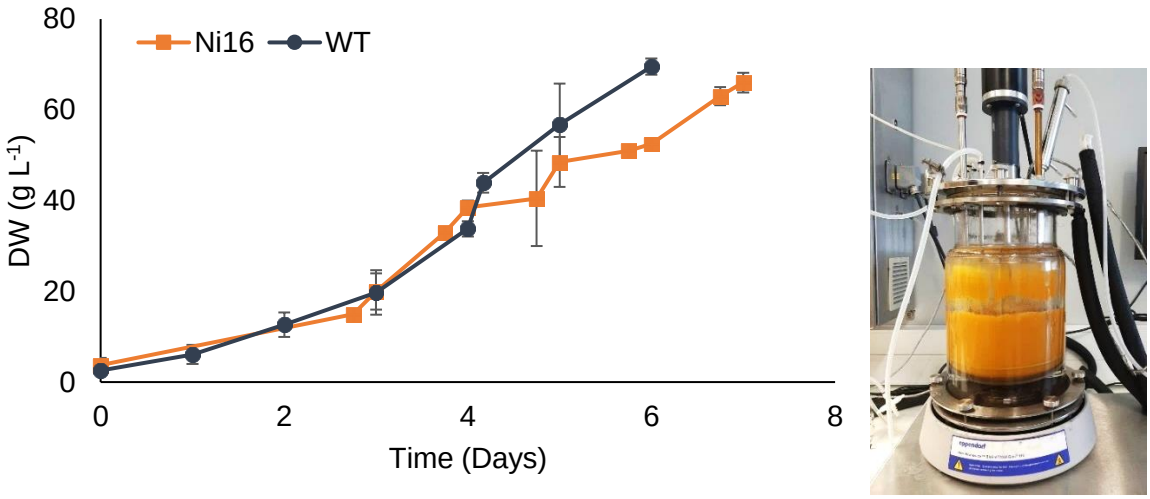


Figure 3.19 - Growth curves of *S. rubescens* WT *vs* the Ni16 mutant in a 7-L fermenter. Data points on each day are displayed as mean ± SD (*n*=3).

Table 3.12 - Biomass concentration (g L^{-1}), average productivity (g L^{-1} d^{-1}), and specific growth rate (d^{-1}) of *S. rubescens* WT and Ni16 in 7-L fermenter. Different letters indicate significant differences between strains, $p < 0.05$. Values are given as mean ± SD (*n* = 3).

Strain	Biomass concentration	Average productivity	Specific growth rate	Reference
WT	69.5 ± 1.8[a]	8.63 ± 0.22[a]	0.58 ± 0.03[a]	[191]
Ni16	66.0 ± 2.1[a]	8.89 ± 0.53[a]	0.54 ± 0.07[a]	This study

There are few reports in the literature regarding the heterotrophic growth of *Scenedesmus* in fermenters. The biomass concentration, productivity and specific growth rate achieved in the 7-L reactor are in accordance with the previously reported values for this WT strain [191]. The highest biomass concentration and productivity have been reported by Jin et al. [265] for the species *Scenedesmus acuminatus*, which reached 286 g L^{-1} and 91.44 g L^{-1} d^{-1}, respectively, in a 7.5-L fermenter. These authors suggest that feeding glucose at a steady but low concentration range (0-5 g L^{-1}) increases the efficiency of substrate-to-biomass conversion. On the other hand, Flórez-Miranda et al. [256] reached a lower biomass concentration and productivity of 18 g L^{-1} and 2.61 g L^{-1} d^{-1}, respectively. However, the same authors reported a higher growth rate (0.74 d^{-1}), when compared to the present study, in a 6-L batch bioreactor inoculated with *Scenedesmus incrassatulus* grown in a medium containing 30 g L^{-1} of glucose. Moreover, a second stage of photoinduction was carried out to induce lutein accumulation, which enabled them to reach 1.49 mg g^{-1} DW and 3.10 mg L^{-1} d^{-1} of lutein content and productivity, respectively [256]. In comparison, the profile reported in the present work for strain Ni16 displayed half of the lutein productivity, whereas the WT strain attained twice than that reported for *S. incrassatulus*.

3.3.4 Conclusions

Considering the values reported in the literature and the ones obtained in this experiment, it is clear that through the optimisation of culture medium, conditions and cultivation mode, *S. rubescens* has the potential to reach higher biomass and lutein productivities, both with the WT and Ni16. Although this species is not approved as a novel food in Europe yet, *Scenedesmus vacuolatus* was recently approved and *S. rubescens* is a great candidate to be considered in upcoming approvals. Nevertheless, few microalgal strains have been approved as novel foods in Europe (www.algae-novel-food.com) (EU, 2017/2470) and the application processes are lengthy and highly costly.

Overall, the two mutants isolated in this work, Ni16 and DPA23, are promising strains and interesting alternative protein sources for food and feed applications.

4

ISOLATION AND SELECTION OF PROTEIN-RICH MUTANTS OF *CHLORELLA VULGARIS* BY FLUORESCENCE-ACTIVATED CELL SORTING WITH ENHANCED BIOSTIMULANT ACTIVITY TO GERMINATE GARDEN CRESS SEEDS

This chapter was adapted from the following published research paper:
M. Trovão, L. Schüler, H. Pedroso, A. Reis, G. E. Santo, A. Barros, N. Correia, J. Ribeiro, G. Bombo, F. Gama, C. Viana, M. Costa, S. Ferreira, H. Cardoso, J. Varela, J. Silva, F. Freitas and H. Pereira, "Isolation and Selection of Protein-Rich Mutants of *Chlorella vulgaris* by Fluorescence-Activated Cell Sorting with Enhanced Biostimulant Activity to Germinate Garden Cress Seeds," *Plants*, vol. 13, no. 2441, 2024, doi: 10.3390/ plants13172441

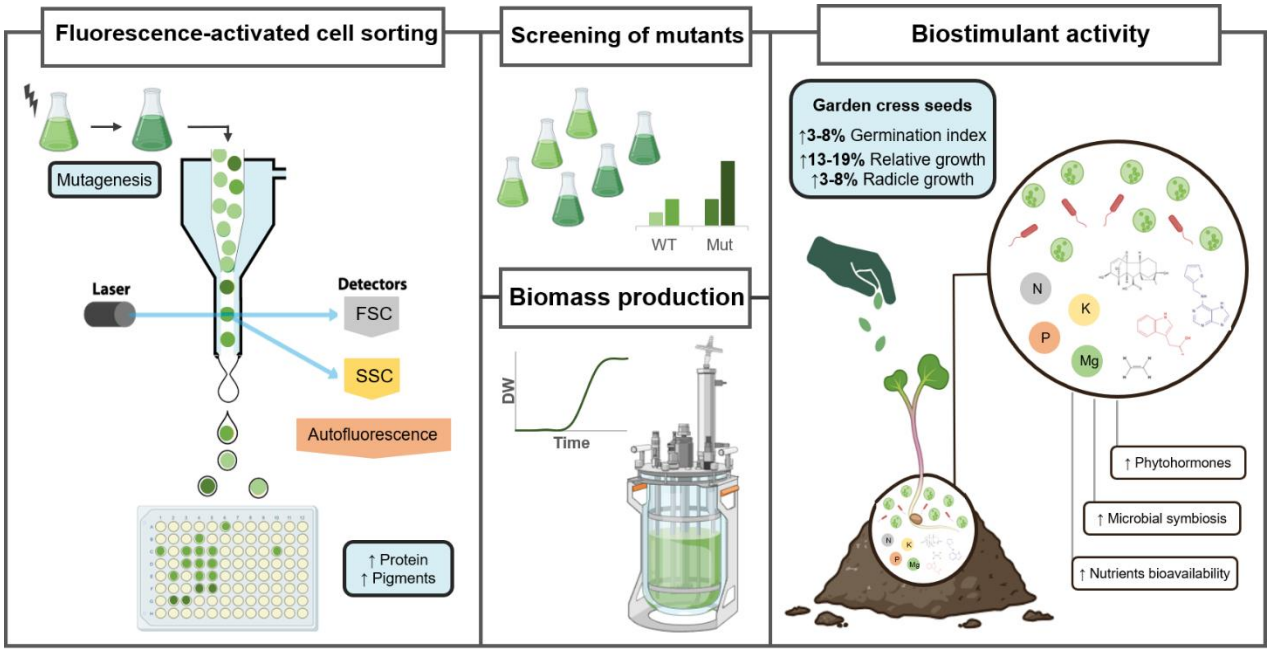
ABSTRACT

Microalgae are a promising feedstock with proven biostimulant activity that is enhanced by their biochemical components (e.g., amino acids and phytohormones), which turns them into an appealing feedstock to reduce the use of fertilisers in agriculture and improve crop productivity and resilience. Thus, this work aimed to isolate protein-rich microalgal mutants with increased biostimulant activity. Random mutagenesis was performed with *Chlorella vulgaris*, and a selection of protein-rich mutants were sorted through fluorescence-activated cell sorting (FACS), resulting in the isolation of 17 protein-rich mutant strains with protein contents 19–34% higher than that of the wildtype (WT). Furthermore, mutant F4 displayed a 38%, 22% and 62% higher biomass productivity, growth rate and chlorophyll content, respectively. This mutant was then scaled up to a 7 L benchtop reactor to produce biomass and evaluate the biostimulant potential of this novel strain towards garden cress seeds. Compared to

water (control), the germination index and the relative total growth increased by 7% and 19%, respectively, after the application of 0.1 g L−1 of this bioproduct, which highlights its biostimulant potential.

Keywords: biostimulants; fluorescence-activated cell sorting; microalgae; protein; random mutagenesis; selection method.

GRAPHICAL ABSTRACT



4.1 Introduction

One in three people struggle with moderate to severe food insecurity worldwide [266]. Nowadays, food and feed production do not meet global demands for these commodities, so more productive and resistant crops are required [267]. The abusive usage of chemical fertilisers, pharmaceuticals, antibiotics and pesticides, either in agriculture or in meat/fish production, contributes to a build-up of water and land contamination as well as eutrophication, pest resistance and increased incidence of human illnesses [268–271].

Plant biostimulants are an important resource for shifting traditional agricultural practices into more sustainable, safe, and productive processes. These compounds and/or extracts enhance plant growth by improving nutrient uptake, root development and crop resilience while reducing the reliance on chemical fertilisers and synthetic pesticides [267]. Microalgae are an under-exploited resource that are of interest either as a feedstock to develop food products and feed formulations or as a product to address the sustainability of existing processes, namely by bioremediation and by their potential as

biopesticides, biostimulants and immunity-boosters, both in agriculture and aquaculture [4, 179, 272, 273].

Microalgal biocompounds, such as amino acids, polysaccharides, phenolic compounds and minerals, and phytohormones, namely auxins, cytokinins, ethylene and gibberellins, have been reported as potent biostimulants and/or biopesticides to improve crop performance, quality and stress tolerance [274–278]. In addition, *C. vulgaris* has been reported as one of the dominating species with biostimulatory activity [275, 276, 279]. Particularly, protein- and amino acid-based biostimulant application has been reported to have many positive effects on several plant species, as reviewed by Sun et al. [280]. In addition, molecules with high antioxidant potential, such as chlorophyll and carotenoids, have protective effects that might contribute to plants' enhanced growth [281]. The anti-oxidative, anti-microbial and immunomodulatory activities of these compounds promote seed germination, alleviate the impact of environmental stress factors, such as high salinity, drought and contaminants, boost crops' productivity and might decrease the need to apply chemical fertilisers, namely by playing a role as osmolytes, aid in heavy metal detoxification, increase plants' absorption of soil micronutrients and improve the enzymatic antioxidant defence machinery of plant cells [280].

Notwithstanding, many microalgal wildtype (WT) strains often do not present the desired traits for industrial production, either in terms of growth performance, lack of robustness, colour or low target compound content [183]. For that reason, several approaches have been used lately to improve wildtype strains, namely random mutagenesis. Although a vast library of mutants might be obtained through this approach, it is very laborious and time-consuming to identify and select the phenotypes of interest. Up until now, few methods have been developed in this regard, namely the use of metabolic pathway inhibitors, visual appearance or autofluorescence of colonies and fluorescence-activated cell sorting (FACS) [183]. Moreover, the few high-throughput strategies available have been developed mostly for the selection of mutants with faster growth, higher lipid content and improved pigment content [16, 37, 55, 111, 117]. Thus, there is a scarcity of selection strategies for other target compounds of interest, such as proteins and amino acids.

In this context, a novel approach was attempted in this study. According to Malerba et al. [282], it is possible to establish a correlation between standard flow cytometric properties, such as side scatter (SSC), forward scatter (FSC) and red fluorescence, derived from the pigments (chlorophyll) of microalgal cells and cell nitrogen quota. Based on the model by these authors, it was hypothesised that cell nitrogen quota would also have a correlation with cell protein content. Therefore, cells of a larger size (as estimated by FSC), higher complexity (as estimated by SSC) and higher chlorophyll content (≈ higher red autofluorescence) were selected in this work using FACS [17].

Chlorella vulgaris is one of the few industrial species of microalgae with the ability to grow heterotrophically, which contributes to the fact that some of the highest biomass productivities reported for microalgae cultivation were achieved with this species [192, 283]. Moreover, this species accumulates high protein contents that can go from 20 to 64% of dry weight (DW) [218, 220].

In this work, a novel selection strategy that resorts to FACS was established to isolate protein-rich mutants of *C. vulgaris*. The mutants generated were characterised and compared between them

and with the wildtype. Based on the growth performance and protein and pigment content, the most promising mutant was selected to be scaled up in a 7-L benchtop fermenter for biomass production. Finally, the biostimulant activity of the produced biomass was compared to that of a commercial algae-based biostimulant and the phytohormone gibberellic acid (GA) in germination trials of garden cress seeds.

4.2 Materials and Methods

4.2.1 Microalgae Strain and Mutagenesis

Chlorella vulgaris wildtype strain 8 (8WT) was obtained from the Allmicroalgae Natural Products S.A. culture collection from cryopreserved aliquots stored in liquid nitrogen (−196 °C). The heterotrophic medium (HM) described by Barros et al. [192] was used to cultivate this strain, with 30 mM ammonium sulphate and 20 g L^{-1} glucose.

The dose-response curve of ethyl methanesulfonate (EMS) was established by exposing the 8WT strain to concentrations from 0 to 300 mM of EMS (1.90×10^8 cells mL^{-1}) according to the protocol described by Trovão et al. [249] and in section 3.1.2.2. In order to maximise the generation of strains with single mutations in their genomes, a concentration of 200 mM of EMS was selected for further rounds of mutagenesis, allowing close to a 10% survival rate obtained when different concentrations of EMS were assayed (Figure 3.9, subchapter 3.2). Upon mutagenesis, cells were recovered by resuspending in the respective diluted HM medium (1:2) and incubated overnight.

4.2.2 Isolation of Protein-Rich Strains by FACS

The fluorescence-activated cell sorting (FACS)-based screening procedure performed was based on the model reported by Malerba et al. [282] that correlates flow cytometric properties (red fluorescence, side scatter (SSC) and forward scatter (FSC)) with intracellular nitrogen quota. The mutagenized *C. vulgaris* cells were acquired in a Becton Dickinson FACS Aria II (BD Biosciences, Erembodegem, Belgium) equipped with a blue, violet and red laser and FACSDiva (version 6.1.3) software. The fluorescence signal of chlorophyll was obtained by applying a filter at 695/40 nm after excitation with the blue laser (488 nm). Cells were gated first for higher complexity (SSC) and then for those emitting higher levels of fluorescence and higher size (FSC), which were sorted onto 96-well microplates containing 250 µL of HM medium and incubated at 30 °C. From the wells that presented cell growth after 15 days, cultures were transferred to Petri dishes containing plate-count agar (PCA). Both the microplates and PCA plates were incubated in the dark at 30 °C.

4.2.3 Screening of FACS Mutants

4.2.3.1 Growth Performance

The mutants isolated on PCA plates were then transferred to 250-mL Erlenmeyer flasks with 50 mL of HM medium to compare their growth performance with that of the wildtype strain. *C. vulgaris* 8WT and FACS-selected mutants were cultivated at 30 °C in an orbital incubator (ArgoLab® shaker SKI 4, Capri, Italy) at 200 rpm. PIPES buffer at 50 mM was added to the medium to keep the pH at 6.5.

Growth assays, sampling and daily analysis were conducted as described in section 3.1.2.5. as well as biomass productivity and growth rate calculations.

The correlations established between OD_{600} and the dry weight (*DW*) of *C. vulgaris* 8WT (Equation (10); R^2 = 0.992) and the established mutant 8F4 (Equation (11); R^2 = 0.976) were the following:

$$OD_{600} = DW \div 0.4255 \quad (10)$$

$$OD_{600} = 2.6258 \times DW \quad (11)$$

Finally, samples were collected by centrifugation at 4500× *g* for 15 min (Hermle® Z300 centrifuge, Gosheim, Germany), and the biomass was frozen at −20 °C.

4.2.3.2 Biochemical Analysis of the Biomass

The frozen samples stored previously were lyophilised in a Coolvacuum, Lyomicron (Barcelona, Spain), and stored in a desiccator for the quantification of protein and pigments' contents at a later step. These analyses were carried out as described in sections 3.1.2.6.1. and 3.1.2.6.2., respectively.

4.2.4 Biomass Growth and Protein Production (7 L Fermenter)

The selected mutant 8F4, cultivated initially in a 250-mL Erlenmeyer, was scaled-up to a 1-L Erlenmeyer flask and then to a 7-L benchtop fermenter (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany) with an initial working volume of 3 L. This strain was also cultivated with HM medium at 30 °C. In addition, glucose (500 g L^{-1}) was supplied in fed-batch mode to ensure a concentration range between 0.1 and 20 g L^{-1}, and ammonia (24%) was used to keep a pH of 6.5. Non-limiting dissolved oxygen was guaranteed by keeping the airflow around 1 vvm and by increasing the agitation rate from 200 to 1200 rpm throughout growth.

Daily samples were collected aseptically to analyse the OD_{600} and/or DW, to measure the offline pH, to observe the culture in the microscopy and to store biomass, after centrifugation for 3 min at 2547× *g* (Hermle® Z 300 centrifuge, Wehingen, Germany), to quantify protein content posteriorly.

4.2.5 Biostimulant Activity (*In Vitro* Assays)

The seed germination bioassay was used to determine the biostimulant activity of the F4 strain according to Zucconi et al. [284]. Garden cress (*Lepidium sativum* L., World of Flowers Sp., Poland) seeds were used as a model species.

Water suspensions of the dried biomass were prepared at 9 concentrations, namely, 0.01; 0.05; 0.1; 0.25; 0.5; 0.75; 1.0; 1.5 and 2.0 g L^{-1}. Sterile deionised water was used as the negative control. Two positive controls were used, gibberellic acid (GA) at 0.00087 g L^{-1} and a commercial algae-based biostimulant product (Algaman B, Hubel Verde, Portugal) at 2.0 g L^{-1}.

For each treatment, 5 replicates of 10 seeds were placed in Petri dishes with 2 Whatman No 1 filter papers, and 11 mL of either treatment was added. The seeds were placed in a growth chamber under controlled conditions of temperature (20 ℃) and ventilation (40%) in the dark and were left to germinate for 3 days. Afterwards, the length of the radicle and the young stem were measured for each seedling using a digital calliper (iGaging® CoolantCal IP67, USA).

Finally, the germination index (GI), relative growth of the radicle (RGR) and relative total growth (RTG) were calculated according to the following equations:

$$\mathrm{GI}\ (\%) = \frac{\#\ \text{germinated seeds (sample)} \times \text{radicle lenght (sample)}}{\text{Mean}\ \#\ \text{germinated seeds (negative control)} \times \text{Mean radicle lenght (negative control)}} \times\ 100 \quad (12)$$

$$\mathrm{RGR}\ (\%) = \frac{\text{radicle lenth (sample)}}{\text{Mean radicle lenth (neagtive control)}} \times\ 100 \quad (13)$$

$$\mathrm{RTG}\ (\%) = \frac{\text{Total lenght seedling (sample)}}{\text{Mean total lenght seedling (negatiove control)}} \times\ 100 \quad (14)$$

4.2.6 Statistical Analysis

All the experiments were carried out in biological triplicates, except the in vitro biostimulant activity assays, which were performed with five replicates. The results, expressed by mean ± standard deviation, were analysed by one-way Analysis of Variance (ANOVA), followed by Tukey's HSD post hoc test, with a confidence interval of 95% (XLStat software, v2401.16.0, Microsoft® Excel®).

4.3 Results and Discussion

4.3.1 Mutagenesis and Isolation of Protein-Rich Mutants by FACS

The mutagenized cells of *C. vulgaris* were acquired in the cytometer after the recovery period. To select mutants with higher protein and chlorophyll contents, two gates were set, P1 and P2 (Figure 4.1).

The first gate, P1, was set to a higher side scatter (SSC) signal, while the second gate, P2, was set to a higher forward scatter (FSC) and a higher chlorophyll autofluorescence (PerCP-Cy5-5-A) signal. This fluorescence-activated cell sorting (FACS) procedure allowed the isolation of 17 mutants of *C. vulgaris* with increased cell complexity, larger cell size/volume and higher chlorophyll autofluorescence.

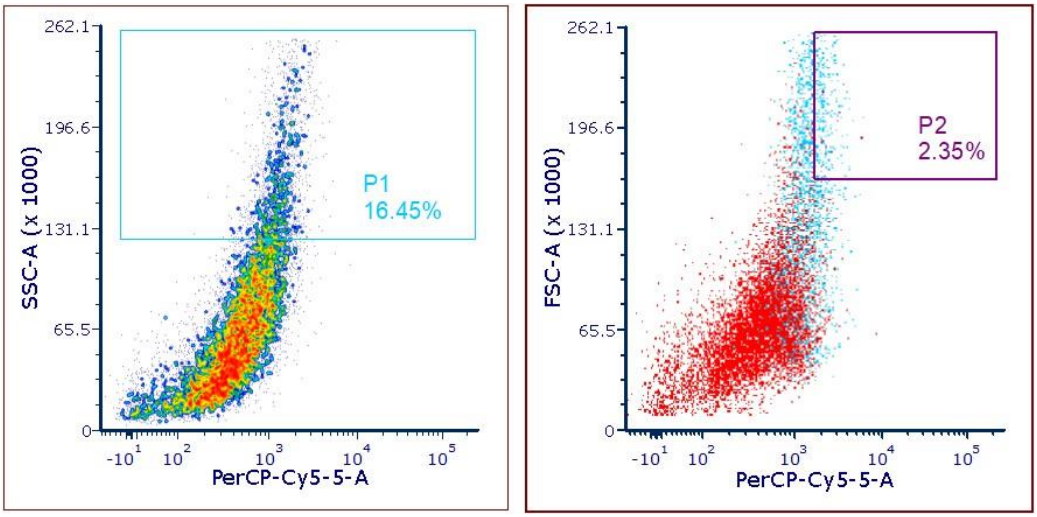


Figure 4.1 - Fluorescence-activated cell sorting (FACS) procedure to isolate protein- and chlorophyll-rich mutants of *C. vulgaris*. The first gate, P1, was applied by combining the inner cell complexity (side scatter—SSC) and chlorophyll autofluorescence (PerCP-Cy5-5-A) and the other gate, P2, was applied by combining the cell size/volume (forward scatter—FSC) with chlorophyll autofluorescence (PerCP-Cy5-5-A).

The optical properties detected by flow cytometers have been extensively correlated with cell features across different microalgae and cyanobacteria species [16]. Although it is an oversimplification, the forward scatter (FSC) detector is often considered proportional to cell size/volume. In contrast, side scatter (SSC) is considered a good proxy for cell internal complexity/granularity. The red (auto)fluorescence signal is proportionally correlated to the total pigment concentration of the cells, particularly chlorophyll [16, 145, 282]. In addition, several strategies that resort to fluorescent dyes have been used to identify and isolate species with higher contents of high-value compounds, such as lipids and carotenoids [16, 183]. However, no strategy has been developed to distinguish and measure the protein content of living cells. Usually, the protein content of microalgae is quantified by indirect or direct methods, either by quantifying the nitrogen content, for example by elemental analysis [193], or by digestion protocols and colorimetric reactions such as Kjeldahl's [285], Lowry's [286] and Bradford's [287] methods, which are often inaccurate and time-consuming [282]. Unlike lipids, which can be stained, for example, with the solvatochromic dye BODIPY [36], there is no standard procedure to stain proteins without compromising the cell viability of the microalgae and disrupting cell membranes.

Nonetheless, Malerba et al. [282] reported a method that correlates the optical properties of flow cytometry with cell nitrogen quota to monitor phytoplankton populations, since nitrogen limitation is known to affect the physiological and morphological aspects of cells. These authors established a model with high accuracy (R^2 = 0.9; Prob (F) < 0.0001) whose most important variable was red fluorescence,

which explained 77% of the variability of the total cell nitrogen, which increased to 87% when combined with SSC and went up to 90% when also combined with FSC. This method allowed them to establish a quantifiable proxy for cell nitrogen quota in a reliable and non-destructive manner across four species (*Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus obliquus* and *Tetraëdron* sp.). Based on this model and this correlation with optical properties, it was hypothesised that the mutants isolated with a higher cell nitrogen quota (Figure 4.1) would have a higher protein content.

4.3.2 Screening of Mutants

4.3.2.1 Growth Performance and Protein Content

The 17 mutants isolated by FACS were named following the respective well position (in the 96-well plate) to which they were sorted. The growth performance of these mutants was compared with that of the wildtype (WT). Their respective growth parameters, biomass productivity and growth rate, as well as their protein content, are shown in Table 4.1.

Table 4.1 - Biomass productivity (r_p) in g L^{-1} d^{-1}, growth rate (μ) in d^{-1}, protein content in % of dry weight (DW) and protein productivity (PP) in g L^{-1} d^{-1} of the wildtype (WT) and the 17 mutants of *C. vulgaris*, isolated by FACS. Results are shown as mean ± SD, $n = 3$. Different letters indicate significant differences ($p < 0.05$) between strains. The most promising mutants and the WT are highlighted in green.

Strain	r_p (g L^{-1} d^{-1})	μ (d^{-1})	Protein (% DW)	PP (g L^{-1} d^{-1})
WT	1.51 ± 0.05 ^{b}	1.05 ± 0.02 ^{d}	32.1 ± 1.1 ^{c}	0.56 ± 0.05 ^{b}
A6	1.54 ± 0.32 ^{b}	1.05 ± 0.06 ^{d}	31.8 ± 0.8 ^{c,d}	0.51 ± 0.03 ^{b,c,d}
B4	1.26 ± 0.02 ^{b,e}	0.99 ± 0.01 ^{c,d}	29.8 ± 1.1 ^{c,d}	0.46 ± 0.02 ^{b,c,d}
C1	1.39 ± 0.02 ^{b,c}	1.05 ± 0.01 ^{c}	29.9 ± 0.8 ^{c,d}	0.47 ± 0.06 ^{b,c,d}
C3	1.54 ± 0.05 ^{b}	1.07 ± 0.02 ^{c}	29.2 ± 0.7 ^{c,d}	0.53 ± 0.02 ^{b,d}
C4	1.52 ± 0.10 ^{b}	1.04 ± 0.02 ^{c}	31.0 ± 1.4 ^{c,d}	0.68 ± 0.12 ^{a,b,d}
C5	1.88 ± 0.13 ^{a,b}	1.12 ± 0.02 ^{b,c}	38.2 ± 0.4 ^{b}	0.66 ± 0.01 ^{a,b,d}
C10	1.49 ± 0.13 ^{b}	1.07 ± 0.01 ^{c}	30.8 ± 0.9 ^{c,d}	0.50 ± 0.02 ^{b,c,d}
D3	1.40 ± 0.03 ^{b,d}	1.05 ± 0.01 ^{c}	33.3 ± 0.4 ^{c}	0.50 ± 0.01 ^{b,c,d}
D4	1.35 ± 0.10 ^{b,d}	1.05 ± 0.01 ^{c}	30.3 ± 0.4 ^{c,d}	0.46 ± 0.04 ^{b,c,d}
D5	1.36 ± 0.08 ^{b,d}	1.05 ± 0.02 ^{c}	28.0 ± 0.7 ^{c,d}	0.44 ± 0.02 ^{b,c,d}
E2	0.84 ± 0.04 ^{e}	0.95 ± 0.01 ^{d}	39.1 ± 0.2 ^{b}	0.43 ± 0.03 ^{b,c,d}
E4	1.24 ± 0.15 ^{b,d}	1.02 ± 0.04 ^{c,d}	27.9 ± 1.5 ^{c,d}	0.39 ± 0.01 ^{d}
E5	1.22 ± 0.04 ^{b,d}	1.02 ± 0.01 ^{c,d}	30.3 ± 0.7 ^{c,d}	0.43 ± 0.02 ^{b,c,d}
F4	2.08 ± 0.17 ^{a}	1.28 ± 0.05 ^{a}	38.1 ± 0.5 ^{b}	0.64 ± 0.03 ^{a,b}
F5	1.88 ± 0.08 ^{a,b}	1.26 ± 0.03 ^{a}	38.2 ± 1.1 ^{b}	0.63 ± 0.02 ^{a,b}
G2	1.67 ± 0.18 ^{b}	1.23 ± 0.01 ^{a}	43.0 ± 1.7 ^{a}	0.60 ± 0.03 ^{a,b}
G3	1.55 ± 0.10 ^{b}	1.05 ± 0.01 ^{c}	35.6 ± 1.7 ^{b,c}	0.60 ± 0.05 ^{a,b}

From the 17 mutants, only mutant E2 displayed a significantly lower biomass productivity and growth rate (0.84 ± 0.04 g L^{-1} d^{-1} and 0.95 ± 0.01 d^{-1}) when compared to the WT (1.51 ± 0.05 g L^{-1} d^{-1} and 1.05 ± 0.02 d^{-1}). On the other hand, three mutants, F4, F5 and G2, exhibited higher growth rates (1.23–1.28 d^{-1}) than the WT, but only mutant F4 presented significantly higher biomass productivity (2.08 ± 0.17 g L^{-1} d^{-1}), which corresponded to a 38% increase. All mutants displayed protein contents that averaged 31% of their dry weight (DW), which was similar to that of the WT (32.14 ± 1.07% DW). However, the mutants C5, E2, F4, F5 and G2 displayed significantly higher protein contents (38.05–42.98% DW), which represented an improvement of 19–34% as compared to the WT. Regarding protein productivity, only mutant E4 (0.39 ± 0.01 g L^{-1} d^{-1}) exhibited a significantly lower value compared to that of the WT (0.56 ± 0.05 g L^{-1} d^{-1}). The highest values were achieved by mutants C4, C5, F4, F5, G2 and G3, between 0.60 and 0.68 g L^{-1} d^{-1}. The growth curves of the mutants mentioned above are shown in Figure 4.2.

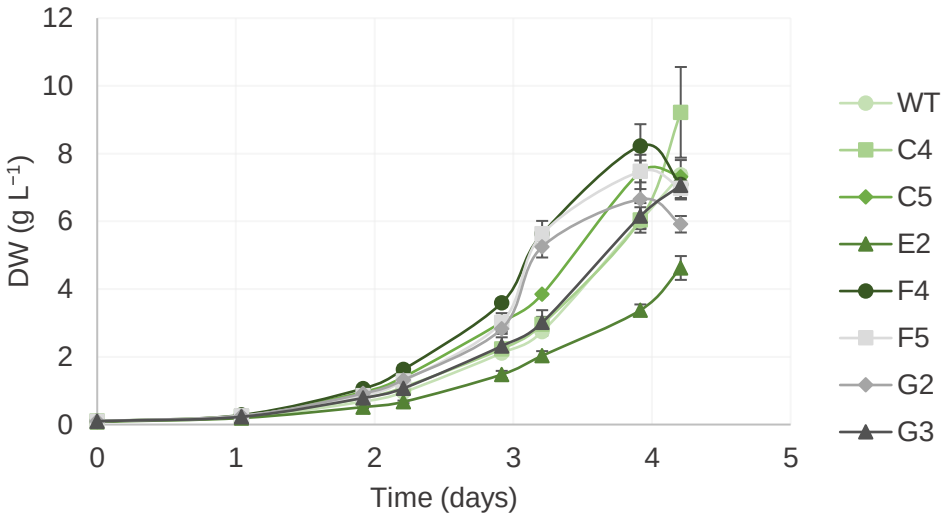


Figure 4.2 - Growth curve of *C. vulgaris* WT and mutants C4, C5, E2, F4, F5, G2 and G3 in 250-mL Erlenmeyers in heterotrophic conditions. Data points on each day are displayed as mean ± SD (*n* = 3).

At this scale, most reports found in the literature for *C. vulgaris* cultivated in heterotrophic conditions presented lower growth rates, between 0.55 and 0.79 day^{-1}, compared to all the strains screened here, while biomass productivities fell within a similar range, 1.65–1.99 g L^{-1} d^{-1} [217–220]. However, upon random mutagenesis, both impaired and improved growth performances of the generated mutants have been reported, depending on the improvement target, mutagen and selection method used. For example, Schüler et al. [19] developed a yellow mutant of *C. vulgaris* that displayed a growth performance equivalent to the WT and a white mutant that grew slower than the WT. The yellow and white strains showed a 30 and 60% increase in protein content, respectively (39.5 and 48.8% of dry weight (DW), respectively), as compared to the WT, even though that was not the primary target of the mutagenesis. Conversely, aiming for different improvement targets, several reports of improvements in the growth performance of several microalgal species after mutagenesis have been published [249]. Kim et al. [200] reported a 1.3-fold improvement in the growth rate of *C. vulgaris* after combining mutagenesis

with ethyl methanesulfonate (EMS) with FACS-based selection in order to improve carotenoid content. Improved growth performance and the biodegradative potential of petroleum was also reported by Eregie et al. [236] by applying UV-radiation to mutagenize *Scenedesmus vacuolatus*, which also led to a 2-fold increase in chlorophyll content, a 1.2-fold increase in carotenoids and 1.4-fold increase in the protein content. In addition, Liu et al. [288] generated an *Auxenochlorella pyrenoidosa* mutant by atmospheric room temperature plasma mutagenesis with a 31% increase in protein content (44.22% DW), with no detectable chlorophyll *b* and a 118-fold decrease in chlorophyll *a* content, comparing to the WT, without significant differences regarding growth performance.

4.3.2.2 Chlorophyll and Carotenoid Profiles

The pigment profiles of the WT and the mutants that displayed improved growth performance and protein content, F4 and G2, were analysed (Table 4.2).

Table 4.2 - *Chlorella vulgaris* wildtype's (WT) and F4 and G2 mutant strains' chlorophyll content (mg g^{-1} of DW) as determined by Ritchie's method and carotenoid concentrations (mg g^{-1} of DW) as determined by HPLC. n.d.—not detected; <LOQ—below limit of quantification. Results are shown as mean ± SD, n = 3. Different letters indicate significant differences (p < 0.05) between strains.

	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Neoxanthin	Violaxanthin	Lutein	*β*-Carotene
WT	0.30 ± 0.03 [a]	0.18 ± 0.02 [a]	0.48 ± 0.05 [a]	0.36 ± 0.01 [a]	0.17 ± 0.01	0.53 ± 0.01 [a]	0.74 ± 0.02 [a]
F4	0.54 ± 0.01 [b]	0.24 ± 0.01 [c]	0.78 ± 0.01 [c]	0.20 ± 0.01 [b]	<LOQ	0.59 ± 0.04 [a]	0.37 ± 0.05 [b]
G2	0.48 ± 0.03 [b]	0.21 ± 0.01 [e]	0.69 ± 0.04 [c]	0.22 ± 0.03 [b]	n.d.	0.82 ± 0.08 [b]	0.67 ± 0.07 [a]

Regarding the pigments' profile, the WT strain presented lower total chlorophyll (0.48 ± 0.05 mg g^{-1} of DW) and lutein (0.53 ± 0.01 mg g^{-1} of DW) contents when compared to the mutant strains F4 and G2. Mutant strain F4 displayed the highest chlorophyll content (0.78 ± 0.01 mg g^{-1} of DW), a 62% increase compared to the WT. Additionally, mutant G2 exhibited a 55% increase in lutein content (0.82 ± 0.08 mg g^{-1} of DW), as compared to the WT.

The chlorophyll contents obtained in this study (Table 4.2) were significantly lower than other values reported for this species under heterotrophic conditions [19, 192, 249], which might be related to the strain used and/or the efficiency of the extraction. In resemblance to growth performance, chlorophyll and carotenoid enhancements and decays have both been reported upon mutagenesis, also depending on the objective [118, 124, 138, 198, 199, 235]. Chlorophyll-deficient mutants in some reports displayed improved growth performance, and were impaired in others [139, 198, 249]. Regarding chlorophyll increments, Nakanishi and Deuchi [113] presented a three-fold increase in chlorophyll content, along with increased halotolerance, upon the UV-mutagenesis of *C. vulgaris* and selection by colour of the colonies generated. By resorting to UV-mutagenesis, Vigeolas et al. [289] also isolated a mutant of *Tetradesmus obliquus* with a 2.2-fold higher chlorophyll and protein content, but with an impaired doubling rate, upon Nile red fluorescence-based screening for cells with higher lipidic contents. In addition, Xi et al. [223]

also generated mutants of *T. obliquus* by $^{12}C^{6+}$ Ion Beam mutagenesis and selected a strain with 33% and 48% higher chlorophyll *a* and carotenoids contents, respectively, through chlorophyll fluorescence, even though improving photosynthetic efficiency and lipid content were the original goals. Furthermore, random mutagenesis can alter the carotenoids profile, as Kim et al. [200] demonstrated by generating a mutant of *C. vulgaris* to accumulate violaxanthin.

4.3.3 Biomass Growth and Protein Production

Mutant strain F4 exhibited the best growing performance compared to the other mutants, as well as the most interesting pigment profile, along with a 19% improvement in protein content. Thus, F4 was selected for scale-up in a 7-L benchtop reactor to generate enough biomass to assay its biostimulant potential (Figure 4.3).

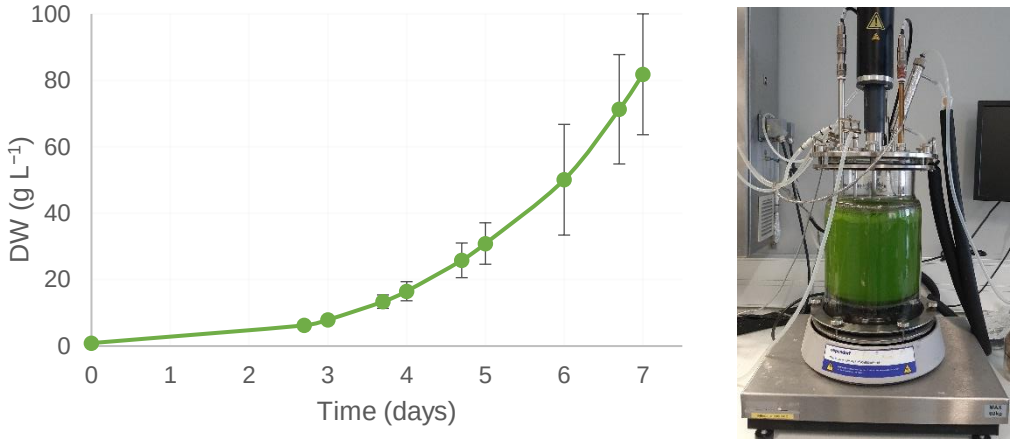


Figure 4.3 - Growth curve of *C. vulgaris* mutant F4 in a 7-L reactor in heterotrophic conditions throughout 7 days. Data points on each day are displayed as mean ± SD (*n* = 3).

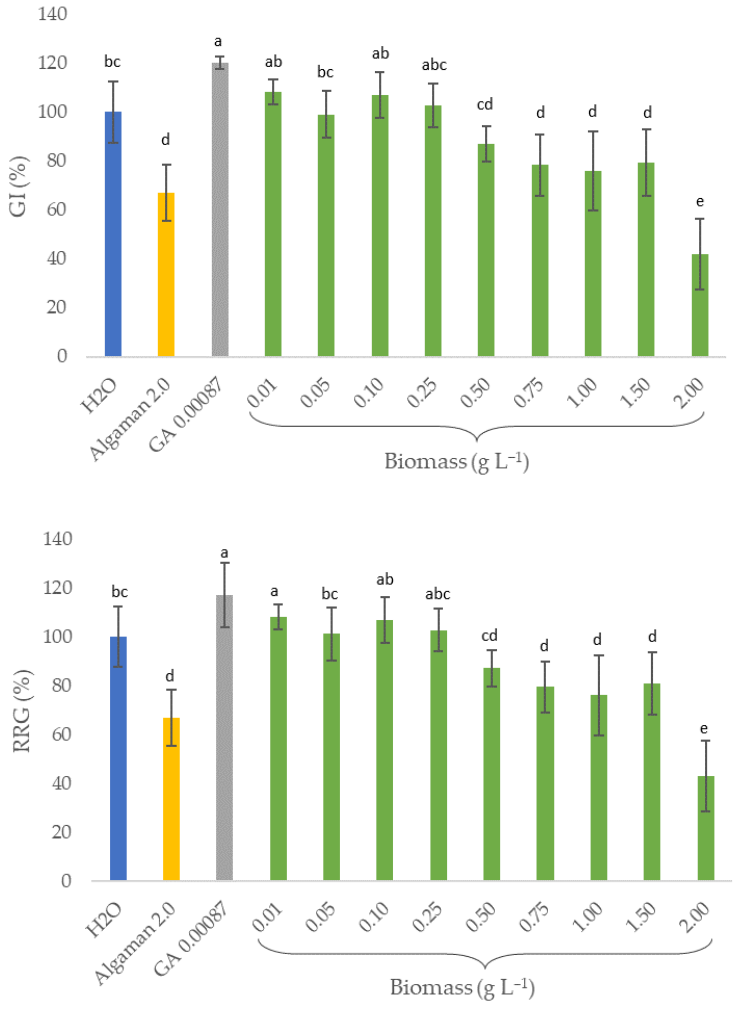
Mutant F4 reached 81.9 ± 18.2 g L^{-1} of DW in 7 days in the 7-L reactor, with a biomass productivity of 13.9 ± 3.6 g L^{-1} d^{-1}, and a specific growth rate of 0.66 ± 0.13 d^{-1} (Figure 4.3). The final protein content attained was 33.1 ± 1.2% of DW, which comes with a protein productivity of 3.9 ± 0.7 g L^{-1} d^{-1} (Figure 4.3). In the first 2 days of cultivation, a lag phase was observed, a period that could potentially be shortened through process optimisation, along with an increase in growth rate and protein content, similar to what was achieved in the laboratory screening trials.

The growth performance of the F4 strain in the 7-L reactor (Figure 4.3) achieved a biomass productivity higher than most values that have been reported for this species, between 1.7 and 3.2 g L^{-1} d^{-1}, [19, 217–220]. Still, it falls short of the value reported by Barros et al. [192], namely 27.3 g L^{-1} d^{-1}. In addition, the protein content achieved was lower than the value obtained in the screening assay, 38.1% DW (Table 4.1). In the literature, lower and higher protein content values are found, ranging between 20 and 64% of DW [218, 220], as reviewed recently by Trovão et al. [249]. However, the F4 strain's protein productivity is higher than most values reported due to the high biomass productivity

obtained. It is also noteworthy that both growth performance and protein productivity still have a great margin of improvement, which would require the optimisation of the culture medium, abiotic factors, cultivation and feeding mode, as well as eventually performing a two-stage process to enhance protein production [227, 290]. Furthermore, higher productivities, both of biomass and target compounds, as well as growth rates, might be achieved upon the scaling-up of the process, as it has been pointed out for *C. vulgaris* and other species, such as *S. rubescens* [191, 192].

4.3.4 Biostimulant Activity (In Vitro Assays)

The whole biomass of mutant F4 obtained in the 7-L fermenter was applied to garden cress seeds, whose germination index (GI), relative radicle growth (RRG) and relative total growth (RTG) were compared to those of water, a commercial algae-based biostimulant (Algaman) and gibberellic acid (GA), as shown in Figure 4.4.



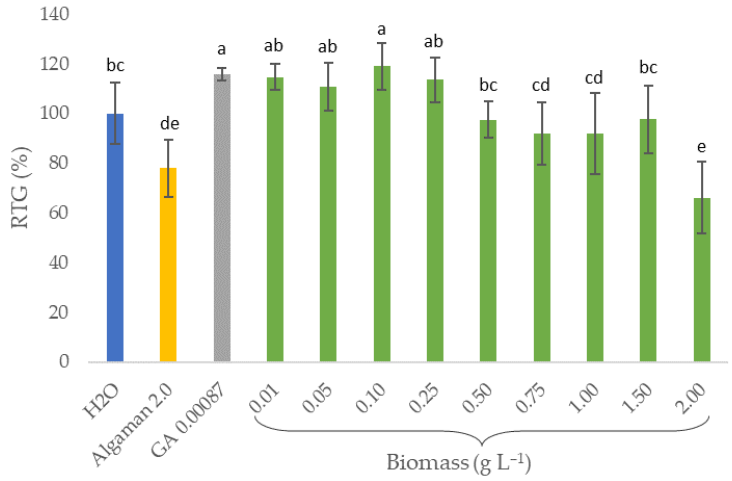


Figure 4.4 - Germination index (GI, %); relative radicle growth (RRG, %); and total relative growth (RTG, %) for the *C. vulgaris* F4 strain at different concentrations: 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 g L^{-1}. H_2O, sterile distilled water, was used as the negative control. Algaman and gibberellic acid (GA) were used as positive controls at the concentrations of 2.0 and 0.00087 g L^{-1}, respectively. Bars represent the mean value ± SD, n = 5. Different letters indicate significant differences (p < 0.05) between treatments.

Regarding the positive controls, GA increased the germination by 20%. However, Algaman led to a 30% decrease in the GI. A similar behaviour was obtained for RRG and RTG. Treatments with 0.01, 0.1 and 0.25 g L^{-1} of the F4 strain biomass enabled the same response as the GA for the three parameters measured, with an improvement in RRG between 3 and 8%, RTG between 13 and 19% and GI between 3 and 8%, as compared to those of water. Concentrations equal to or higher than 0.5 g L^{-1} of microalgal biomass significantly impaired the three parameters under study concerning the biostimulant activity on garden cress seeds.

Regarding the biostimulant activity assays (Figure 4.4), similar results were reported by Morillas-España et al. [291], which attained an increment of 3.5% of the GI of watercress seeds also when using 0.1 g L^{-1} of *C. vulgaris*, highlighting the biostimulant capacity of this species. However, these authors applied this microalgal extract after cell wall disruption by sonication, while in the present study, non-disrupted biomass was applied directly instead. In addition, these authors also reported the promotion of root formation in soybean seeds, a cytokinin-like effect in a cucumber expansion test and the formation of chlorophyll in wheat leaves after treatments with *C. vulgaris* extract. On the other hand, Gitau et al. [292] treated a *Medicago truncatula* model plant with live algae cells of *Chlorella*, which led to larger leaves, more flowers/pods, increased fresh biomass and more robust plants compared to the control. Alling et al. [293] tested the biostimulating effects of both the algal biomass (intact vs. disrupted cells) and supernatant (after cultivation) of *C. vulgaris* on tomato and barley seeds. Intact cells and their supernatant enabled up to a 25% higher germination percentage, higher GI and earlier germination by 0.5–1 day when evaluated against seeds treated with *S. obliquus* and the negative control (water). Martini et al. [294] also reported improved development of maize roots when plants were treated with *C. sorokiniana*, compared to the untreated negative control, under stress conditions, such as nitrogen depletion. In addition, these authors suggested that the absence of pretreatment of the biomass enables the establishment of a more sustainable process, since the physical treatment of cells that they

performed (partial disruption with glass beads before freeze-drying) had a limited effect on their biostimulant properties compared to the untreated freeze-dried biomass. Finally, Gharib et al. [295] recently reported the impact of microalgal extracts (obtained through methanol extraction, solvent evaporation and resuspension in water) of several species, including *C. vulgaris*, as biostimulants on common bean plant growth, yield and antioxidant capacity. The most promising results were obtained with extract concentrations between 0.5 and 1.0%, which improved root and shoot length, number and area of leaves, weight per plant, seed index and yield per plant, as well as reduced content of oxidative stress markers, among other positive effects. Besides the biostimulant potential of aqueous suspensions of *C. vulgaris*, other effects have been reported recently, namely as a biocontrol agent/biopesticide against *Fusarium oxysporum* to protect spinach [296], while the biopesticide properties of *C. sorokiniana* have also been reported against the strawberry pathogen *Phytophthora cactorum* [297].

Although the beneficial effect of microalgal biomass as a biostimulant and bioprotective agent has been reported by several authors, as reviewed by Mrid et al. [298], it would be interesting to study further which compounds provide these effects and the underlying mechanisms, namely by identifying and quantifying phenolic compounds, phytohormones, amino acids and polysaccharides, for example. While some of the mechanisms of these compounds' biostimulant activity has been reported, others have not been investigated comprehensively. For example, pigments, such as carotenoids, are precursors of known phytohormones, such as strigolactones and abscisic acid [299, 300]. It would be worth studying the effect of these molecules on plants' growth and resistance to stress.

Finally, most of these bioactive microalgal compounds are intracellular, and *Chlorella* spp. and other species are known for having a recalcitrant cell wall. Although there are already a few studies comparing the usage of intact vs. disrupted cells or even supernatant, as discussed above, it would be important to further study the effect of different biomass treatments on the composition of microalgal extracts for this application. Such treatments could include different disruption methodologies, namely high-pressure homogenisation, pulsed electric fields and enzymatic and acid or alkali hydrolysis [301, 302]. In addition, the conditions applied in these processes must be optimised to avoid compromising the bioactivity of the target compounds.

4.4 Conclusions

This is the first study that described the usage of a high-throughput technology, fluorescence-activated cell sorting, to develop a pipeline to generate and select protein-rich mutants. The unavailability of appropriate and effective selection methodologies to isolate mutants with the desired target phenotypes is one of the most significant limitations of random mutagenesis. There are many strategies reported regarding the improvement in pigments, lipidic and carbohydrate contents of both wildtype and mutant microalgal strains for several biotechnological applications, but no work has been developed concerning protein content, which is one of the components of microalgal biomass with the greatest potential for agricultural and cosmetic applications. This work not only presents a novel selection

strategy to target a combination of high protein and pigments contents, but also unveils the potential of the isolated mutant of *Chlorella vulgaris* F4 for an interesting application as a plant biostimulant. This mutant exhibited a 38% higher biomass productivity, 62% higher chlorophyll content and 19% higher protein content when compared to the wildtype (WT).

The biomass obtained from the scale-up of this strain enhanced the germination index and the relative total growth of garden cress seeds by 7% and 19%, respectively, when 0.1 g L^{-1} was applied, which highlight its biostimulant potential. As for future perspectives, it would be interesting to further analyse this biomass to understand which components provide this effect, such as the amino acid profile and the identification and quantification of phytohormones and their precursors. Additionally, downstream treatments of the biomass produced should also be investigated to further enhance the biostimulant capacity, namely by disrupting cells and releasing bioactive compounds. Finally, this scale-up process should be further optimised to achieve higher biomass and protein productivities, namely by optimising abiotic conditions, culture medium and cultivation and feeding strategies.

5

IMPROVING THE HETEROTROPHIC MEDIA OF THREE *CHLORELLA VULGARIS* MUTANTS TOWARD OPTIMAL COLOUR, BIOMASS AND PROTEIN PRODUCTIVITY

This chapter was adapted from the following submitted research paper (to the journal Bioresource Technology):
M. Trovão, M. Cunha, G. Espírito Santo, H. Pedroso, A. Reis, A. Barros, N. Correia, L. M. Schüler, M. Costa, S. Ferreira, H. Cardoso, M. Ventura, J. Varela, J. Silva, F. Freitas and H. Pereira, "Improving the heterotrophic media of three *Chlorella vulgaris* mutants toward optimal colour, biomass and protein productivity," *Bioresour. Technol.*, 2024

ABSTRACT

The high production costs and unappealing sensory properties still limit the widespread commercialisation of microalgae feedstocks. Therefore, this work focused on fine-tuning the heterotrophic medium composition to cultivate novel green, yellow, and white *Chlorella vulgaris* mutant strains. Screening assays were carried out to select the most significant factors, and different nutrient concentrations were optimised by modelling biomass and protein productivity, specific growth rate, and colour, via response surface methodology. The biomass and protein productivities achieved by these strains were improved by up to 70% and 94%, respectively. Additionally, biomass colour was correlated with medium composition for the first time, allowing the improvement of the yellow and white mutant colorations by 20%. Overall, the findings of this study are vital to overcoming the challenges of the biobased industry, allowing the enhancement of the cost-effectiveness, attractiveness, and nutritional profiles of microalgae-based products in different markets and applications.

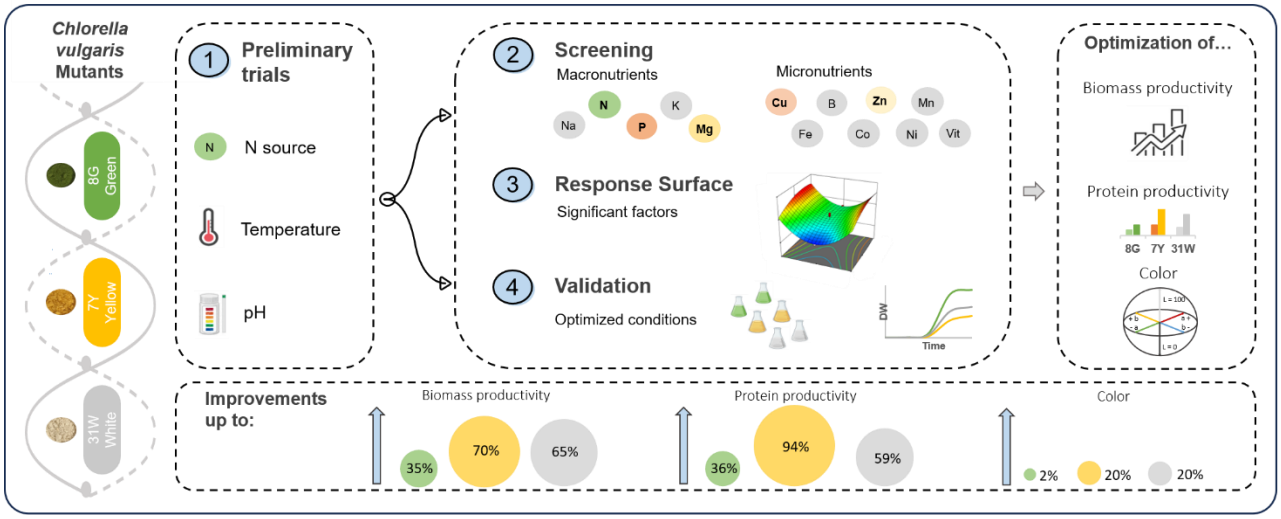
Keywords: Biomass colour; Biomass and protein productivity; Culture medium optimisation; Microalgae; Random mutagenesis; Surface Response Methodology.

Highlights

- Green (8G), yellow (7Y) and white (31W) strains were generated by mutagenesis;

- Culture's media were optimised by surface response methodology;
- Biomass productivity was improved by 35, 70, and 65% in mutants 8G, 7Y, and 31W;
- Protein productivity was improved by 36, 94, and 59% in mutants 8G, 7Y, and 31W;
- Yellow and white colours were improved by 20% in mutants 7Y and 31W;

GRAPHICAL ABSTRACT



5.1 Introduction

Microalgae are considered an interesting alternative feedstock, owing to their rich nutritional composition and reduced environmental impact [272, 273]. The diversity of microalgae is expressed in the variety of compounds they produce, including essential lipids, such as omega-3 fatty acids, high-quality vegan protein, including all the essential amino acids and other important compounds, such as vitamins, namely vitamin B12, iron, zinc and antioxidant pigments [180, 181, 303]. The inclusion of microalgal biomass was shown to improve the bioaccessibility and digestibility of some nutrients and/or the composition of food products [181, 304].

Nevertheless, the industrial production and widespread commercialisation of microalgae are challenged not only by their high-costs of production and processing, but also by unappealing sensory properties, like the green colour, "grassy" flavour, and "fishy" smell, caused by the presence of chlorophyll [9, 182, 305]. Overcoming these challenges is of vital importance for the competitiveness of microalgal products in the food and feed markets, which relies on designing an appropriate bioprocess, starting with strain improvement and process optimisation. Colour is of particular relevance in marketing, as it influences consumer´s perceptions towards a product or service, including food [9]. Therefore, its quantification for optimisation purposes is of paramount significance.

Different approaches have been used to improve microalgal strains, such as random mutagenesis [183]. Regarding process optimisation, there is a plethora of abiotic factors and nutrients that considerably affect biomass and target biocompound´s productivity. Abiotic factors like pH and temperature influence several processes of microalgal metabolism [23, 306]. Additionally, the sources and concentrations of nutrients such as N, P, Na, K, Mg, Ca, trace elements (e.g., Cu, B, Zn, Mn, Mo, Ni, and Fe) and vitamins can also impact the growth and biochemical composition of microalgae [23, 307, 308]. The one-variable at a time (OVAT) approach to optimise a process involves changing one of the variables under study while all others are kept constant and then assessing how the response is influenced by the changed variable, which is highly time- and resource-consuming compared to other approaches [309]. Furthermore, interactions between variables are difficult to estimate systematically [309]. Alternatively, Design of Experiments (DoE) overcomes these limitations by creating models for the optimisation of each response [310]. In this case, the workflow usually starts by screening for the most significant factors with fractional factorial experimental designs followed by the determination of the optimal values for a subset of factors with response surface designs, which enables the estimation of interactions and quadratic effects [310, 311].

Considering that the success of microalgae-based products for food and feed applications relies on their nutritional quality, organoleptic traits, and economic viability of the production and processing pipeline, this work focused on the optimisation of the abiotic factors (pH and temperature) and medium composition, using a DoE methodology, for a green (8G), a yellow (7Y) and a white (31W) mutant of *C. vulgaris*, all generated by random mutagenesis. In this context, the main goals of the optimisation endeavor were to: i) maximize biomass productivity and growth rate; ii) maximize protein productivity; iii) improve the colour of the biomass. To the authors' knowledge, this is the first report of biomass optimisation and protein productivity related to the colour of heterotrophic microalgae.

5.2 Materials and Methods

5.2.1 Microalgal strains and cultures' maintenance

Axenic *Chlorella vulgaris* cultures were obtained from Allmicroalgae Natural Products S.A. culture collection. The wildtype strains, 7, 8, and 31, were retrieved from cryopreserved aliquots stored in liquid nitrogen (–196 °C).

The three mutants studied throughout this work were generated by random mutagenesis with ethyl methanesulfonate (EMS), following the protocol reported by Trovão et al. [249] and in section 3.1.2.2. The green mutant, 8G, was obtained as described by Trovão et al. [312], and in the chapter 4, by fluorescence-activated cell sorting (FACS). The wildtype strain 7 was mutagenized to select the yellow mutant, 7Y, with 2.5 mM of nicotine, as reported in the subchapter 3.2. and the white mutant, 31W, was attained through 2-round mutagenesis, as described in the same subchapter.

The inoculum of each mutant strain was maintained in PCA plates that were then passed to 250-mL Erlenmeyers, with 50 mL of a proprietary standard heterotrophic medium described by Barros et al. [192] (HM-medium), which will be referred to later as the control condition.

5.2.2 Growth assessment

Growth assays, sampling and daily analysis were conducted as described in section 3.1.2.5. as well as biomass productivity and growth rate calculations.

The *DW* was estimated by establishing a correlation with OD_{600} for each *C. vulgaris* mutant: 8G (Equation 15; R^2 = 0.9757), 7Y (Equation 16; R^2 = 0.9815) and 31W (Equation 17; R^2 = 0.9906):

$$OD_{600} = 2.6258 \times DW \quad (15)$$

$$OD_{600} = 1.8025 \times DW \quad (16)$$

$$OD_{600} = 1.9248 \times DW \quad (17)$$

At the end of each assay, pellets were frozen at −20 °C after centrifuging at 4500 *g* for 5 min (Hermle® Z300 centrifuge, Gosheim, Germany). Afterwards, samples were freeze-dried in a Coolvacuum, Lyomicron (Barcelona, Spain), and stored at −20 °C for biochemical analysis at a later time.

5.2.3 Laboratory trials

All trials were carried out in triplicate, except in the screening and optimisation assays, where a single replicate per experimental condition was used (excluding the central points of the DoE design).

In all the experimental trials, PIPES buffer was added at 75 mM to maintain pH at 6.5. Cultures were kept in the dark, at 30 °C and 200 rpm, in an orbital shaker (Agitorb 200IC, Norconcessus®, Ermesinde, Portugal).

5.2.3.1 Preliminary assays

To determine the optimal temperature, pH, and nitrogen source for each mutant strain, *C. vulgaris* 8G, 7Y, and 31W strains were cultivated in the conditions summarized below (Table 5.1).

Temperature was tested at 26, 28, and 30 °C, at pH 6.5 in HM-medium. The pH of this medium was set at pH 5.5, 6.5, and 7.5 by adding PIPES buffer to the respective conditions, in HM-medium, at 30 °C. Regarding the nitrogen source trial, ammonium sulphate (A), sodium nitrate (N), or urea (U) were tested at a N concentration of 57 mM for strain 8G, whereas 38 mM was used for strains 7Y and 31W assays, at pH 6.5 and 30 °C.

Table 5.1 - Conditions of the preliminary trials carried out with 8G, 7Y and 31W *C. vulgaris* strains: temperature, pH and nitrogen source.

Factor	Conditions tested		
T (°C)	26	28	30
pH	5.5	6.5	7.5
N source	Ammonium	Nitrate	Urea

5.2.3.2 Screening assays

Two screening assays were performed to understand which macro- and micronutrients significantly affected the biomass productivity, growth rate, and protein content (PC) of the three mutants. In both assays, a DoE approach was followed by using a randomized two-level fractional factorial experimental design matrix of resolution IV, set up in Design Expert v11.1.2.0. The set of variables (*i.e.*, factors) in each assay was tested on two levels (Table 5.2).

Table 5.2 - Factors and levels used in the macro- and micronutrient screening assays for each mutant strain of *C. vulgaris* (8G, 7Y, and 31W).

Factor	**Levels**					
Strain	**8G**		**7Y**		**31W**	
			Macronutrient screening			
[N] (mM)	20.0	80.0	20.0	80.0	20.0	80.0
[P] (mM)	20.0	80.0	20.0	80.0	20.0	80.0
[Ca] (mM)	0.50	4.00	0.40	2.00	0.40	2.00
[Na]/[K]	0.30	0.90	0.30	0.90	0.30	0.90
[Mg] (mM)	3.00	12.0	3.00	12.0	3.00	12.0
Nitrogen source	Ammonium	Urea	Ammonium	Urea	Ammonium	Nitrate
			Micronutrient screening			
[Cu] (mM)	0.0020	0.2000	0.0060	0.0600	0.0060	0.0600
[B] (mM)	0.0500	0.5000	0.0500	0.5000	0.0500	0.5000
[Zn] (mM)	0.0300	0.3000	0.0300	0.3000	0.0300	0.3000
[Mn] (mM)	0.0200	0.2000	0.0200	0.2000	0.0200	0.2000
[Mo] (mM)	0.0005	0.5000	0.0050	0.5000	0.0050	0.5000
[Ni] (mM)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
[Fe] (mM)	0.0100	1.0000	0.0500	0.5000	0.0500	0.5000
[Vitamin solution]	0.2500×	2.5000×	0.2500×	2.5000×	0.2500×	2.5000×

These levels corresponded to a low and a high concentration of a specific numeric factor, except for categorical factors, where the two levels corresponded to different categories (*i.e.,* nitrogen sources). For each screening assay, the experimental design consisted of 16 experimental runs plus 4 central points, totalling 20 runs (Appendix A1 and A2).

The micronutrient composition of the standard HM-medium was kept the same in all runs of the macronutrient screening, while the macronutrient concentrations remained constant in the runs of the micronutrient screening. Additionally, the carbon source (glucose) concentration in all media was set at 30 g L^{-1}.

5.2.3.3 Optimisation assays

An optimisation assay was performed for each strain, according to the most significant 4 factors pointed out in the screening assays, to maximize the biomass productivity (r_p), growth rate (μ), and protein productivity (PP) as well as improving the coloration (C) of the biomass of the three mutants. The final aim of the trials was to obtain a darker green hue for mutant 8G, a more intense yellow colour for 7Y, and a whiter tone for 31W).

A response surface methodology was followed by using a central composite inscribed design, in Design Expert v11.1.2.0. For the mutant 8G, the concentrations of N, P, Zn, and Mo were included in the optimisation design, concentrations of N, P, Zn and Cu were optimised for mutant 7Y and, for mutant 31W, the concentrations of N, P, Ca and Mg were selected (Table 5.3). The experimental design consisted of a total of 30 experimental runs, 16 factorial, 8 axial, and 6 central points. The concentrations of the nutrients that were not included in the optimisation were set to the same concentration as the HM-medium. Additionally, the glucose concentration of all media was set at 30 g L^{-1}.

Table 5.3 - Factors and levels tested in the optimisation assay for each mutant strain of *C. vulgaris* (8G, 7Y and 31W).

Strain	**8G**			**7Y**			**31W**		
Factor (mM)	**Levels**								
	Low	**High**	**Mean**	**Low**	**High**	**Mean**	**Low**	**High**	**Mean**
[N]	10.00	90.00	50.00	20.0	80.0	50.0	10.0	90.0	50.0
[P]	10.00	90.00	50.00	20.0	80.0	50.0	10.0	90.0	50.0
[Zn]	0.050	0.250	0.15	0.01	0.10	0.06	-	-	-
[Mo]	0.025	0.125	0.08	-	-	-	-	-	-
[Cu]	-	-	-	0.01	0.10	0.06	-	-	-
[Ca]	-	-	-	-	-	-	0.50	2.50	1.50
[Mg]	-	-	-	-	-	-	3.00	15.00	9.00

5.2.3.4 Validation assays

A validation assay was carried out for each strain to compare the optimised medium obtained with the standard HM-medium (control). The optimised medium was formulated in this assay according to the models obtained in the previous optimisation assay. The concentrations of the remaining nutrients (not included in the optimisation assays) were at the same concentration as in the HM-medium. In the validation of the optimised medium for mutant 8G, only one condition was tested against the control, since the conditions to maximize growth and protein and to reach optimal colour were in accordance. The several conditions tested for mutant 7Y provided different yellow tones and different results concerning biomass and protein productivities. To obtain a whiter colour of the biomass, the optimised conditions for mutant 31W were the opposite of the conditions to reach higher productivities of biomass and protein. Thus, several conditions were tested to analyse what would be the most advantageous combination (Table 5.4).

Table 5.4 - Validation of the optimised vs control (Ctl) conditions for each *C. vulgaris* mutant (8G, 7Y and 31W): respective concentrations (mM) of the optimised factors. Glu - glucose.

Strain	Solution	Concentrations (mM)							Other
		[N]	[P]	[Zn]	[Mo]	[Cu]	[Ca]	[Mg]	
8G	**1**	70	50	0.2	0.075	-	-	-	-
	Ctl 1	57	60	0.1	0.001	-	-	-	-
7Y	**1**	76	50	0.1	-	0.055	-	-	-
	2	76	50	0.1	-	0.055	-	-	Feed Glu until N depletion
	3	30	30	0.1	-	0.055	-	-	-
	4	30+30	30+30	0.1	-	0.055	-	-	-
31W	**1**	67	70	-	-	-	2	9	
	2	67	70	-	-	-	2	9	Feed Glu until N depletion
	3	30	70	-	-	-	2	9	-
	4	30+30	70	-	-	-	2	9	-
	5	30+10+10+10	70	-	-	-	2	9	-
	Ctl 2	38	63	0.2	-	0.0024	0.4	7.7	-

5.2.4 Biochemical characterisation

5.2.4.1 Protein content

The protein content was determined as described previously in section 3.1.2.6.1.

5.2.4.2 Colour

The colour of the samples was measured with a Chroma Meter (CR-400-410; Konica Minolta; Nieuwegein; Netherlands) and a Colour Data Software CM-S100w (SpectraMagic^{TM}NX). In this case, the *L*a*b** colour space, defined by the Comission Internationale de l'Eclairage (CIE) was used (Observer – 2 degrees; Illuminant - C). *L** corresponds to the brightness of the sample (S), ranging from 0 to 100; *a** to the green-red coordinate and *b** to the yellow-blue coordinate, both with numerical values ranging between -120 and 120. The standard was a white plate, conveyed by the manufacturer, with the C-illuminant having the following values of *L**, *a** and *b**, respectively: 83.8, 0.3185 and 0.3250.

5.2.5 Statistical analysis

Statistical analyses were performed using the R statistical package (v. 4.0.5) for the preliminary and validation assays. Experiments were carried out in biological triplicates, and results were expressed as mean ± standard deviation. Results were analysed by One-way ANOVA, followed by Tukey HSD *post-hoc* multiple comparisons test at a probability level of 0.05.

For the screening and optimisation assays, statistical analyses were performed with the Design-Expert software (v.11.1.2.0), and a significance of 5% was also considered. The biochemical characterisation of these samples was carried out in analytical duplicate replicates due to the absence of biological replicates in these trials.

Data was analysed for each response variable in the screening assays, namely r_p, μ, PC, and C. The significance of the explanatory variables of the selected factorial model was assessed by ANOVA as well as the selected models for each response of the optimisation trials (the same as in the screening trials plus protein productivity). The best-fitting model was selected for each response, whether linear, quadratic, 2-factor interaction, cubic, or a modified version of one of those.

The adequacy of all these models was also evaluated according to the correlation coefficient (R^2), the adjusted and predicted R^2, and the "Diagnostics" section of Design-Expert software.

5.3 Results and Discussion

5.3.1 Preliminary assays

Chlorella vulgaris mutants 8G, 7Y, and 31W were tested at different temperatures, pH, and with different N sources to establish the optimal parameters towards the conciliation of maximal biomass productivity (r_p), growth rate (μ), and protein content (PC) (Table 5.5).

The responses analysed concerning the temperature variation were not unanimous among the three mutants. Regarding biomass productivity, mutant 8G did not display differences among the tested temperatures, while the highest productivity in mutant 7Y was achieved at 28 °C (2.70 ± 0.03 g L^{-1} d^{-1}) and for mutant 31W at 30 °C (2.22 ± 0.07 g $L^{-1}d^{-1}$). Concerning growth rate, the highest values were achieved at 28 and 30 °C in mutant 8G, at 26 and 28 °C for mutant 7Y, and 30 °C for mutant 31W. In

regard to protein content, mutant 8G displayed the highest value at 28 °C (39.3 ± 0.4% of DW), mutant 7Y at 28 °C and 30 °C (33.6-34.6% of DW), and mutant 31W at 26 and 30 °C (39.8-41.2% of DW). Since there was no clear optimal temperature for all the responses simultaneously, the temperature of 30 °C was selected, which also corresponds to the temperature at which the mutants were generated.

Table 5.5 - Biomass productivity (r_p) in g L^{-1} d^{-1}, growth rate (μ) in d^{-1}, and protein content (PC) in % of DW of the three mutants of *C. vulgaris*, 8G, 7Y, and 31W, tested at 3 temperatures, 3 pH, and with 3 N sources (A – ammonium; N – nitrate; U – urea). Different letters represent statistical differences between the three conditions tested (T, pH, and N source) for each response (r_p, μ, PC) for each mutant, *i.e.*, if a response has the same letter under different conditions (of T or pH or N source), there were no significant differences in that response (r_p, μ, PC) for that mutant.

		T (°C)			pH			N source		
		26	**28**	**30**	**5.5**	**6.5**	**7.5**	**A**	**N**	**U**
8G	r_p	1.85±0.02^{a}	1.74±0.01^{a}	1.85±0.11^{a}	2.20±0.07^{a}	2.36±0.05^{a}	2.18±0.05^{a}	2.36±0.05^{a}	2.09±0.07^{b}	2.29±0.01^{a}
	μ	1.25±0.01^{b}	1.30±0.02^{a}	1.31±0.01^{a}	1.15±0.01^{b}	1.17±0.01^{ab}	1.14±0.01^{bc}	1.17±0.01^{a}	1.13±0.01^{b}	1.13±0.01^{b}
	PC	35.2±0.9^{b}	39.3±0.4^{a}	37.0±0.9^{b}	30.4±0.2^{a}	29.3±1.1^{ab}	27.1±0.9^{b}	29.3±1.1^{a}	32.5±1.4^{a}	29.2±1.0^{a}
7Y	r_p	2.50±0.03^{b}	2.70±0.03^{a}	1.93±0.09^{c}	0.85±0.06^{a}	0.75±0.03^{a}	0.42±0.11^{b}	0.75±0.03^{a}	0.37±0.04^{c}	0.64±0.03^{b}
	μ	1.30±0.00^{a}	1.32±0.02^{a}	1.05±0.01^{b}	0.83±0.02^{a}	0.80±0.02^{a}	0.66±0.06^{b}	0.80±0.02^{a}	0.65±0.03^{b}	0.77±0.03^{a}
	PC	32.2±1.1^{b}	33.6±0.5^{ab}	34.6±0.4^{a}	34.9±0.6^{a}	35.1±0.1^{a}	31.0±1.6^{b}	35.1±0.1^{a}	27.8±3.9^{a}	32.3±2.1^{a}
31W	r_p	1.71±0.15^{b}	1.65±0.06^{b}	2.22±0.07^{a}	2.06±0.03^{ab}	2.48±0.23^{a}	1.99±0.05^{b}	2.48±0.23^{a}	1.48±0.12^{b}	2.27±0.06^{a}
	μ	1.02±0.02^{b}	1.01±0.01^{b}	1.09±0.01^{a}	1.03±0.02^{b}	1.10±0.01^{a}	1.01±0.03^{b}	1.10±0.03^{a}	0.88±0.02^{b}	1.04±0.03^{a}
	PC	39.8±1.0^{a}	41.2±0.5^{a}	33.8±0.5^{b}	33.9±0.1^{a}	32.1±0.7^{b}	35.1±0.3^{a}	32.1±0.7^{b}	36.3±0.6^{a}	36.2±0.3^{a}

Concerning pH, mutant 8G did not present differences in biomass productivity and growth rate between pH 5.5 to 7.5. For mutant 7Y, both parameters were higher at pH values of 5.5 and 6.5 (0.75-0.85 g $L^{-1}d^{-1}$; 0.80-0.83 d^{-1}, respectively), and there were no differences in biomass productivity for mutant 31W between pH 5.5 and 6.5, while the highest growth rate was attained at pH 6.5 (1.10 d^{-1}). In addition, the protein content was higher at pH values of 5.5 and 6.5 for mutant 8G and 7Y, while for mutant 31W it was higher at 5.5 and 7.5. Given this, a pH value of 6.5 was selected as the optimal condition for the three mutants.

Finally, the N source that ensured the highest biomass productivity and growth rate was ammonium for the three mutants, although some values did not present significant differences when compared to urea. In regard to protein content, the three N sources tested enabled to attain similar protein contents, 29.2-32.5% and 27.8-35.1% of DW, in mutants 8G and 7Y, respectively, while mutant 31W achieved the highest values when cultivated with nitrate and urea, 36.2-36.3% of DW. Since ammonium enabled an improved growth performance among the three mutants, it was selected as one of the N sources to maintain for the three strains. Urea was also included in the screening trials of 8G and 7Y, and nitrate was included in the trials of 31W instead.

Temperature and pH are critical factors that affect microalgal metabolism, enzymatic reaction rates, and the solubility of nutrients and gases (particularly oxygen), which greatly impact algae growth [313]. The optimal temperature and pH heavily depend on the species used and the goal of the cultivation, as it is possible to deduce by analysing examples from literature (Appendix A3). *Chlorella vulgaris* is commonly cultivated at 28-30 °C under heterotrophic conditions [313, 314], a similar range to the optimal temperatures obtained in the present study. In this work, 30 °C was the temperature selected, not only based on the results obtained for each response, but also because lower cultivation temperatures require more energy to cool down the fermentation broth, which greatly impacts production costs, particularly at larger scales [315]. Regarding pH, the optimal range for microalgal heterotrophic growth is generally between pH 6 and 7 [316]. Concerning the impact of these factors on protein content of microalgal cells, information in literature is scarce. Nonetheless, Xie et al. [317] reported higher protein contents (48-53% of DW) at 28 and 32 °C and at pH 2-5.5 for *Euglena gracilis*.

Nitrogen is also a major macronutrient, since it contributes to the synthesis of amino acids and subsequently proteins. Although microalgae can metabolize different nitrogen sources, ammonium is often preferred, as evidenced in this study, since its uptake requires less energy and for the fact that it can be readily used by the cells without additional reactions, while nitrate and urea need to be converted into ammonium first [23]. Moreover, it has been suggested that ammonium provides a higher proportion of total essential amino acids to total amino acids, including in *C. vulgaris* [317].

5.3.2 Screening assays

Screening assays allow for a reduction of the number of factors in the subsequent optimisation trials by removing variables that, in this case, have not had a significant impact on the biomass productivity, growth rate, and protein content. In

Figure 5.1, a visual representation of the results is shown. A more detailed summary of the complete screening trials analysis, namely which factors significantly impacted each response variable (p-value < 0.05) is also displayed in Appendix A4 and A5).

Overall, the factors that affected growth significantly were the concentration of N, P, Zn, Cu, B, Mo, Fe and vitamin solution and N source in the case of mutant 8G, the concentration of N, P, Ca, Cu and N source, and Zn was marginally significant for 7Y growth and the concentration of N, P, Ca, Mg, Cu, Zn, Ni and N source for mutant 31W (

Figure 5.1A and B). In addition, the factor that most impacted protein content was N concentration across the three screenings, as well as the N source and the concentration of Cu in the case of 8G, the concentration of the vitamin solution in the case of 7Y, and the concentration of P, B, Mn and vitamin solution for mutant 31W, but these with a much less significant effect (

Figure 5.1C).

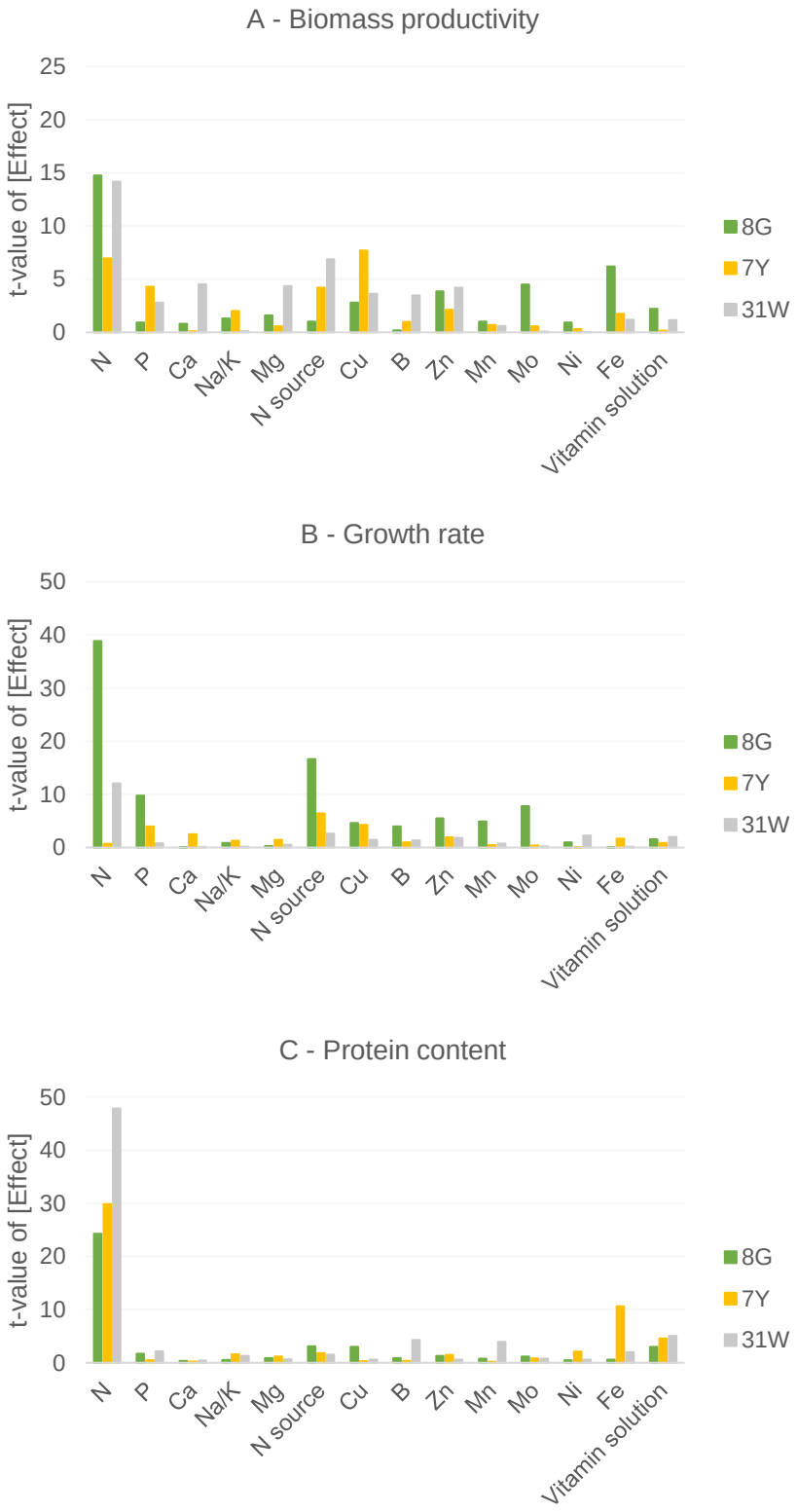


Figure 5.1 - Statistical results obtained in Design Expert v.11.1.2.0: *t*-value of effect, retrieved from Pareto Charts for the variables tested in the macro- and micronutrient screening assays (two-level fractional factorial design) for the green (8G), yellow (7Y) and white (31W) *C. vulgaris* mutants.

The three mutants showed significantly improved growth with ammonium instead of urea or nitrate (p-value < 0.05). However, mutant 8G achieved higher protein content with urea. Despite this, the impact of the N source on growth rate was more significant than the effect on protein content. Thus, N source was excluded as a factor, and the three mutants were optimised using only ammonium as the N source. Then, 4 numeric factors were selected for each optimisation, considering the factors with a more significant effect overall. Likewise, the concentrations of N, P, Zn and Mo were the factors selected for the optimisation of mutant 8G; the concentrations of N, P, Cu and Zn for mutant 7Y; and the concentration of N, P, Ca and Mg for mutant 31W.

Kim et al. [290] also performed a study on the optimisation of heterotrophic cultivation of *Chlorella* sp. HS2 towards improved biomass concentration and productivity. In the screening phase (Plackett-Burman Design), the concentrations of N and P were also identified as two of the three key nutrients to optimise. On the other hand, Ward and Rehmann [318] reported an optimisation for mixotrophic *C. vulgaris* cultivation, where glucose, nitrate, and magnesium were included in a Box-Behnken design. Thus, in these two reports, N, P, and Mg were also significant factors as in the present study.

5.3.3 Optimisation assays

The three mutants' cultivation conditions were optimised towards improved biomass productivity, growth rate, protein productivity, and colour. The equations of the models obtained for each response (Appendix A6) as well as all the statistical parameters of the models obtained for mutant 8G (Appendix A7), 7Y (Appendix A8) and 31W (Appendix A9) are summarized in the Appendix The results reported for each response is discussed in the following sections, separately.

5.3.3.1 Growth performance

Some of the results obtained for the green, 8G (Figure 5.2A), yellow, 7Y (Figure 5.2B), and white mutant, 31W (Figure 5.2C) are shown in the figures below, regarding biomass productivity and growth rate.

The biomass productivity model of mutant 8G showed that this parameter was affected by the concentrations of N and Zn and by the quadratic terms of N, P, and Mo, while the growth rate was only significantly affected by the quadratic term of P. Therefore, improved biomass productivity and growth rate were predicted to achieve the maximum, around 2.7 g L^{-1} d^{-1} and 1.24 d^{-1}, respectively, with the highest concentrations of N (60-70 mM) and Zn (0.18-0.20 mM) and intermediate concentrations of P (40-60 mM) and Mo (0.07-0.08 mM) (Figure 5.2A).

The concentration of N significantly affected both responses for mutant 7Y. Higher concentrations of N (60-70 mM) allowed maximum biomass productivity and growth rate ~2.7 g L^{-1} d^{-1} and 1.01 d^{-1}, respectively, according to the models obtained (Figure 5.2B). Moreover, intermediate concentrations of Cu, between 0.05-0.06 mM, also allowed to achieve improved growth parameters. Finally, Zn also significantly affected biomass productivity, so that higher values between 0.08-0.10 mM led to increased growth.

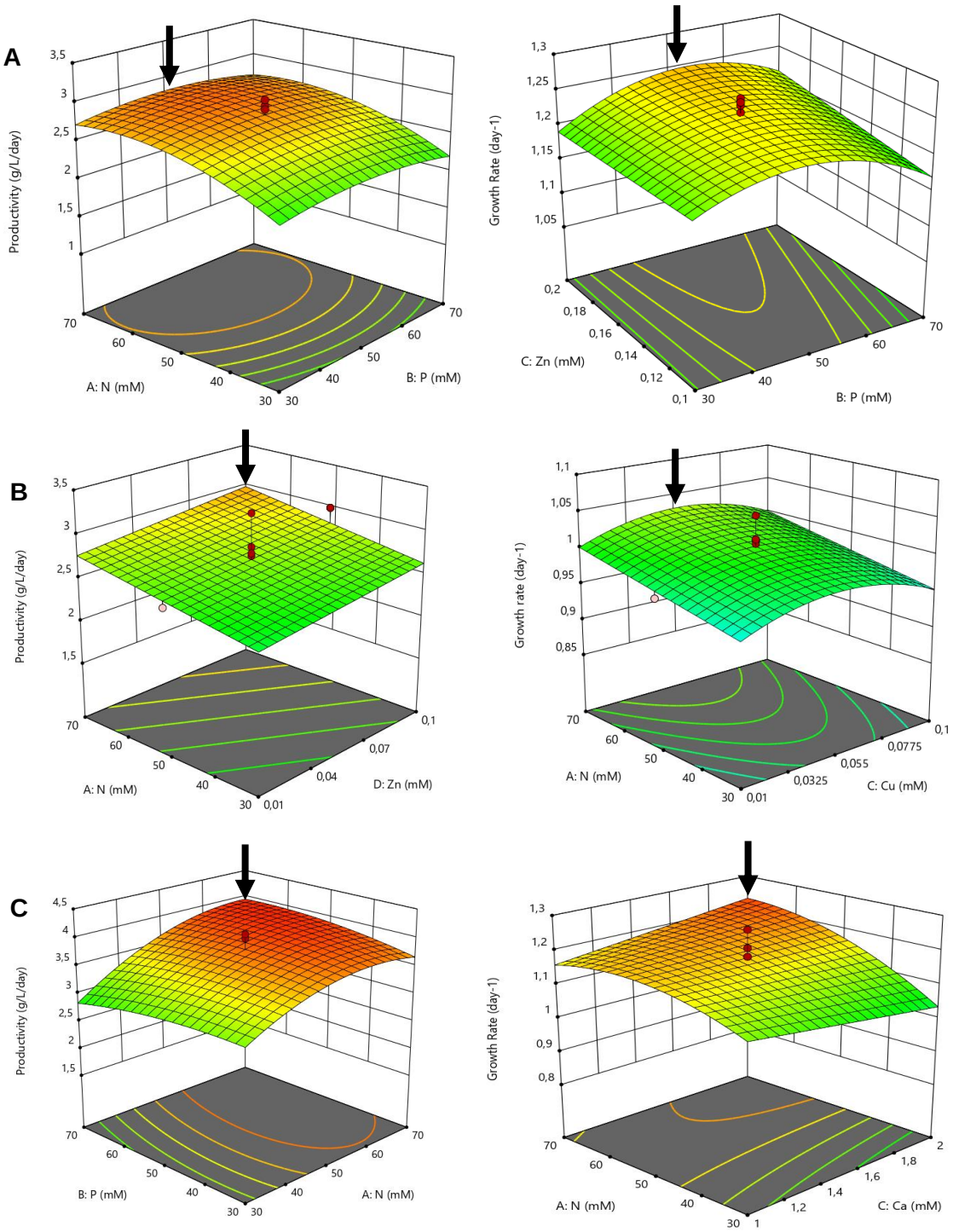


Figure 5.2 - Model Graphics obtained in Design-Expert software (v.11.1.2.0): 3D surface plots of biomass productivity (g L^{-1} d^{-1}) and growth rate (d^{-1}) predicted for mutant 8G (A), 7Y (B) and 31W (C) of *C. vulgaris* within the range of the nutrient concentration tested in the optimisation trial and the interaction between those 2 factors, considering the average value of the remaining factors tested. The black arrows indicate the conditions in which the highest values of that response would be obtained according to that model. Cooler colours (blue-green) indicate suboptimal conditions, yellow represents intermediate values and warmer colours (orange-red) indicate optimal conditions for that response.

Mo, Cu, and Zn are beneficial for living organisms at low concentrations but yield negative effects at high concentrations. For example, Mandal et al. [319] reported enhanced production of reactive oxygen species (ROS) and antioxidant enzymes through the supplementation of Mo (between 0.03-0.09 µM). On the other hand, the optimal Cu concentration predicted here (55 µM) was significantly higher than the values reported in literature, ranging from 0.01 to 0.4 µM [219, 290, 320]. Li et al. [321] used a Cu concentration of 0.063 mM, close to the optimal concentration determined for mutant 7Y, but achieved a biomass productivity of 0.61 g L^{-1} d^{-1} and a specific growth rate of 0.44 d^{-1}, around 5- and 2-fold lower than the predictions of the models generated in this study, respectively, albeit with *Chlorella protothecoides*. On the other hand, Giordano et al. [322] reported a similar behaviour to that was observed here with the variation of Cu concentration, for the cell density of *Chlorella* sp. in heterotrophic conditions. The highest cell concentration was obtained at 0.002 mM of Cu, but at higher concentrations of this metal, the former values rapidly decreased, at least by half. The range of Zn concentrations found in literature, 0.0008 to 0.001 mM, encompasses concentrations lower than the lowest concentration tested in this study, 0.01 mM [219, 290, 320]. Nevertheless, Shi et al. [320] cultivated *C. sorokiniana* using a heterotrophic regime with a higher Zn concentration, 0.31 mM, achieving a growth rate similar to the values obtained in the present models, but with a lower biomass productivity.

The biomass productivity of mutant 31W was impacted by the concentration of N, by the quadratic terms of N and P, and by their interaction (Figure 5.2C). In addition, the N concentration, the respective quadratic term, and the interaction of Ca with N and P, significantly affected the growth rate (Figure 5.2C). Since it was more beneficial to have Ca between 1.6-2.0 mM, according to the interaction with N, then P concentration should be between 60-70 mM, according to the interaction with Ca. In resemblance to the green and yellow mutant, N concentrations between 60-70 mM provided the highest biomass productivity (3.9 g L^{-1} d^{-1}) and growth rate (1.25 d^{-1}).

In the present work, increasing concentrations of N resulted in higher biomass productivities, when comparing the low (20 mM) and high (70 mM) N levels. The optimal N concentration predicted here falls within the range of concentrations found in the literature to cultivate *Chlorella* sp. heterotrophically with values ranging from 10 to 94 mM [219, 290, 320]. Similar results were obtained by [227]. Although these authors used a different N source (sodium nitrate) to cultivate *C. vulgaris*, they also achieved higher DW with higher N concentrations, between 3 and 24 mM. However, under optimal N concentration, the highest DW they achieved was less than 3.5 g L^{-1} over 9 days of cultivation, thus presenting a biomass productivity around 10-fold lower than the maximum reported here. Shen et al. [323] reached higher values by cultivating *C. protothecoides*, also in heterotrophic conditions, with 60 mM of N (potassium nitrate), attaining 13 g L^{-1} in 8 days, a productivity still 2-3 times lower than the maximum obtained here. In the present study, the biomass productivities and growth rates reported are more than twice the values mentioned above, which could be due to differences in the cultivation methodologies, namely the use of different species and strains, temperatures, glucose concentrations, nitrogen sources and nitrogen concentrations.

While N concentration was consistently more beneficial for growth at the maximum, the three mutants did not present a similar behaviour among the different P concentrations. For mutant 8G,

intermediate concentrations were preferable (Figure 5.2A), while for mutant 31W, the highest concentrations were more favourable (Figure 5.2C). On the other hand, for mutant 7Y, it was not a significant factor for optimal growth.

It is noteworthy that the range of P concentrations found in the literature to cultivate *Chlorella* sp. in heterotrophic conditions, between 0.2 to 9 mM, is much lower than the lowest concentration used in this study (20 mM) [219, 290, 320, 324]. Li et al. [324] reported that excess P in heterotrophic conditions, decreased the cell density of *C. regularis* by 40 % when cells grew on medium containing 8 mM of P compared to 1.5 mM, causing growth inhibition and cell damage. Overall, these results indicated that while P is an important nutrient for microalgal cell growth, it may result in sub-optimal growth performance if supplied at too high or too low concentrations.

5.3.3.2 Protein productivity

Figure 5.3 presents the protein productivity results obtained for the green, 8G (A), yellow, 7Y (B), and white 31W (C) mutants.

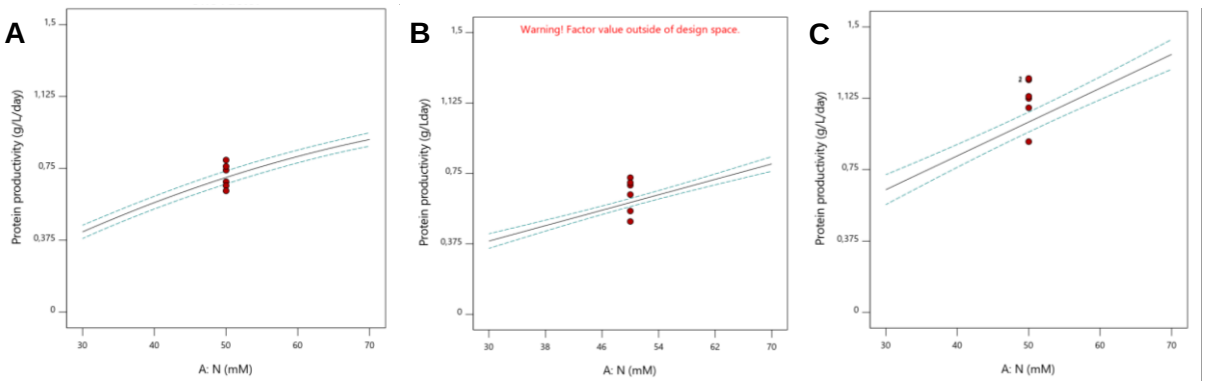


Figure 5.3 - Model Graphics obtained in Design-Expert software (v.11.1.2.0): one-factor plots of protein productivity (g L^{-1} d^{-1}) showing the linear effect of changing the concentration of a single factor, N concentration (mM), considering average values of the remaining, for mutant 8G (A), 7Y (B) and 31W (C) of *C. vulgaris*.

Considering the models obtained for each mutant, maximum concentrations of N, around 70 mM, have led to the maximum protein productivities of 0.9, 0.7, and 1.4 g L^{-1} d^{-1}, with mutants 8G, 7Y, and 31W, respectively (Figure 5.3), and predicted protein contents around 40% of the DW (data not shown). These results are in line with the optimal conditions verified for maximum biomass productivities and growth rates, not only because maximum N concentrations were preferable for the four responses, but also because if higher biomass productivities are achieved, higher protein productivities will be attained. Additionally, in the model of protein content of mutant 31W (data not shown), Ca and its interaction with N and Mg also revealed significant effects ($p < 0.05$), with higher concentrations of Ca having a beneficial effect on protein content. On the contrary, Husseini et al. [325] found no differences between

the protein content of *C. vulgaris* cultures pretreated with increased concentrations of $CaCl_2$ and without Ca supplementation. However, Ca was not significant for the protein productivity of this strain.

On the other hand, nitrogen is crucial in the synthesis of amino acids and proteins [23]. Xie et al. [227] also showed an increment in protein content of *C. vulgaris* at higher N concentrations (but with nitrate instead of ammonium). However, this increment was only observed at concentrations between 3 and 15 mM, which have led to protein contents of 25 and 40% of the DW, respectively. According to these authors, with higher N concentrations, the protein content actually began to decrease slightly. However, in the present study, the protein content and productivity always increased with increasing N concentrations (Figure 5.3). It is also noteworthy that if N depletion usually leads to lipid and/or starch accumulation [318, 326], as reported for *Chlorella* [327], it is not surprising that an over-compensation leads to protein accumulation, as also suggested by [227]. Despite the different results found, N is undoubtably one of the nutrients with more influence on protein content of microalgal cells.

Protein contents from 20 to 64% of the DW were reported for *Chlorella* cultivated in heterotrophic conditions [249]. In addition, the quality of *Chlorella* protein, concerning, for example, the amino acid composition, is similar to other protein sources such as fish, eggs, and soybean [220, 328]. It is also noteworthy that *Chlorella* protein might have an added-value since the content of other important micronutrients, as iron and vitamin B12, are 10-100-fold higher than in meat, fish, and soy [329–331].

Despite the fact that lower protein contents were obtained here, compared to the maximum of 64% reported, protein productivity should also be considered, since those authors attained a biomass productivity of 1.65 g L^{-1} d^{-1} [220], which would mean a protein productivity of 1.1 g L^{-1} d^{-1}, while in the present work, the biomass productivities were more than twice that, which enabled the achievement of a maximum protein productivity of 1.4 g L^{-1} d^{-1}, which is 27% higher than what was reported by those authors.

5.3.3.3 Colour

Through the field of colorimetry, it is possible to quantify colours by attributing numerical values to different components according to colour systems [332]. The CIE *L*a*b** system is considered a standard for colour measurement and it has been used in various food applications, as Cairone et al. [333] discussed in their review.

Four variables of colour characterisation were analysed, *L**, *a** and *b**, but only the most significant for each mutant was used to create the respective model: *L** in the case of mutant 8G, *b** for mutant 7Y, and *a** for mutant 31W. In Figure 5.4, it is possible to observe the colour of the samples as well as the colour measurements of the optimisation trials (Appendix A10). Colour optimisation of the green, 8G, yellow, 7Y, and white, 31W, mutants, is graphically represented in Figure 5.5.

Mutant 8G presented a darker green colour (lower *L**) when higher concentrations of N and lower concentrations of Zn were used. On the other hand, the interactions between N and Mo, P and Zn, P and Mo, and Zn and Mo were all significant ($p < 0.05$). P and Mo had a reversed behaviour, when the highest concentrations of Mo were used, the lowest concentrations of P should be applied and vice-versa. Regarding mutant 7Y, N and the quadratic term of Zn affected the yellow colour, so that higher

concentrations of N and the lowest or the highest Zn concentrations tested were more beneficial for a stronger yellow colour (higher b*). Finally, for mutant 31W the colour was predicted to be whiter and/or less green (higher a*) as lower concentrations of N and P were used.

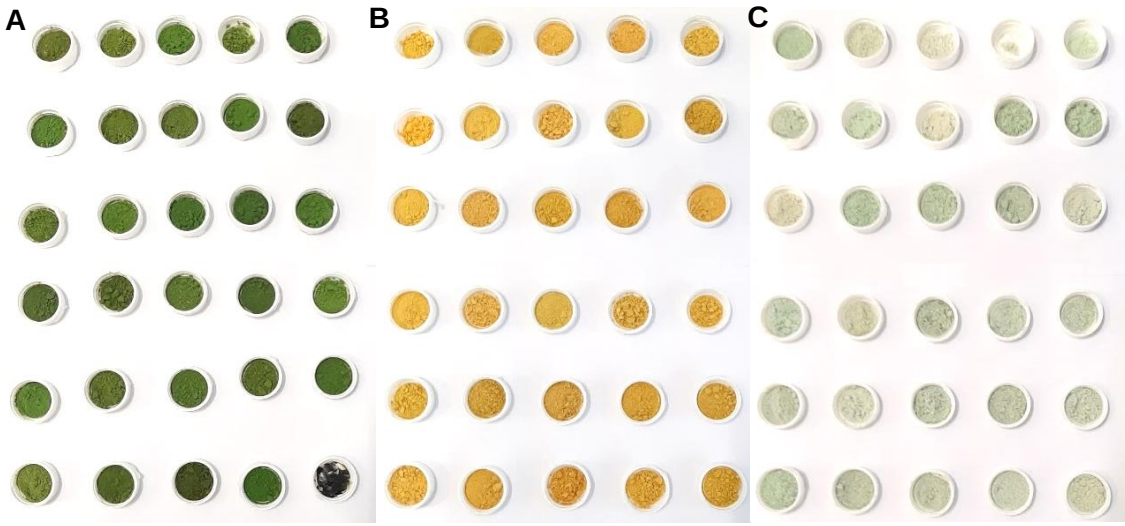


Figure 5.4 - Freeze-dried samples with different coloration of the 30 runs of the optimisation trial of the green 8G (A), yellow 7Y (B) and white 31W (C) mutants of *C. vulgaris*.

N and P are both macronutrients crucial not only for microalgal growth and metabolism but also for pigment synthesis [308, 334]. In addition, the chlorophyll molecule contains four pyrrole groups each with a N atom that form a ring around Mg, responsible for the stabilization of the molecule [334, 335] and ammonium (N source used) is a precursor of glutamate and α-ketoglutarate, which are in turn precursors of chlorophyll biosynthesis [220]. Thus, N content inevitably affects chlorophyll synthesis, so that N deficiency and/or starvation has been reported to cause up to 50% of chlorophyll content reduction in *Chlorella* sp. [336, 337]. This is in accordance with the models obtained for the colour of the green and white mutant, whose green and white colour were correlated with high and low concentrations of N, respectively. A similar behaviour has been reported for the effect of P in the chlorophyll accumulation of *C. pyrenoidosa*, which might be associated with the requirement of P for ATP synthesis and metabolic processes, such as chlorophyll synthesis [338]. On the other hand, increased Zn concentrations led to decreasing chlorophyll contents, as it has been reported previously for several species, including *C. vulgaris* [339].

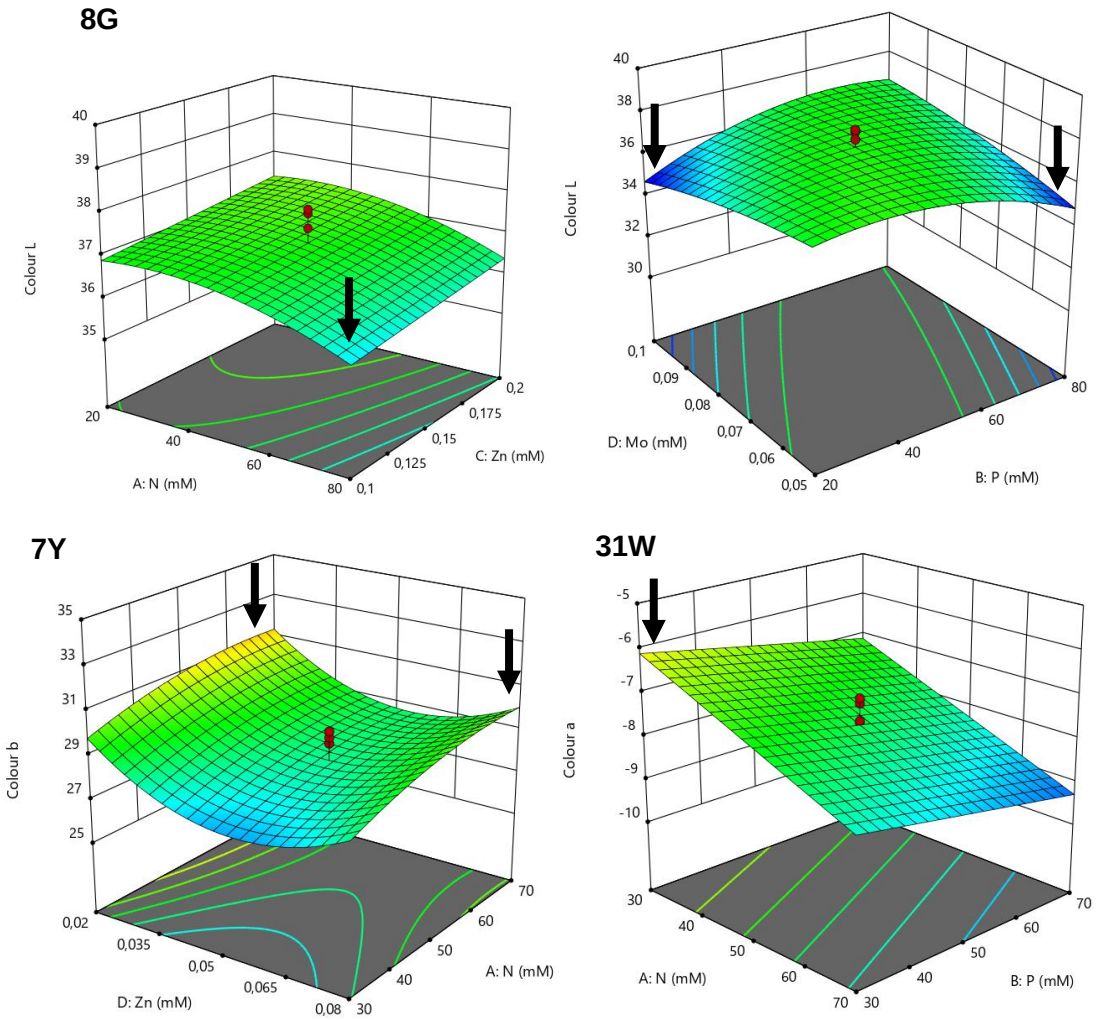


Figure 5.5 - Model Graphics obtained in Design-Expert software (v.11.1.2.0): 3D-surface plots of colour: L* (brightness/darkness coordinate) predicted for mutant 8G, b* (yellow/blue coordinate) predicted for mutant 7Y and a* (green/red coordinate) predicted for mutant 31W of *C. vulgaris* within the range of the nutrient concentration tested in the optimisation trial and the interaction between those 2 factors, considering the average value of the remaining factors tested. Cooler colours (blue-green) indicate lower values, yellow represents intermediate values and warmer colours (orange-red) indicate higher values. The darker green colour aimed for mutant 8G is represented by the cooler colours, corresponding to lower L* values (darker), a stronger yellow colour is represented by warmer colours for mutant 7Y, corresponding to higher b* values (more yellow) and a whiter tone in the case of mutant 31W is also represented by the warmer colours, corresponding to higher a* values (less green).

5.3.4 Validation assays

The optimised conditions and the models obtained for each mutant were compared to the standard medium (control condition) to validate the results and the values attained for each response are shown in Table 5.6.

As predicted by the models obtained in the optimisation of the three mutants, higher concentrations of N and the combination of the remaining factors enabled to achieve higher biomass productivities, with improvements of 35% for mutant 8G, 9-70% for mutant 7Y, and 30-65% for mutant 31W. The highest value was 4.03 ± 0.05 g $L^{-1}d^{-1}$, achieved by mutant 7Y. Growth rate followed the same trend but less

pronounced as it is an intrinsic feature of a species. In addition, protein content and productivity also followed this same trend, with maximum improvements by up to 61% and 94%, respectively. The highest protein content was also reached by mutant 7Y, with 40.07 ± 1.12% of DW, and the highest protein productivity was attained by mutant 31W, with 1.29 ± 0.03 g $L^{-1}d^{-1}$. The colour of mutant 8G did not present significant differences between the control and optimised medium condition, while the yellow mutant 7Y presented a 15% and 20% stronger yellow colour (*b** coordinate) under the conditions of solutions 1 and 2, respectively, compared to the WT. Finally, the white mutant 31W achieved the whitest tone under the conditions of solution 3, with 20% lower green colour (*a** coordinate) compared to the WT.

Table 5.6 - Biomass productivity (r_p) in g L^{-1} d^{-1}, growth rate (μ) in d^{-1}, protein content (PC) in % of DW, protein productivity (PP) in g L^{-1} d^{-1} and colour (C) – L* (brightness/darkness coordinate), b* (yellow/blue coordinate) and a* (green/red coordinate) of the three mutants of *C. vulgaris*, 8G, 7Y and 31W, respectively and the respective % of change of each solution (S) comparing with the control (Ctl) condition (before optimisation). The most interesting solutions have the line highlighted in the table with the colour of the respective mutant: green (8G), yellow (7Y) and white (31W). Different letters indicate statistical differences between the control condition and the solution(s) tested for each response (r_p, μ, PC, PP and C) for each mutant.

	S	r_p	Change (%)	μ (d^{-1})	Change (%)	PC (%)	Change (%)	PP (%)	Change (%)	C	Change (%)
8G	1	2.62 ± 0.11^{a}	+35	1.06 ± 0.01^{a}	+12	34.62 ± 0.71^{a}	+12	0.95 ± 0.05^{a}	+36	38.01 ± 0.41^{a}	-2
	Ctl	1.94 ± 0.04^{b}	-	0.95 ± 0.00^{b}	-	30.80 ± 0.73^{b}	-	0.70 ± 0.02^{b}	-	38.59 ± 0.26^{a}	-
7Y	1	3.17 ± 0.05^{b}	+34	1.10 ± 0.02^{b}	-4	40.07 ± 1.12^{a}	+57	1.28 ± 0.06^{a}	+94	32.47 ± 0.25^{a,b}	+15
	2	4.03 ± 0.05^{a}	+70	1.16 ± 0.02^{a}	+1	30.27 ± 0.93^{b}	+19	1.21 ± 0.04^{a,b}	+83	33.90 ± 2.02^{a}	+20
	3	2.58 ± 0.08^{c}	+9	1.14 ± 0.01^{a,b}	0	24.00 ± 0.44^{c}	-6	0.53 ± 0.02^{c}	-20	28.16 ± 0.47^{c}	0
	4	3.64 ± 0.10^{b}	+54	1.16 ± 0.02^{a}	+1	32.10 ± 1.78^{b}	+26	1.07 ± 0.06^{b}	+62	29.29 ± 1.54^{b,c}	+4
	Ctl	2.37 ± 0.08^{c}	-	1.15 ± 0.01^{a,b}	-	25.45 ± 0.12^{c}	-	0.66 ± 0.01^{c}	-	28.28 ± 0.58^{c}	-
31W	1	2.76 ± 0.12^{b}	+30	1.11 ± 0.00^{b}	+1	37.35 ± 2.28^{a}	+60	1.07 ± 0.07^{b}	+41	-7.23 ± 0.40^{a}	+12
	2	3.50 ± 0.05^{a}	+65	1.12 ± 0.01^{b}	+2	30.08 ± 0.10^{b}	+29	0.96 ± 0.01^{c}	+26	-7.64 ± 0.14^{a,b}	+18
	3	2.01 ± 0.00^{c}	-5	1.09 ± 0.00^{b}	-1	18.05 ± 0.79^{d}	-23	0.53 ± 0.02^{e}	-30	-5.20 ± 0.31^{d}	-20
	4	2.86 ± 0.02^{b}	+35	1.11 ± 0.01^{b}	+1	36.53 ± 0.30^{a}	+57	1.06 ± 0.01^{b}	+40	-6.87 ± 0.34^{a}	+6
	5	3.36 ± 0.03^{a}	+58	1.16 ± 0.00^{a}	+6	38.24 ± 0.71^{a}	+61	1.29 ± 0.03^{a}	+59	-6.74 ± 0.28^{a}	+4
	Ctl	2.12 ± 0.04^{c}	-	1.10 ± 0.01^{b}	-	23.33 ± 0.46^{c}	-	0.76 ± 0.02^{d}	-	-6.48 ± 0.31^{a,c}	-

Previous reports highlighted improved growth performances after medium optimisation. For example, Kim et al. [290] optimised the concentrations of nitrate and phosphate and were able to increase the biomass productivity of *Chlorella* sp. by 210% (from 0.84 to 2.59 g L^{-1} d^{-1}) compared to the unoptimized medium of that study. The biomass productivities achieved in this study were also higher than the productivity attained by Liang et al. [340], 0.15 g L^{-1} d^{-1}, that also cultivated *C. vulgaris* heterotrophically. At this scale, Schüler et al. [19] also cultivated *C. vulgaris*, yielding biomass productivities between

2.45 and 3.23 g L^{-1} d^{-1}, while Cheng et al. [341] were able to attain 3.50 g L^{-1} d^{-1} with *C. protothecoides*. Similarly, Cheng et al. [341] and Xiong et al. [342] reported lower specific growth rates (0.44 and 0.58 d^{-1}, respectively) than the values obtained in this work. Compared to the present study, O'Grady and Morgan [343] reported a considerably higher growth rate in *C. protothecoides* (2.30 d^{-1}). This suggested that further optimisation may be achieved, namely by scaling up the process, as supported by the productivities reported by Barros et al. [192] of 27.5-31.9 g L^{-1} d^{-1}, in 0.2- and 5-m^3 reactors. Protein content might possibly be further improved also upon scale-up by including, for example, two-stage processes and optimizing feeding strategy as suggested by Xie et al. [227], which reached 44.3% of DW with an N overcompensation two-stage strategy. While the latter was the only study found regarding the optimisation of protein with *C. vulgaris*, no study has been found concerning the optimisation of the colour of the biomass, only of the pigments content, as discussed above.

5.4 Conclusions

The heterotrophic medium of three mutant strains of *C. vulgaris* was significantly optimised by response surface methodology. Although the N concentration was the most significant factor affecting protein content and productivity, as well as biomass colour, the concentrations of P, Zn, Mo, Cu, Ca and Mg also significantly impacted the established models. Overall, this work identified the optimum nutrient composition for the precise cultivation of heterotrophic *C. vulgaris*, leading to improved biomass and protein productivities as well as improved colour of the final product, which is crucial to ensure the successful commercialisation of microalgae-based products in the food market.

6

HETEROTROPHIC CULTIVATION OF *CHLORELLA VULGARIS* YELLOW MUTANT ON INDUSTRIAL SIDESTREAMS: MEDIUM FORMULATION AND PROCESS SCALE UP

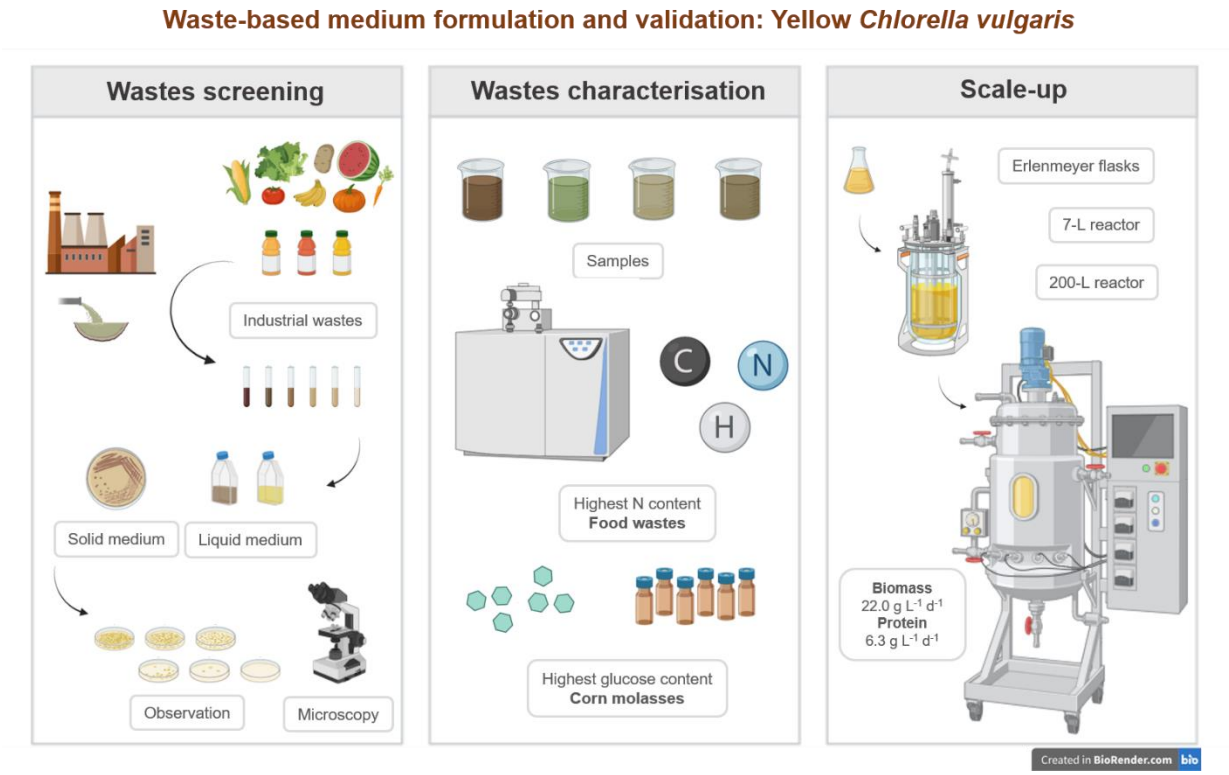
This chapter was adapted from the following submitted research paper (to the Journal of Environmental Chemical Engineering):
M. Trovão, A. Barros, A. Machado, A. Reis, G. Espírito Santo, H. Pedroso, N. Correia, M. Costa, S. Ferreira, H. Cardoso, J. Varela, J. Silva, H. Pereira and F. Freitas, "Heterotrophic cultivation of yellow mutant of *Chlorella vulgaris* on sidestreams: medium formulation and process scale up," *J. Environ. Chem. Eng.*, 2024

ABSTRACT

Microalgal protein is a promising feedstock to complement and/or replace other protein sources. Besides requiring less land and water usage, microalgae production is a more sustainable process, especially if industrial sidestreams are used as nutrient sources. Additionally, the heterotrophic cultivation of microalgae, such as *Chlorella vulgaris*, enables the achievement of higher biomass productivity and lower areal footprint than autotrophic cultivation. Chlorophyll-deficient strains of *C. vulgaris*, such as the yellow strain 7Y, provide microalgal biomass with improved sensory properties. In line with this, a waste-based medium (WM) was formulated to cultivate this strain, aiming at maximum biomass-productivity. In this context, several industrial sidestreams were screened, and two food wastes and corn molasses were selected for their high nitrogen and glucose concentrations, respectively. The waste-based medium formulated was compared to the inorganic optimised medium at laboratory scale in Erlenmeyer flasks and 7-L reactors. The results obtained in the 7-L fermenters revealed that both conditions achieved similar biomass productivities and growth rates of approximately 14 g L^{-1} d^{-1} and 0.8 d^{-1}, respectively. The biomass and protein productivities were further enhanced by supplying a higher nitrogen concentration in the feeding solution when the process was scaled-up to 200-L reactors, reaching 22 and 6.3 g L^{-1} d^{-1}, respectively, thus validating the developed industrial waste-based medium for the efficient cultivation of *C. vulgaris* under heterotrophic conditions.

Keywords: Biomass and protein productivity; Heterotrophic cultivation; Industrial sidestreams; Microalgae; Scale-up.

GRAPHICAL ABSTRACT



6.1 Introduction

The current agricultural practices that come with land, water and air contamination, greenhouse gas emissions, ecosystem degradation and land clearing, must be replaced by the implementation of more sustainable practices [178, 344, 345]. In this context, microalgae arise as natural sources of not only protein but also lipids (including omega-3 fatty acids), carbohydrates, vitamins (e.g., B_{12}), micronutrients (e.g., iron), and antioxidant pigments, thus making them an interesting alternative feedstock for nutritional applications with reduced environmental impact [10, 180, 181, 303]. In addition, microalgal protein from several genera, including *Chlorella*, fits the requirements reported by the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO) [248], containing all the essential amino acids in relevant quantities, being also comparable to other protein sources, such as soy.

Nonetheless, it is challenging to create such products considering the sensory properties of microalgal biomass unleashed by the presence of chlorophyll, such as the fishy smell, green colouration and grassy flavour [7, 19, 181, 249]. Chlorophyll-deficient mutants with yellow or white colorations, for

example, have been isolated through random mutagenesis and selection with metabolic inhibitors of pigments' pathways to attain an improved colour of the biomass, odour and taste [19, 249]. After strain selection, the optimisation of the whole bioprocess (e.g., cultivation conditions, culture medium, among others) might also be carried out to develop an appealing product and to maximize productivity.

Additionally, the production costs might be further decreased by replacing chemical reagents with sidestreams, while contributing to the bioremediation of such industrial byproducts and towards a more sustainable process [240, 272, 273].

Finally, since microalgal biomass is more costly to produce than traditional feedstocks, it is critical to scale up the processes to reach economic feasibility while increasing the value of the biomass. This valorisation can be achieved by developing more appealing products, as mentioned above, but also through other differentiation factors, namely the enrichment of target nutrients to claim the biomass as a source of those, assuring the dietary reference values requirements (DRVs) established by the EFSA [346] and/ or differentiating certifications, for instance, the European organic certification (Regulation EU 2018/848).

Regarding the usage of sidestreams, many reports can be found in the literature about microalgae cultivation with different wastes and effluents. However, most studies have been performed in photoautotrophic or mixotrophic conditions. Few studies have been published reporting cultivation in heterotrophic conditions with sidestreams, namely with sugarcane molasses, crude glycerol, swine wastewater, among others [347–349], which reached low biomass concentrations if no pre-treatment was applied, compared to the highest values reported in the literature for *Chlorella vulgaris* [192]. Higher biomass concentrations have been reported when pre-treatments, such as enzymatic hydrolysis, were carried out before [350]. In addition, since an organic carbon source is used in heterotrophic cultivation, more challenges arise concerning contaminations throughout the process, which prevents the success of waste-based fermentations due to the hardship of securing the sterilisation of these feedstocks.

Many industries, particularly the food and beverage industry, end up wasting a large portion of their production. This year, one-fifth of the all food produced worldwide was wasted [351]. These sidestreams are often overlooked and thrown away. However, they represent a rich source of nutrients, which might actually be useful to produce more food for an increasing population with increasing demands, namely by using these feedstocks to cultivate microalgae. Moreover, from the sustainability point of view, it would be important to reuse wastes at a local level, for example by installing microalgae production facilities near those sources.

This work focused on the development of an appealing microalgal product for food applications, based on a more sustainable production process and biomass valorisation. Thus, a waste-based (WM) medium was developed and compared with the optimised inorganic medium (IM) to cultivate a yellow *Chlorella vulgaris* mutant strain in heterotrophic conditions, aiming at maximizing biomass productivity. To the authors knowledge, this is the first report on the use of sidestreams with no pre-treatment to reach biomass productivities and concentrations comparable to the values achieved under cultivation with inorganic medium as well as an organic-certified medium formulation to cultivate this species under heterotrophic conditions.

6.2 Materials and Methods

6.2.1 Microalgal strains and culture medium

Chlorella vulgaris wildtype axenic inoculum was obtained from Allmicroalgae Natural Products S.A. culture collection, from cryopreserved aliquots stored in liquid nitrogen (–196 °C). The yellow *Chlorella vulgaris* mutant strain, 7Y, was obtained as described in subchapter 3.2. The heterotrophic medium (HM-medium) described by Barros et al. [192], containing 40 mM of ammonium sulphate and 20 g L^{-1} of glucose, was used to cultivate these strains.

6.2.2 Sidestreams screening

6.2.2.1 Sterilisation trials

Several sidestreams, namely corn steep powder (Baolingbao Biology ®), corn steep liquor (Nutropam®), corn (Copam®), beetroot (Greenstim®) and sugarcane molasses (Sidul® and RAR®), apple pulp (Sumol Compal®), cheese whey (Lactogal®), vinasses (Vinaza®) and food wastes (confidential source), were subjected to a standard sterilisation procedure: autoclaving 50 mL in 100-mL bottles, at 121 °C for 40 min. The occurrence of precipitation of each sample was analysed, as well as their sterility. Precipitation was evaluated macroscopically, and a classification was given according to the presence of solids in suspension: "-", no precipitate; "+", some precipitation and solids in suspension; "++", significant precipitation and solids in suspension. The viscosity of each sidestream was also observed and classified as: "o", low viscosity; "oo", intermediate viscosity"; "ooo", high viscosity. After autoclaving, a sample of 1.1 mL was taken from each sidestream, from which 1 mL was added to 9 mL of liquid broth (nutrient broth, HiMedia) and the remaining 100 µL were spread onto plates with solid medium (plate count agar, VWR). The samples in liquid broth and the plates were incubated for 3 days at 30 °C (INCU-Line, VWR), after which a sample was taken to observe contamination macroscopically and by optical microscopy in the case of the liquid samples (Axio Scope A1®, Carl Zeiss Microscopy GmbH, Oberkochen, Germany), in contrast phase to more easily identify eventual contaminations.

6.2.2.2 Chemical characterisation

6.2.2.2.1 Elemental analysis (C, H, N)

The C, H and N content of each sidestream was quantified by elemental analysis (Vario EL III®, Elementar Analyser System; GmbH, Hanau, Germany) according to the manufacturer's instructions (the samples were weighed into aluminium microcapsules, in duplicate). Liquid samples were freeze-dried in a Coolvacuum, Lyomicron (Barcelona, Spain) before weighting and encapsulation.

6.2.2.2.2 Sugar composition

The sugars' concentration and composition of each sidestream were analysed by HPLC, with a CarboPac PA10 column (Dionex, Sunnyvale, CA, USA) equipped with an amperometric detector, as reported by Rodrigues et al. [352]. A solution of anhydrous 99% D-(+)-glucose (Fisher Chemical) and 99% D-(+)-xylose (Sigma-Aldrich) ranging between 1 to 100 ppm was used as standard, and NaOH at 4 mM was used as eluent at 30 °C using a flow rate of 0.9 mL/min.

6.2.2.2.3 Elemental analysis by Inductively Coupled Plasma - Atomic Emission Spectroscopy

According to the sidestreams' screening, corn molasses was selected as the carbon source, and food wastes 1 and 2 were selected for their higher nitrogen content, whose sterilisation was also validated. To further characterise these sidestreams and the concentration of essential micro- and macronutrients, the following elements were quantified by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES), from Horiba Jobin-Yvon, France, model Ultima: B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, N, Ni, P, Zn. For this, samples were previously hydrolysed at 70 °C for 1 h after dilution of 1:4 with H_2SO_4, cooled down to room temperature and filtered before the analysis.

Considering these compositions, a waste-based medium (WM) was formulated by adjusting the concentration of each sidestream to reach a concentration of each nutrient as close as possible to the concentrations of the optimised inorganic medium (IM).

6.2.3 Waste-based medium formulation and validation

6.2.3.1 Growth performance under different carbon sources

The growth performance of strain 7Y was compared under cultivation with two carbon sources, with a 50-mL working volume in 250-mL Erlenmeyer flasks. This trial was carried out in biological triplicates with HM-medium, at 30 °C, at pH 6.5, with PIPES buffer at 50 mM, in an orbital incubator set at 200 rpm (ArgoLab® shaker SKI 4, Carpi, Italy). Dextrose, synonym of glucose (Dextropam), at 500 g L^{-1} was compared to corn molasses, with a final concentration of 20 g L^{-1} of glucose in the medium.

6.2.3.2 Comparison of growth performance with inorganic optimised medium vs waste-based medium

The growth performance of strain 7Y was compared under cultivation with the respective inorganic optimised medium (IM), reported in Chapter 5, and the waste-based medium (WM), with a 50-mL working volume in 250-mL Erlenmeyer flasks. The WM condition was tested with and without Ni supplementation, at the same concentration as in the IM condition, 0.675 μM (stock solution of $NiCl_2.6H_2O$ at 1 mM), since the WM did not contain Ni in its composition. This trial was carried out using biological triplicates in an orbital incubator set at 200 rpm (ArgoLab® shaker SKI 4, Carpi, Italy) at 30 °C, a pH of 6.5, and PIPES buffer at 50 mM.

6.2.3.3 Process validation and scale-up

The growth trials of this yellow strain were validated in 7-L fermenters (New Brunswick BioFlo® CelliGen®115; Eppendorf AG, Hamburg, Germany) with a working volume of 5 L to compare the IM and WM medium when higher cell concentrations were achieved as well as adapting the feeding strategy to the in-house automation control system. Thus, the seed for the 200-L trials was also obtained in these 7-L bench-top reactors.

For the IM condition, trials in the 7-L reactors were conducted in the same conditions as described by Schüler et al. [19]. Briefly, glucose (500 g L^{-1}) and ammonia (24% m/m) were supplied in fed-batch mode to maintain a concentration of 1-20 g L^{-1} and a pH of 6.5, respectively. Silicon-based antifoam was added when necessary.

For the WM condition, both in the 7-L and 200-L reactors, besides using the waste-based basal-medium (WM) instead of the inorganic (IM), fed-batch was performed by replacing glucose with corn molasses and ammonia with food waste 1 (5:1 v/v in the 7-L reactors and 4:1 v/v in the 200-L reactors). Vegetable oil-based antifoam 3001 was added when necessary.

In both IM and WM conditions, growth was carried out at 30 °C, and aseptic sampling was performed twice a day to monitor the culture's growth. In addition, the air inlet flowrate was increased gradually to keep approximately at 1 vvm, and the agitation was increased from 100 to 1200 rpm in the 7-L reactors, and from 100 to 450 rpm in the 200-L reactors, to ensure a sufficient supply of oxygen. A constant dissolved oxygen (DO) value feeding strategy (DO-stat) was used to control substrate concentration throughout growth [353], [354].

6.2.4 Growth assessment

Growth assays, sampling and daily analysis were conducted as described in section 3.1.2.5. as well as biomass productivity and growth rate calculations. In addition, the same correlation between DW and OD_{600} for mutant 7Y mentioned in section 5.2.2. was used.

At the end of each trial, samples were centrifuged at 4500 *g* for 15 min (Hermle® Z300 centrifuge, Gosheim, Germany), the supernatant was discharged, and the resulting pellet was frozen at −20 °C. The obatined pellets were freeze-dried in a Coolvacuum, Lyomicron (Barcelona, Spain) and stored in a desiccator for posterior biochemical analysis.

6.2.5 Protein content

The protein content was determined as described previously in section 3.1.2.6.1.

6.2.6 Statistical analysis

All assays were carried out in biological triplicates and results were analysed as described in section 4.2.6.

6.3 Results and Discussion

6.3.1 Sidestreams screening

6.3.1.1 Sterilisation trials

Several sidestreams were evaluated for the formation of a precipitate upon autoclaving, the sterilisation effectiveness and the samples viscosity, whose results are shown in Table 6.1.

Table 6.1 - Precipitation level of each sidestream was classified as no precipitate (-), some precipitation and solids in suspension (+) and significant precipitation and solids in suspension (++) according to what was observed in each solution after autoclaving for 40 min, at 121 °C. The viscosity of each solution was classified similarly as low viscosity (o), intermediate viscosity (oo) and high viscosity (ooo). Sterilisation effectiveness of each sidestream was classified as sterile (√) and not sterile (X). It is also mentioned if each product holds an organic certification (OC). VO – vegetable oil-based; n.a. – not applicable.

Sidestream	Precipitation	Viscosity	Sterilisation	OC
Corn steep powder	++	oo	√	No
Corn steep liquor	++	ooo	X	No
Corn molasses	-	o	√	No
Beatroot molasses	+	o	√	Yes
Sugar cane Molasses 1	+	ooo	√	No
Sugar cane Molasses 2	+	ooo	√	No
Apple pulp	+	oo	√	No
Cheese whey	++	o	√	No
Vinasses 1	+	ooo	X	Yes
Vinasses 2	+	ooo	X	Yes
Vinasses 3	+	ooo	X	Yes
Food waste 1	-	o	√	Yes
Food waste 2	-	o	√	Yes
Antifoam 1414 (PO)	-	oo	√	No
Antifoam 3001 (VO)	+	oo	√	Yes
Antifoam 4000 (VO)	++	oo	√	Yes
Antifoam 4010 (VO)	++	oo	√	Yes
Antifoam 4020 (VO)	++	oo	√	Yes

Corn steep powder and liquor and the different molasses tested are abundant sidestreams worldwide and are considered byproducts rich in sugar and with great potential as carbon sources. Apple pulp, cheese whey and vinasses are abundant local sidestreams in Portugal, which also impacts

transportation costs. Apple pulp is richer in carbon, while cheese whey and vinasses also have high nitrogen content, besides carbon. Food wastes are also very abundant worldwide and rich in a plethora of nutrients, depending on their origin. According to the Food Waste Index Report of United Nations Environment Programme and the World Food Programme, one-fifth of the food produced for human consumption was wasted in 2024 [351]. Using these sidestreams as feedstocks for food production, in this case, microalgal biomass, enables the re-utilisation of these nutrients and partially addresses the current unsustainable food waste that contributes to environmental issues and food insecurity worldwide. Additionally, some of the sidestreams selected for further testing hold an organic certification, which might enhance the value of microalgal products in the market if a fully organic production process is developed and established.

The more solids in suspension, organic matter, and viscosity a solution presents, the more difficult it is to sterilise [355]. Marques et al. [356] also indicated the presence and concentration of organic carbon as a potential factor promoting microbial growth. For these reasons, it was not possible to effectively sterilise corn steep liquor and vinasses by this standard autoclaving procedure (Table 6.1), which prevents the usage of these substrates in axenic fermentations without further treatments. Interestingly, Marques et al. [356] tested different treatments on different food waste formulations and autoclaving, also at 121 °C for 40 minutes as in this work, effectively eliminated microbial contamination, followed by ohmic treatment (20 kHz, 95 °C) that eliminated contamination in 6 out 7 food wastes, while conventional heating (95 °C for 30 seconds) only eliminated 57% of microbial contamination.

The non-organic antifoam (1414) was the clearer solution, followed by 3001, which was selected to carry out this study, since it presented a less pronounced deposit compared to 4000, 4010 and 4020. Nevertheless, all of them were effectively sterilised.

6.3.1.2 Chemical characterisation

6.3.1.2.1 Elemental analysis and sugar composition

The most important macronutrients in microalgae cultivation are carbon and nitrogen, so an elemental analysis of all sidestreams was carried out to identify those with the highest potential to be used as C and N sources (Table 6.2). Additionally, the sugar composition of each sidestream was determined.

All sidestreams presented a C content between 30-40%, except the food wastes that had much lower values, around 16% (Table 6.2). Regarding the N content of the samples, the highest values (23-30%) were displayed by the latter, so those food wastes were selected for further analysis as potential nitrogen sources. Since the other substrates displayed similar carbon contents, further analyses were also carried out to select the most suitable for the cultivation of *C. vulgaris*, namely the sugar composition, in particular glucose content.

Several sidestreams have been used as carbon sources, but not exclusively, since most lack other important nutrients, to cultivate *Chlorella*, as it is reviewed in Appendix A11, being the most common, crude glycerol and sugarcane molasses for heterotrophic cultivation (Appendix A12).

Table 6.2 - Elemental composition (C, H, N) and sugar composition (including glucose, galactose and fructose) of different sidestreams. Different letters indicate significant differences ($p < 0.05$) between sidestreams. n.a. – not applicable; n.d. – not detected.

	Elemental analysis (%)			Sugar composition (g L^{-1})		
Sidestream	**C**	**N**	**H**	**Glucose**	**Galactose**	**Fructose**
Corn steep powder	$36.27 \pm 0.18^{a,b}$	7.55 ± 0.04^{a}	6.13 ± 0.09^{a}	1.09 ± 0.02^{a}	n.d.	n.a.
Corn steep liquor	$34.54 \pm 0.24^{a,b}$	5.67 ± 0.05^{a}	6.30 ± 0.09^{a}	1.77 ± 0.04^{a}	0.34 ± 0.01^{a}	n.a.
Corn molasses	$35.49 \pm 0.21^{a,b}$	$0.31 \pm 0.08^{a,b}$	6.60 ± 0.05^{a}	501.72 ± 13.00^{c}	n.d.	n.a.
Beatroot molasses	45.10 ± 0.68^{d}	$9.85 \pm 0.04^{a,c}$	$9.54 \pm 0.34^{a,b}$	$0.02 \pm 0.02^{a,b}$	n.d.	n.a.
Sugar cane Molasses 1	$34.58 \pm 0.13^{a,b}$	$0.06 \pm 0.00^{a,b}$	5.77 ± 0.06^{a}	78.09 ± 1.25^{b}	n.d.	n.a.
Sugar cane Molasses 2	$36.23 \pm 0.43^{a,b}$	$0.02 \pm 0.02^{a,b}$	6.26 ± 0.15^{a}	37.66 ± 1.57^{d}	n.d.	n.a.
Apple pulp	$41.82 \pm 1.46^{c,d}$	$0.12 \pm 0.01^{a,b}$	7.69 ± 0.34^{a}	5.8 ± 0.39^{a}	$<2.00^{b}$	$14.95 \pm 0.53^{*}$
Cheese whey	$39.35 \pm 0.13^{a,c}$	1.62 ± 0.03^{a}	6.56 ± 0.02^{a}	0.10 ± 0.00^{a}	0.32 ± 0.03^{a}	n.a.
Vinasses 1	$35.57 \pm 0.60^{a,b}$	4.31 ± 0.12^{a}	6.94 ± 0.20^{a}	1.14 ± 0.03^{a}	n.d.	n.a.
Vinasses 2	$34.91 \pm 0.21^{a,b}$	2.08 ± 0.02^{a}	6.05 ± 0.14^{a}	7.87 ± 0.03^{a}	n.d.	n.a.
Vinasses 3	$36.61 \pm 0.32^{a,c}$	4.27 ± 0.18^{a}	7.02 ± 0.36^{a}	1.02 ± 0.03^{a}	n.d.	n.a.
Food waste 1	$16.44 \pm 1.12^{d,e}$	22.88 ± 3.82^{d}	$5.57 \pm 1.20^{a,c}$	n.a.	n.a.	n.a.
Food waste 2	$15.87 \pm 0.30^{d,e}$	30.06 ± 0.12^{d}	7.25 ± 0.21^{a}	n.a.	n.a.	n.a.

***** Retrieved from Pereira et al. [357]

It has been well-established that glucose is one of the preferred and more efficient carbon sources for *Chlorella* spp. under heterotrophic metabolism [348, 358, 359]. Other monomeric sugars such as fructose and galactose or more complex sugars such as sucrose might be converted to glucose through hydrolytic treatments, namely enzymatic, though that would impact production costs and time. Different approaches have also been reported more recently, namely by combining the cultivation of *C. pyrenoidosa* with other symbiotic microorganisms, such as yeasts, that convert, for example, sucrose to glucose [360, 361].

Since glucose is the preferred C source for *C. vulgaris*, corn molasses, which exhibited the highest glucose concentration (Table 6.2), 501.72 ± 13.00 g L^{-1}, without further treatment, was selected as the most suitable substrate to replace dextrose used in the inorganic-based medium formulation. To the authors knowledge, the use of this substrate for *C. vulgaris* cultivation is reported here for the first time. Together with corn steep liquor, sugarcane molasses have also been used to cultivate this

microalga heterotrophically. However, this is a more sucrose-rich resource as reported by Mirzaie et al. [347]. Other microalgal species, namely *C. protothecoides* [350], *C. zofingiensis* [362] and *Chlorella* sp. [363] (Appendix A11) have also been cultivated in sugarcane molasses. Under these conditions, maximum DW of 70.9 g L^{-1} was achieved by Yan et al. [350], with this carbon (and nitrogen) source, with *C. protothecoides.*

Starch and cellulose are both polymers of glucose. While cellulose has *β*-1,4 glycosidic bonds between monomers, which are very difficult to hydrolyse, starch consists of an amylose skeleton with *α*-1,4 bonds between glucose monomers that branch into amylopectin through *α*-1,6 glycosidic bonds [364]. Plants, fruits and seeds exhibit various types of starch that might vary in size, composition, among other features, which, when fully hydrolysed, either by acidic or enzymatic mechanisms, is split into glucose [364]. Corn is one of the most abundant crops worldwide, and it is used for dextrose production through hydrolysis of the starch fraction. The hydrolysate obtained is then concentrated until saturation, cooled down and sent to crystallizers [365]. In these crystallizers, water circulates to induce crystal formation, and the crystallisation water gives origin to a by-product, that will be called from now on as corn molasses [365, 366]. Figure 6.1 illustrates this process with sugar cane, instead of corn.

Corn molasses is a by-product of this process with high concentrations of glucose, as demonstrated by the HPLC results. In addition, this sidestream might be rich in other nutrients, which will be discussed further.

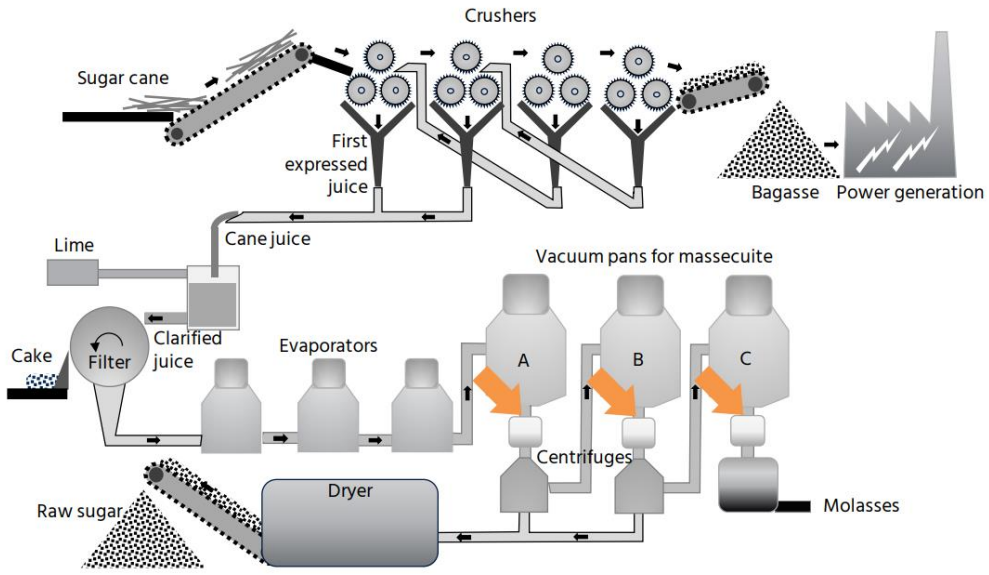


Figure 6.1 - Schematic representation of the process of production of sugarcane molasses. In FOSS Application note AN 5424 Rev. 4 NIRS™ DS2500 Massecuite [367].

6.3.1.2.2 Inductively Coupled Plasma - Atomic Emission Spectroscopy

ICP-AES was carried out to determine the concentration of several elements present in the selected N sources (Food Waste 1 and Food Waste 2) and C source (corn molasses) (Table 6.3).

The Food Waste 1 was the richest in N, while Food Waste 2 presents a higher concentration of P. Corn molasses are mostly a C source, as mentioned before, but it also exhibited almost 5 mM of P and 24 mM Na (Table 6.3). Nickel was the only element that was not detected in any of these side-streams.

Table 6.3 - Elemental analysis to determine the concentration of each nutrient (mM) by ICP, except for nitrogen (* Product datasheet values). n.d. - not detected. n.a. – not applicable.

Element	Food Waste 1	Food Waste 2	Corn molasses
P	369.06	1377	4.9719
K	669.11	0.3877	0.4427
Na	16.573	3.0666	24.446
Mg	0.2115	0.0930	0.0144
Ca	0.0631	0.0996	0.0087
Cu	0.0002	0.0002	0.0002
B	0.0102	0.1380	0.0028
Zn	0.0023	0.0017	n.d.
Mn	0.0075	n.d.	n.d.
Mo	0.0003	0.0003	0.0002
Ni	n.d.	n.d.	n.d.
Fe	0.0193	0.0097	0.0014

Other food wastes with different origins have been analysed by other authors through the same methodologies (elemental analysis and ICP-AES) and compositions vary significantly. For example, Marques et al. [356] analysed seven food wastes, and the concentrations of Ca, Fe, K, Mg, Na and P were around 5, 1000, 10-10000, 25, 1000-10000 and 40000-120000 times lower than the concentrations obtained in this study (Table 6.3).

Based on the composition of the inorganic optimised medium (IM), a waste-based medium was formulated by adjusting the concentration to add each sidestream selected: 0.5% of food waste 1, 3.5% food waste 2, 4% corn molasses and 0.8% food waste-based supplement, rich in trace elements, magnesium and calcium. The only element that was not detected in any of the sidestreams was nickel, although it is included in the IM-medium and HM-medium compositions.

6.3.2 Waste-based medium formulation and validation

6.3.2.1 Growth performance under different carbon sources

Commercial glucose and corn molasses were tested as C sources in terms of growth performance of the yellow *C. vulgaris* strain, 7Y, in shake flask assays (Table 6.4).

Standard glucose and corn molasses led to similar biomass productivity, growth rate, maximum DW and protein contents of the yellow strain 7Y. The values obtained in the present study are similar to those reported for this species and also for this particular mutant strain at this scale (around 2 g L^{-1} d^{-1}, 1 d^{-1} and 30-40% DW of protein content as discussed in the previous Chapter 5. However, standard glucose is almost twice the price of corn molasses (Table 6.4). Since carbon supply is one of the most significant costs in heterotrophic production and microalgal products struggle to compete with other plant-based cheaper products, corn molasses was selected to carry out further work [240]. In addition, regarding the sustainability of the process, corn molasses is a more sustainable resource, given it is an undervalued sidestream from an industrial process. On the other hand, if the goal is to increase the value of the final product, the biomass might be produced entirely with organic certified products, so that the final product also holds that certification. Thus, the previously mentioned carbon sources might be replaced by glucose with organic certification. However, it entails increasing production costs around 3-fold. This change would enable this microalgal biomass with organic certification, since the remaining reagents of the WM-medium formulated in this study also hold this certification.

Table 6.4 - Biomass productivity (r_p), growth rate (μ), maximum DW (X_{max}), and protein content of DW, of *C. vulgaris* 7Y. Different letters indicate statistical differences between using the standard glucose and corn molasses for each response. A price comparison between the two carbon sources is also mentioned.

Condition	r_p (g L^{-1} d^{-1})	μ (d^{-1})	X_{max} (g L^{-1})	Protein (%)
Standard glucose	1.93 ± 0.10[a]	1.05 ± 0.01[a]	7.30 ± 0.35[a]	35.08 ± 0.70[a]
Corn molasses	2.40 ± 0.28[a]	1.09 ± 0.03[a]	9.06 ± 1.02[a]	38.26 ± 1.53[a]

Interestingly, a recent study screened several by-products as potential alternative carbon sources to cultivate Chlorellaceae and Scenedesmaceae microalgae [368]. These authors reported comparable maximum growth rates (1.44 d^{-1}) and biomass productivities, when glucose was replaced by peppermint or liquorice candies manufacturing residues and presented a preliminary evaluation that would allow to reduce the operational costs by up to 85.6% [368]. However, these tests were carried out to a maximum volume of 2 L and the other essential nutrients were provided through inorganic reagents.

6.3.2.2 Comparison of growth performance with inorganic optimised medium vs waste-based medium

The yellow *C. vulgaris* 7Y strain was cultivated with media with different compositions, namely, IM medium, WM-medium and WM-medium supplemented with Ni, aiming to compare the growth performance and protein content of the produced biomass, in shake flask assays (Figure 6.2).

Regarding growth performance, there were no significant differences among the conditions tested, neither for biomass productivity and growth rate, which was 2.7-2.8 g L^{-1} d^{-1} and 1.3 d^{-1},

respectively. The IM condition led to protein content of 50.2 ± 0.8% of DW by the end of the trial, a slightly lower content than what was achieved under the WM and WM+Ni conditions (~54% DW). Pleissner et al. [369] reported a lower growth rate of 1.1 d^{-1} and a protein content of 13.1% DW, with *C. pyrenoidosa* cultivated with a different food waste-based medium.

There are few reports of using food waste to cultivate *Chlorella*. In autotrophic conditions, maximum DWs of 0.86 and 1.7 g L^{-1} have been achieved by *C. vulgaris* with food waste digestates [370, 371]. Under heterotrophic conditions, Pleissner et al. [369] reported a growth rate of 1.1 d^{-1} and a maximum DW of 20 g L^{-1} achieved with *Chlorella pyrenoidosa* cultivated with a food waste hydrolysate, obtained by fungal hydrolysis of fresh rice, noodles, meat and vegetables collected from canteens, and subsequent centrifugation and filtration. This hydrolysis led to a recovery of 31.9 g glucose, 0.28 g free amino nitrogen, and 0.38 g phosphate per 100 g of food waste, while Lau et al. [218] attained 95.8 g L^{-1} glucose, 0.8 g L^{-1} phosphate, 1.5 g L^{-1} FAN and 21.1 mg L^{-1} nitrate in the final hydrolysate, with a similar process applied to bakery and food wastes. These authors reached 4 g L^{-1} of DW, with a growth rate of 0.8 d^{-1} with *C. vulgaris*. Zheng et al. [372] achieved a similar DW of 4.7 g L^{-1} with a university's cafeteria food waste hydrolysate, but with *Chlorella sorokiniana*. More recently, Marques et al. [356] tested several food wastes, coming from fruits, vegetables, dairy, bakery, meat, and fish, but with high C contents, between 32-40%, and low N contents, around 1-2%, on the contrary of the food wastes tested in this work. These authors reported the highest growth rate, 0.82 d^{-1} with for *C. vulgaris*, with a food waste obtained by solubilisation of strawberry, pear, mango, banana, tomato, lettuce, pepper, liquid and solid yogurt and bread wastes and subsequent sedimentation and decantation. Furthermore, the glucose concentration of the latter, around 42%, was not affected by pre-treating the food waste through autoclaving, conventional heating and ohmic heating.

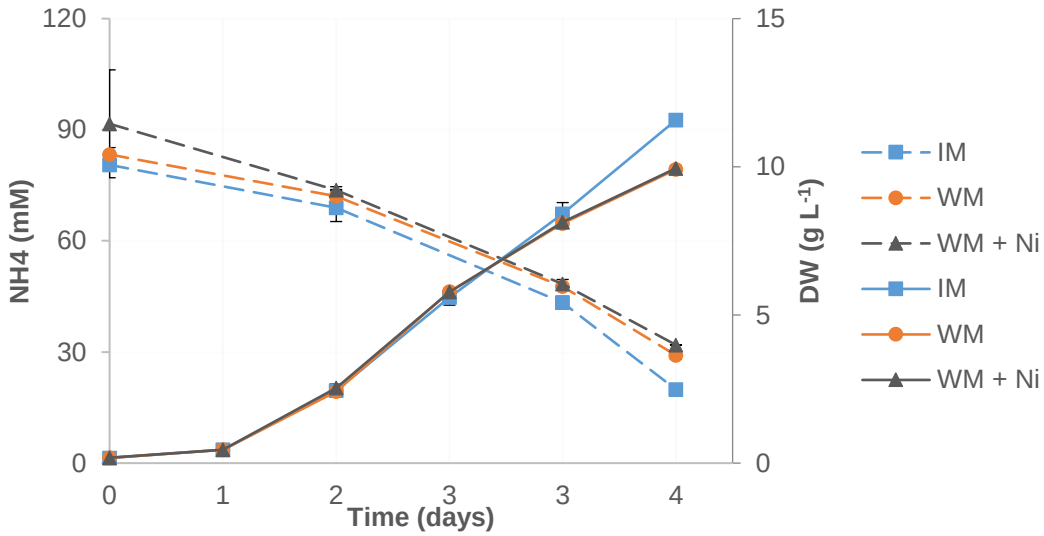


Figure 6.2 - Growth curves of *C. vulgaris* yellow strain 7Y, cultivated heterotrophically in 250-mL Erlenmeyer flasks for 4 days with the inorganic optimised medium (IM), waste-based medium (WM) and waste-based medium

supplemented with Ni (WM + Ni), at the same concentration of IM. Dashed lines represent ammonium consumption and solid lines represent dry weight throughout time. Results are shown as mean ± SD, *n*=3.

A review of these results, as well as cultivation of *C. vulgaris* with other sidestreams, was carried in Appendix A12.

Thus, at this scale, the waste-based medium formulation was validated, and it was concluded that no Ni supplementation was required. For *C. vulgaris*, similar ranges of biomass productivity under heterotrophic conditions have been reported at this scale, ranging from 0.15 to 3.23 g L^{-1} d^{-1} [19, 340]. Concerning protein content, in heterotrophic conditions, a wide range of values (from 20% up to 64% DW) have also been reported for this species [192, 218, 220], as reviewed by Trovão et al. [249].

Moreover, for this particular mutant yellow strain, a maximum value of biomass productivity of 4.03 g L^{-1} d^{-1}, 1.16 d^{-1} of growth rate and 40% DW of protein have been described previously (Chapter 5) after optimisation of the inorganic medium by surface response methodology, so that the biomass productivity was inferior in this case. In contrast, the growth rate and protein content were significantly higher.

6.3.2.3 Process validation and scale-up

Considering the previous results, strain 7Y was cultivated under IM medium and WM-medium in fed-batch mode in 7-L reactors (Figure 6.3), which was further scaled-up to 200-L reactors (Figure 6.4).

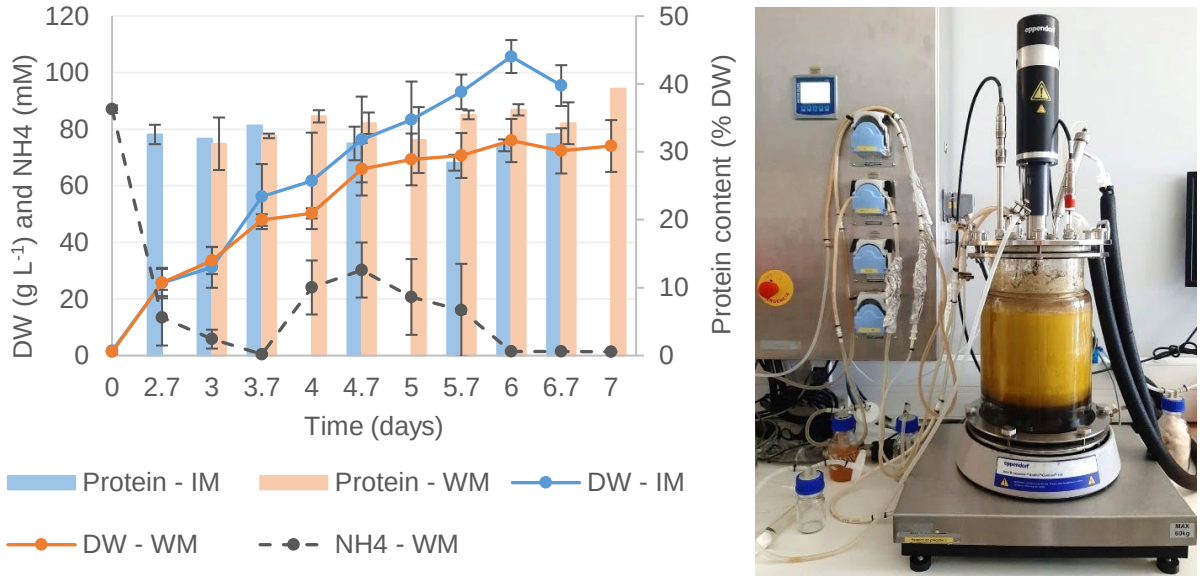


Figure 6.3 - Growth curves and protein contents throughout growth of *C. vulgaris* yellow strain 7Y (on the left), cultivated heterotrophically in 7-L bench-top reactors (on the right) with the inorganic optimised medium (IM) and waste-based medium (WM). The dashed line represents ammonium consumption of the WM-medium throughout time. Results are shown as mean ± SD, *n*=3.

The waste-based medium (WM) reached a biomass productivity and growth rate similar to that of the inorganic medium (IM), approximately 14 g L^{-1} d^{-1} and 0.8 d^{-1}, respectively. While productivity increased by 5-fold compared to the Erlenmeyer flasks trials, the growth rate decreased considerably. Maximum DW of 105.7 ± 5.8 and 76.0 ± 7.6 g L^{-1} were attained around day 6, with the IM-medium and WM-medium, respectively. However, WM-medium appears to have slowed down the growth on day 5, possibly by depletion of some nutrient(s), namely N as it is possible to observe in Figure 6.3 by the exhaustion of ammonium. Therefore, the N concentration in the feed solution of WM-medium was increased by 25% for the 200-L trials, which allowed to increase biomass and protein productivities and growth rate up to 22.0 ± 3.1 g L^{-1} d^{-1}, 6.3 ± 0.6 g L^{-1} d^{-1}, and 1.0 ± 0.1 d^{-1}, respectively (Figure 6.4). However, there were only significant differences concerning protein productivity ($p < 0.05$).

Additionally, it is possible that the absence of Ni affected growth at this scale, due to the high-cell concentrations achieved, compared to those obtained in the Erlenmeyer flasks trial. In the future, it would be worth it to analyse all the concentrations of the remaining nutrients in the broth to find out if there is a need for further supplementation of specific elements, such as Ni, to further maximize growth.

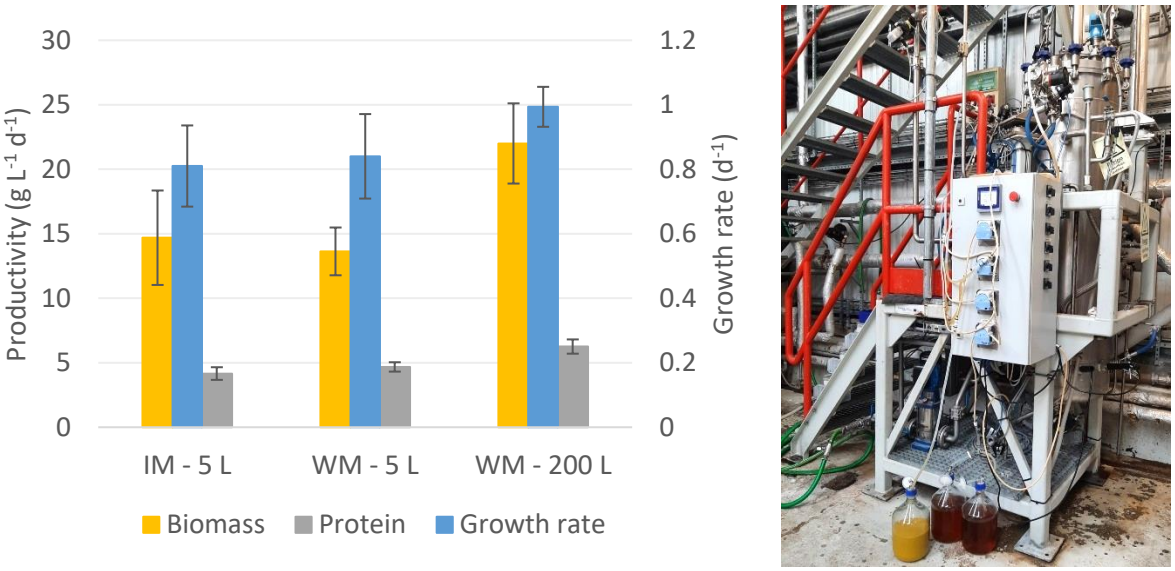


Figure 6.4 - Biomass productivities, growth rates and protein productivity of *C. vulgaris* yellow strain 7Y (on the left), cultivated heterotrophically in 7-L and 200-L reactors (on the right) with the inorganic optimised medium (IM) and waste-based medium (WM). Results are shown as mean ± SD, *n*=3.

Regarding protein, contents around 50% were achieved at the Erlenmeyer scale, with maximum DWs of approximately 10 g L^{-1}, whereas at the 7 L and 200 L scale, protein contents around 40% and 30% of DW were obtained (data not shown), respectively, which might be possibly explained by the shortage of N supply. Ammonium concentrations measured throughout growth in 200-L reactors ranged between 40 to 3 mM (data not shown), indicating that at these high growth rates and cell concentrations, it might be necessary to increase the concentration of N, either during growth or by the end of growth to maximize biomass productivity and protein content of the cells. In the previous Chapter 5, the model obtained concerning protein productivity (and content) indicated that N concentrations up to 70 mM were

required to maximize these responses. Thus, it would be interesting to further study how N feeding could affect the growth and protein of the culture, and to test N over-compensation strategies, as suggested by Xie et al. [227].

To the authors knowledge, this is the first study that reported the growth of *Chlorella*, under heterotrophic conditions at an industrial scale, with a fully waste-based medium. Other studies have reported cultivation with different sidestreams, such as sugarcane molasses and whey, reaching DWs ranging from 4 to 70.9 g L^{-1}, until benchtop reactors scale (maximum 5 L) [218, 350].

Several factors should be further optimised in reactor cultivation, namely oxygen control, cultivation and feeding strategies, which will lead to higher biomass productivities and growth rates [23, 192, 227]. These optimisations should bring these values closer to the higher biomass productivities achieved with scale-up to 0.2- and 5-m^3 reactors, reported by Barros et al. [192], between 27.5-31.9 g L^{-1} d^{-1}. Nonetheless, these results are promising for this fully waste-based medium under heterotrophic conditions, which also holds an organic certification if the glucose supplied is also certified, which is the first organic cultivation process reported for microalgae under heterotrophic conditions.

6.4 Conclusions

A waste-based medium was developed to cultivate a yellow *C. vulgaris* strain under heterotrophic conditions, based on food wastes, rich in nitrogen, and corn molasses, rich in glucose. This is the first fully waste-based medium that allowed the achievement of biomass productivity and growth rate comparable to the values attained with an inorganic-based medium. Moreover, the biomass and protein productivities were further increased, reaching up to 22.0 ± 3.1 g L^{-1} d^{-1}, 6.3 ± 0.6 g L^{-1} d^{-1}, when the process was scaled up to 200-L reactors, reaching values close to the highest productivities reported for this species. Additionally, the waste-based medium was formulated with organic products, which means the microalgal final product might hold an organic certification if the glucose supplied is certified, reported for the first time under heterotrophic cultivation conditions (to the authors knowledge). Overall, the process developed enables the reutilisation of these sidestreams, rich sources of nutrients that otherwise would be thrown away, and the production of food grade microalgal biomass.

7

CONCLUSIONS AND FUTURE WORK

Algae-based feedstocks are not only promising food and feed sources, particularly as alternative protein sources, but also present great potential as biostimulants to apply to crops and enhance their productivity and resilience. The bio-based industry still faces many challenges affecting microalgal production and commercialisation, namely the high production and processing costs and unappealing organoleptic characteristics of biomass. Accordingly, several strain improvement strategies have been developed to improve the productivity, cost-effectiveness, robustness and sensory properties of microalgae, including random mutagenesis, adaptive laboratory evolution and genetic engineering technologies. The different benefits and limitations of each of these approaches, as well as their applications, were reviewed at the beginning of this work. Depending on the target, a suitable strategy should be followed. Random mutagenesis is a time- and cost-effective approach to generate a wide portfolio of strains, whose main limitation is the lack of adequate selection methods. Random mutagenesis was applied here to generate novel strains without the introduction of foreign genetic material and excluding the commercialisation restrictions imposed on genetically modified organisms. In addition, two novel selection strategies were developed and validated: i) a novel metabolic inhibitor, oxyfluorfen, that directly targets the chlorophyll pathway to generate chlorophyll-deficient and differently-coloured mutants, on the contrary to the standard protocols that targeted the carotenoids' pathway; ii) a novel high-throughput methodology, based on fluorescence-activated cell sorting, targeting cell's protein content (and pigments) to isolated protein-rich mutants, instead of lipidic and pigments' contents as most of the existing protocols, without compromising cells' integrity.

The first strategy allowed the selection of yellow (31Y15) and white (31W62) *C. vulgaris* and brownish (37Y01) *S. rubescens* mutants with 95% and 55% decreased chlorophyll contents, respectively. In contrast, the second strategy enabled the generation of 17 protein-rich mutants, out of which mutant 8GF4 was selected, exhibiting a 38% higher biomass productivity, 19% higher protein content and 62% higher chlorophyll content. Furthermore, other previously established selection strategies, namely the use of metabolic inhibitors of the carotenoids' pathway, such as nicotine and norflurazon, were applied after mutagenesis to select chlorophyll-deficient and differently-coloured strains of *C. vulgaris* and *S. rubescens*. A yellow mutant of *C. vulgaris* (7Y) was selected with nicotine, and a whiter mutant of *C. vulgaris* (31W) was isolated after a second mutagenesis round of the mutant 31W62 and selection with norflurazon. In addition, a darker-green mutant of *S. rubescens* (DPA23) with 40% higher protein productivity than the wildtype was selected with diphenylamine and an orange mutant of *S. rubescens* (Ni16) with 95% decreased chlorophyll content was selected with nicotine. Through these studies, it was also demonstrated that the effect of these chemicals on the metabolic pathways is not as

straightforward as expected. Many reports claim that the use of carotenoid inhibitors, for example, usually leads to carotenoid-hyperproducing strains, while in this study, the opposite was verified.

Mutant Ni16 was rich in lutein, β-carotene and astaxanthin, and displayed an interesting orange colour for food and feed applications. This strain was scaled up to a 7-L benchtop reactor and reached 66 ± 2.1 g L^{-1} of DW and biomass productivity similar to the wildtype (~9 g L^{-1} d^{-1}). Moreover, this strain presented an essential amino acid content of 54.9 g 100 g^{-1} and an essential amino acid index (EAAI) of 1.88, which indicates this is a protein source with superior quality (EAAI ≥ 1), according to the World Health Organization (WHO) and Food and Agriculture Organization (FAO) recommendations. On the other hand, mutant 8GF4 achieved 81.9 ± 18.2 g L^{-1} of DW and a protein productivity of 3.9 ± 0.7 g L^{-1} d^{-1} at the same scale. The biomass of the latter (0.1 g L^{-1}) was applied to garden cress seeds, improving the germination index and the relative total growth by 7% and 19%, respectively, highlighting its potential as a biostimulant. To further enhance the biostimulant potential of this novel strain, it would be interesting to test the effect of different downstream treatments in the future, namely by disrupting cells. Additionally, the compounds that specifically increase biostimulant and bioprotective activity, possibly amino acids, phytohormones, pigments, etc., should be further investigated, namely by identifying such molecules and their mechanism of action. The identification of these chemicals might also unravel novel opportunities, since strain improvement approaches might be studied to increase their content.

To further improve the green (8G), yellow (7Y) and white (31W) mutants of *C. vulgaris*, a novel optimisation pipeline targeting multiple responses was developed. This innovative study described for the first time (to the best of the authors' knowledge) an integrated optimisation of the cultivation conditions of three *C. vulgaris* mutants, aiming at more appealing colours, higher protein contents and higher biomass and protein productivities. Through design of experiments and surface response methodologies, screening assays were carried out to select the most significant factors, and different nutrient concentrations were optimised by modelling these responses not only individually but also considering the interactions between the factors affecting them. This strategy allowed to improve the biomass productivity, specific growth rate, protein content and productivity of these mutants by up to 70, 12, 61 and 94%, respectively. Additionally, a methodology to measure and optimise the coloration of microalgal biomass and a correlation of this response with protein content and biomass productivity was also presented here for the first time (to the authors' knowledge). This method allowed the yellow and white colorations of the mutants to improve by up to 20%. Finally, protein content and productivity, a poorly reported topic in the literature, was deeply discussed here, namely which factors affect it and how.

The development of more sustainable microalgal-production processes not only contributes to higher cost-effectiveness but also might have a role in the bioremediation of industrial sidestreams, which are rich feedstocks of important nutrients for microalgal growth. Therefore, a waste-based medium was formulated to cultivate the yellow strain 7Y of *C. vulgaris*. The most abundant and required nutrients for microalgal growth are N, P and C. Thus, two food wastes, rich in ammonium and phosphate, as well as corn molasses, rich in glucose, were selected in the screening phase, as the sidestreams with more potential to replace the inorganic medium previously optimised. Then, a waste-based medium was formulated by adjusting the concentration of each sidestream to the closest possible concentrations

of each nutrient in the inorganic medium. Both formulations were compared and achieved a similar biomass productivity and growth rate, around 14 g L^{-1} d^{-1} and 0.8 d^{-1}, respectively. These values were further enhanced when this strain was cultivated in 200-L reactors with a higher concentration of nitrogen, which led to an average productivity of 22 g L^{-1} d^{-1}, which is close to the highest productivity values reported for this species. Besides reporting the formulation and validation of the fully-waste-based medium at an industrial scale, with such biomass concentrations, which constitutes a significant progress compared to previous reports, an organic-certified version of this medium for cultivation under heterotrophic conditions was also proposed for the first time (to the author's knowledge) (by supplying organic glucose in fed-batch instead of corn molasses), which might increase considerably the market value of this microalgal product.

Overall, the whole production pipeline of *C. vulgaris* was improved in this work, from strain selection to medium optimisation and scale-up. This study not only targeted multiple responses, but also critically discussed the metabolic pathways involving these factors, compared with the results available in the literature and suggested future possibilities. This work paved the way for the biobased industry to overcome several challenges and achieve significant progress, namely, allowing the improvement of the attractiveness, cost-effectiveness, and nutritional value of microalgal products for different applications. However, there are several points that require additional optimisation. The impact of the dissolved oxygen, oxygen control cascades as well as feeding strategies, on the biomass and protein productivity, and colour, should be further studied, particularly the concentration of nitrogen throughout growth, or even the possibility of a two-stage process with different nitrogen concentrations.

Regarding strain improvement, there are still a number of other tests that could be carried out to further address the current hurdles of this industry. For example, after mutagenesis, reduced temperatures could be applied to select mutants with the same nutritional and organoleptic characteristics as the previously selected ones but with lower optimal cultivation temperatures, to reduce the energetic costs of cooling down the algal broth during the heterotrophic cultivation, and to potentially increase protein yield, as it has been reported previously. Another approach to reduce production costs, could be selecting mutants able to grow in oxygen-limited conditions, to reduce the energy expended by agitation, one of the most significant outlays regarding energy consumption under heterotrophic conditions. On the other hand, after mutagenesis, the cells could be plated onto a medium with alternative carbon sources, such as sucrose, lactose and fructose, to be able to cultivate these strains under cheaper and more abundant industrial sidestreams, such as sugarcane molasses, cheese whey and fruit pulp. Concerning the functional properties of the biomass, such as water solubility, foaming capacity and emulsifying capacity, as required by the food industry to develop novel food products, strain improvement might also be a useful tool. For instance, to enhance the emulsifying capacity, mutants could be selected, for example, under nitrogen depletion or through fluorescence-activated cell sorting, or both combined, to isolate strains with higher lipid contents. Moreover, other selection methodologies might be developed to decrease downstream processing costs. For example, larger cells could be selected to reduce the time and costs of concentrating cells. Finally, it would also be interesting to develop products with enhanced micronutrient content to develop supplements that are richer in iron and/or vitamin B12.

For this, cells might be plated onto media with increasing concentrations of these micronutrients to induce tolerance to such concentrations.

Besides generating novel mutants with different characteristics, it would be interesting to sequence the genotype of such mutants to identify the mutated genes and how different metabolic pathways, including the pleiotropic effects on untargeted pathways, were affected.

The co-cultivation of microalgae with other microorganisms also holds high potential but is still quite under-explored. For example, the use of symbiotic yeast in co-cultivation with microalgae has been reported to convert sucrose to glucose, a substrate more prone to being used efficiently by algae cells. On the other hand, specific bacteria strains could be used to enhance overall biomass productivity and to enhance the content of valuable compounds, such as vitamin B12.

To maximize productivities, different cultivation strategies, as semi-continuous and continuous modes, should also be considered to the detriment of batch mode. The batch process optimized in this work could be adapted to test the operation in semi-continuous mode, which could increase sharply the overall yield, avoid lag phases, work at maximum growth rate, and decrease the down time of the reactors, namely the time and costs spent in cleaning and reactor preparation, while maintaining the characteristics of the biomass.

Another poorly studied topic, which would be important to bring microalgae to the frontline in what comes to food and feed products, and supplements, is the actual demonstration of the effect of consuming such products in humans and animals. Although there are some studies, mostly in animals, reporting the effect of supplementing feed with microalgae, namely immunostimulatory effects, there is scarce information on these effects in humans. The consumption of microalgae by humans should be studied regarding first the bioavailability and bioaccessibility of important nutrients after digestive processes, and then the actual role of microalgae in health issues, such as nutrient deficiency and respective consequences.

Finally, the direct usage of the concentrated biomasses attained in heterotrophic cultivation for different applications, could be further studied to avoid downstream processing costs, though storage and shipping costs would have to be assessed considering the need for refrigerated conditions.

Overall, microalgae are definitely a promising alternative feedstock with great potential for different biotechnological applications, namely in the food and feed industries, cosmetics, pharmaceutics, nutraceutics, textile industry, development of novel materials, among others. Although a lot of knowledge has been acquired in the last decades, the potential of algae is still just starting to unfold. These diverse group of organisms is quite underexplored since very few species have been studied and even fewer met actual practical applications. The species currently produced and commercialised also need further optimisation and scale to reach their full potential in the market. This work attempted to address some of the current hurdles that prevent the success of these microalgal products, presented novel methodologies that might be useful for that purpose and insights into future perspectives.

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APPENDIX

CHAPTER 5

A.1

A1 - Randomized Regular Two-Level Factorial Design: conditions tested for each factor for the 20 runs of the screening of the macronutrients of three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W. The concentrations of the nutrients are indicated in mM. A - ammonium; Ni - nitrate; U - urea.

Fac-tor	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
Run	[N]	[P]	[Ca]	[Na]/[K]	[Mg]	N source	[N]	[P]	[Ca]	[Na]/[K]	[Mg]	N source	[N]	[P]	[Ca]	[Na]/[K]	[Mg]	N source
Strain	8G						7Y						31W					
1	50	50	2.25	0.6	7.5	U	50	50	1.2	0.6	7.5	U	50	50	1.2	0.6	7.5	N
2	50	50	2.25	0.6	7.5	A	50	50	1.2	0.6	7.5	A	50	50	1.2	0.6	7.5	A
3	80	20	4.00	0.9	3.0	A	80	20	2.0	0.9	3.0	A	80	20	2.0	0.9	3.0	A
4	20	80	0.50	0.3	12.0	U	20	80	0.4	0.3	12.0	U	20	80	0.4	0.3	12.0	N
5	50	50	2.25	0.6	7.5	A	50	50	1.2	0.6	7.5	A	50	50	1.2	0.6	7.5	A
6	80	20	0.50	0.9	12.0	U	80	20	0.4	0.9	12.0	U	80	20	0.4	0.9	12.0	N
7	20	80	4.00	0.9	3.0	U	20	80	2.0	0.9	3.0	U	20	80	2.0	0.9	3.0	N
8	80	80	0.50	0.9	3.0	A	80	80	0.4	0.9	3.0	A	80	80	0.4	0.9	3.0	A
9	20	80	4.00	0.3	3.0	A	20	80	2.0	0.3	3.0	A	20	80	2.0	0.3	3.0	A
10	20	20	4.00	0.3	12.0	U	20	20	2.0	0.3	12.0	U	20	20	2.0	0.3	12.0	N
11	80	80	4.00	0.3	12.0	A	80	80	2.0	0.3	12.0	A	80	80	2.0	0.3	12.0	A
12	80	20	0.50	0.3	12.0	A	80	20	0.4	0.3	12.0	A	80	20	0.4	0.3	12.0	A
13	20	80	0.50	0.9	12.0	A	20	80	0.4	0.9	12.0	A	20	80	0.4	0.9	12.0	A
14	80	80	0.50	0.3	3.0	U	80	80	0.4	0.3	3.0	U	80	80	0.4	0.3	3.0	N
15	80	80	4.00	0.9	12.0	U	80	80	2.0	0.9	12.0	U	80	80	2.0	0.9	12.0	N
16	50	50	2.25	0.6	7.5	U	50	50	1.2	0.6	7.5	U	50	50	1.2	0.6	7.5	N
17	20	20	0.50	0.9	3.0	U	20	20	0.4	0.9	3.0	U	20	20	0.4	0.9	3.0	N
18	80	20	4.00	0.3	3.0	U	80	20	2.0	0.3	3.0	U	80	20	2.0	0.3	3.0	N
19	20	20	0.50	0.3	3.0	A	20	20	0.4	0.3	3.0	A	20	20	0.4	0.3	3.0	A
20	20	20	4.00	0.9	12.0	A	20	20	2.0	0.9	12.0	A	20	20	2.0	0.9	12.0	A

A.2

A2 - Randomized Regular Two-Level Factorial Design: conditions tested for each factor for the 20 runs of the screening of the micronutrients of three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W. The concentrations of the nutrients are indicated in mM, except for vitamin solution, in which the concentration of the stock solution used, is presented (x).

Fac-tor	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E	F	G	H
Run	Cu	B	Zn	Mn	Mo	Ni	Fe	Vit	Cu	B	Zn	Mn	Mo	Ni	Fe	Vit	Cu	B	Zn	Mn	Mo	Ni	Fe	Vit
Strain	8G								7Y								31W							
1	0.002	0.500	0.300	0.02	0.00050	0.00010	1.000	2.500	0.060	0.050	0.030	0.20	0.5000	0.00100	0.050	0.250	0.060	0.050	0.030	0.20	0.5000	0.00100	0.050	0.250
2	0.200	0.050	0.030	0.02	0.50000	0.00010	1.000	2.500	0.006	0.050	0.030	0.20	0.0050	0.00100	0.500	2.500	0.006	0.050	0.030	0.20	0.0050	0.00100	0.500	2.500
3	0.002	0.050	0.300	0.20	0.50000	0.00010	0.010	2.500	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375
4	0.002	0.500	0.030	0.02	0.50000	0.00100	0.010	2.500	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375
5	0.101	0.275	0.165	0.11	0.25025	0.00055	0.505	1.375	0.006	0.500	0.300	0.02	0.0050	0.00010	0.500	2.500	0.006	0.500	0.300	0.02	0.0050	0.00010	0.500	2.500
6	0.101	0.275	0.165	0.11	0.25025	0.00055	0.505	1.375	0.006	0.050	0.030	0.02	0.0050	0.00010	0.050	0.250	0.006	0.050	0.030	0.02	0.0050	0.00010	0.050	0.250
7	0.200	0.500	0.030	0.20	0.00050	0.00010	0.010	2.500	0.006	0.500	0.030	0.20	0.5000	0.00010	0.500	0.250	0.006	0.500	0.030	0.20	0.5000	0.00010	0.500	0.250
8	0.200	0.500	0.300	0.20	0.50000	0.00100	1.000	2.500	0.060	0.500	0.030	0.02	0.0050	0.00100	0.500	0.250	0.060	0.500	0.030	0.02	0.0050	0.00100	0.500	0.250
9	0.002	0.050	0.030	0.20	0.00050	0.00100	1.000	2.500	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375
10	0.002	0.500	0.030	0.20	0.50000	0.00010	1.000	0.250	0.006	0.050	0.300	0.20	0.5000	0.00010	0.050	2.500	0.006	0.050	0.300	0.20	0.5000	0.00010	0.050	2.500
11	0.200	0.050	0.030	0.20	0.50000	0.00100	0.010	0.250	0.060	0.050	0.030	0.02	0.5000	0.00010	0.500	2.500	0.060	0.050	0.030	0.02	0.5000	0.00010	0.500	2.500
12	0.002	0.500	0.300	0.20	0.00050	0.00100	0.010	0.250	0.060	0.500	0.030	0.20	0.0050	0.00010	0.050	2.500	0.060	0.500	0.030	0.20	0.0050	0.00010	0.050	2.500
13	0.101	0.275	0.165	0.11	0.25025	0.00055	0.505	1.375	0.060	0.050	0.300	0.20	0.0050	0.00010	0.500	0.250	0.060	0.050	0.300	0.20	0.0050	0.00010	0.500	0.250
14	0.002	0.050	0.300	0.02	0.50000	0.00100	1.000	0.250	0.006	0.500	0.030	0.02	0.5000	0.00100	0.050	2.500	0.006	0.500	0.030	0.02	0.5000	0.00100	0.050	2.500
15	0.200	0.500	0.030	0.02	0.00050	0.00100	1.000	0.250	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375	0.033	0.275	0.165	0.11	0.2525	0.00055	0.275	1.375
16	0.200	0.050	0.300	0.02	0.00050	0.00100	0.010	2.500	0.060	0.050	0.300	0.02	0.0050	0.00100	0.050	2.500	0.060	0.050	0.300	0.02	0.0050	0.00100	0.050	2.500
17	0.200	0.500	0.300	0.02	0.50000	0.00010	0.010	0.250	0.060	0.500	0.300	0.20	0.5000	0.00100	0.500	2.500	0.060	0.500	0.300	0.20	0.5000	0.00100	0.500	2.500
18	0.002	0.050	0.030	0.02	0.00050	0.00010	0.010	0.250	0.006	0.500	0.300	0.20	0.0050	0.00100	0.050	0.250	0.006	0.500	0.300	0.20	0.0050	0.00100	0.050	0.250
19	0.200	0.050	0.300	0.20	0.00050	0.00010	1.000	0.250	0.060	0.500	0.300	0.02	0.5000	0.00010	0.050	0.250	0.060	0.500	0.300	0.02	0.5000	0.00010	0.050	0.250
20	0.101	0.275	0.165	0.11	0.25025	0.00055	0.505	1.375	0.006	0.050	0.300	0.02	0.5000	0.00100	0.500	0.250	0.006	0.050	0.300	0.02	0.5000	0.00100	0.500	0.250

A.3

A3 - Examples of optimization reports under heterotrophic conditions, where the respective optimised factor and result as well as species and goal are referred. (n.a. - not applicable and/or information not found; AS - ammonium sulfate; U - urea; N - nitrate; A - ammonium.

Species	Temperature (°C)	pH	N source	Goal	Results	Other	Reference	Year	DOI
Chlorella protothecoides	n.a.	Tested: 5-8 **Optimal:** 6	n.a.	Maximize growth rate	**pH 6:** 1.08 d^{-1} other pH: 0.82-0.98 d^{-1}	n.a.	Shi *et al.*[373]	2006	10.1002/mnfr.200600037
Chlorella pyrenoidosa	Tested: 35-40 **Optimal:** 40	n.a.	n.a.	Improve harvesting efficiency	1.45 d^{-1}	n.a.	Dai *et al.* [315]	2022	10.3389/fbioe.2022.1072942
Chlorella sorokiniana	n.a.	n.a.	Tested: N, U **Optimal:** N	Maximize protein content	n.a.	n.a.	Chen *et al.* [328]	2023	10.1016/j.biortech.2022.128538
***Chlorella* sp.**	n.a.	n.a.	Tested: A, U, N **Optimal:** N	Improve growth	n.a.	n.a.	Kim *et al.* [290]	2019	10.1038/s41598-019-55854-9
Chlorella vulgaris	Tested: 20-36 **Optimal:** 23	n.a.	n.a.	Maximize lipid productivity	n.a.	Box-Behnken Design	Xie *et al.* [313]	2013	10.4014/jmb.1301.01046
Chlorella vulgaris	n.a.	Tested: 5-8 **Optimal:** 7 and 8	n.a.	Maximize biomass productivity	**pH 7-8:** 0.27-0.30 g L^{-1} d^{-1}	n.a.	Sakarika and Kornaros [374]	2016	10.1016/j.biortech.2016.08.033
Euglena gracilis	Tested: 25-35 **Optimal:** 28 and 32	Tested: 2-7.5 **Optimal:** 2-5.5	Tested: AS, U, N **Optimal:** AS	Increase protein content/yield	48-53% of DW	n.a.	Xie *et al.* [317]	2023	10.3390/md21100519

A.4

A4 - Summarized parameters retrieved from the Pareto chart, Half-normal plot and ANOVA analysis for three responses of the screening of the macronutrients of three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W: biomass productivity, specific growth rate and protein content.

Biomass productivity									
Factors		**8G**			**7Y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[N] (mM)	2.0975	14.7521	<0.0001	0.3381	6.9521	<0.0001	0.2986	14.1591	<0.0001
[P] (mM)	-	0.914361	-	-0.2094	4.30512	0.0012	0.0588	2.78796	0.0192
[Ca] (mM)	-	0.801903	-	-	0.113028	-	0.0955	4.52805	0.0011
[Na]/[K]	-	1.2903	-	-0.0980	2.01464	0.069	-	0.106727	-
[Mg] (mM)	-	1.58578	-	-	0.583598	-	0.0916	4.34321	0.0015
N source	-	0.994261	-	-0.2049	4.2125	0.0015	-0.1450	6.87651	<0.0001
Model									
***R*²**		0.923			0.9101			0.9677	
***p*-value**		<0.0001			<0.0001			<0.0001	
Specific growth rate									
Factors		**8G**			**7Y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[N] (mM)	0.1297	38.8683	<0.0001	-	0.715113	-	0.1377	12.0866	<0.0001
[P] (mM)	-0.0327	9.78131	<0.0001	-0.1142	3.98096	0.001	-	0.797069	-
[Ca] (mM)	-	0.016958	-	0.0713	2.48446	0.0229	-	0.076166	-
[Na]/[K]	-	0.871722	-	-	1.27351	-	-	0.14976	-
[Mg] (mM)	-	0.253715	-	-	1.47873	-	-	0.521288	-
N source	-0.0555	16.6143	<0.0001	-0.1900	6.4369	<0.0001	-0.0299	2.62585	0.0191
Model									
***R*²**		0.9954			0.8367			0.9122	
***p*-value**		<0.0001			<0.0001			<0.0001	
Protein content									
Factors		**8G**			**7y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[N] (mM)	0.9882	24.2777	< 0.0001	-0.0278	29.8287	<0.0001	15.6048	47.8059	< 0.0001
[P] (mM)	-	1.72176	-	-	0.469028	-	-0.7039	2.1563	0.0482
[Ca] (mM)	-	0.362791	-	-	0.250932	-	-	0.406353	-
[Na]/[K]	-	0.558606	-	-	1.58424	-	-	1.27158	-
[Mg] (mM)	-	0.861107	-	-	1.17541	-	-	0.665955	-
N source	0.0967	3.10158	0.0194	-	1.80991	-	-	1.54371	-
Model									
***R*²**		0.9707			0.9817			0.993	
***p*-value**		< 0.0001			<0.0001			< 0.0001	

A.5

A5 - Summarized parameters retrieved from the Pareto chart, Half-normal plot and ANOVA analysis for three responses of the screening of the micronutrients of three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W: biomass productivity, specific growth rate and protein content.

Biomass productivity									
Factors		**8G**			**7Y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[Cu] (mM)	0.1835	2.78982	0.014	0.1620	7.68295	<0.0001	-0.0384	3.62697	0.004
[B] (mM)	-	0.194642	-	-	0.983219	-	0.0369	3.48512	0.0051
[Zn] (mM)	0.2531	3.84826	0.0019	-0.0471	2.13582	0.0724	-0.0445	4.21122	0.0015
[Mn] (mM)	-	1.0098	-	-	0.692582	-	-	0.599267	-
[Mo] (mM)	0.2950	4.48577	0.0006	-	0.583536	-	-	0.072177	-
[Ni] (mM)	-	0.928469	-	-	0.304778	-	-	0.01658	-
[Fe] (mM)	-0.4076	6.19706	<0.0001	-	1.75094	-	-	1.17946	-
[Vit solution] (x)	-0.1458	2.21652	0.0414	-	0.147714	-	-	1.14145	-
Model									
R²		0.8891			0.8915			0.8945	
***p*-value**		<0.0001			<0.0001			<0.0001	
Specific growth rate									
Factors		**8G**			**7Y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[Cu] (mM)	0.0066	4.58792	0.0018	0.0266	4.28846	0.0012	-	1.47184	-
[B] (mM)	-0.0057	3.96321	0.0042	-	0.962773	-	-	1.31039	-
[Zn] (mM)	-0.0079	5.50539	0.0006	-0.0117	1.90697	0.0766	-0.0056	1.79605	0.0885
[Mn] (mM)	-0.0071	4.91065	0.0012	-	0.436652	-	-	0.774053	-
[Mo] (mM)	-0.0112	7.79959	<0.0001	-	0.396595	-	-	0.226127	-
[Ni] (mM)	-	0.974029	-	-	0.0445556	-	0.0070	2.26264	0.0388
[Fe] (mM)	-	0.043707	-	0.0104	1.69069	0.111	-	0.150146	-
[Vit solution] (x)	-	1.59526	-	-	0.872094	-	-0.0061	1.9714	0.0651
Model									
R²		0.9718			0.8245			0.8367	
***p*-value**		<0.0001			0.0025			0.0028	
Protein content									
Factors		**8G**			**7y**			**31W**	
	Coef. E.	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**	**Coef. E.**	***t*-value**	***p*-value**
[Cu] (mM)	-0.4166	2.96735	0.0104	-	0.31288	-	-	0.592752	-
[B] (mM)	-	0.876532	-	-	0.360768	-	0.6943	4.26549	0.0021
[Zn] (mM)	-	1.2709	-	-	1.44865	-	-	0.60921	-
[Mn] (mM)	-	0.747294	-	-	0.183757	-	-0.6442	3.95773	0.0032
[Mo] (mM)	-	1.17135	-	-	0.849166	-	-	0.75008	-
[Ni] (mM)	-	0.454516	-	0.0722	2.13679	0.054	-	0.614099	-
[Fe] (mM)	-	0.603718	-	0.3634	10.5861	<0.0001	-0.3214	1.97481	0.0717
[Vit solution] (x)	0.4188	2.98259	0.0101	0.1567	4.54876	0.0008	0.8188	5.03046	0.0007
Model									
R²		0.8823			0.9788			0.9565	
***p*-value**		<0.0001			<0.0001			0.0002	

A.6

A6 - Equations of actual factors of each model of the three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W for each response analysed: r_p - biomass productivity (g L d^{-1}), μ - growth rate (d^{-1}), PP - protein productivity (g L d^{-1}) and C - colour (models obtained either by L* - brightness coordinate, b* - yellow-blue coordinate and a* - green-red coordinate, based on CIELAB colour space).

Strain	Response	Equation
8G	r_p	+0.64168 +0.061815 N +1.53251 Zn -0.000491 N² -0.000019 P² -10.44263 Mo²
	μ	+1.31102 -1.26790 Zn +0.030431 P*Zn -0.000051 P²
	PP	-0.269175 +0.029529 N -1.58923 Zn -0.000165 N² +6.26966 Zn² -0.782906 Mo²
	C (L*)	+26.15967 +0.105481 N +55.72603 Zn +109.61192 Mo -1.09419 N*Mo -0.250731 P*Zn +1.61354 P*Mo -514.67692 Zn*Mo -0.000413 N² -0.000797 P² -420.44615 Mo²
7Y	r_p	+2.29082 +0.004750 N +2.43643 Zn -10.25807 Cu^2
	μ	+0.978839 +0.000324 N +0.002822 N*Zn -0.881204 Cu^2
	PP	+0.029925 +0.010259 N +0.956390 Zn
	C (b*)	+28.42 -0.5804 N -0.3296 P -0.2946 Cu -0.4631 N*P +0.4456 N*Cu -0.2740 N^2 +0.2797 P^2 + 1.09 D^2
31W	r_p	+1.39953 +0.075554 N +0.000398 N*P -0.000748 N² -0.000210 P²
	μ	+0.738039 +0.014554 N -0.000046 N*Ca +0.000029 P*Ca -0.000114 N²
	PP	+0.111515 +0.017773 N
	C (a*)	-4.23958 -0.054812 N -0.024771 P +0.345833 Ca

A.7

A7 - Parameters of each model obtained for the four responses analysed for the mutant 8G of *Chlorella vulgaris*: biomass productivity, specific growth rate, colour and protein productivity.

Strain	**8G**											
Factors	**Biomass productivity**			**Specific growth rate**			**Colour (L*)**			**Protein productivity**		
	Coef. E.	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**
N	0.2546	55.59	<0.0001	-	-	-	-0.3372	7.43	0.018	0.2602	782.74	<0.0001
P	-	-	-	-	-	-	-	-	-	-	-	-
Zn	0.0766	5.03	0.0343	0.0098	2.73	0.1129	0.2503	6.78	0.0692	0.0146	3.42	0.0775
Mo	-	-	-	-	-	-	-0.0964	3.74	0.4663	-	-	-
N*P	-	-	-	-	-	-	-	-	-	-	-	-
N*Zn	-	-	-	-	-	-	-	-	-	-	-	-
N*Mo	-	-	-	-	-	-	-0.5158	10.24	0.0050	-	-	-
P*Zn	-	-	-	0.012	2.51	0.1277	-0.4067	6.36	0.0213	-	-	-
P*Mo	-	-	-	-	-	-	0.6508	16.3	0.0008	-	-	-
Zn*Mo	-	-	-	-	-	-	-0.6121	14.41	0.0013	-	-	-
N^2	-0.2167	46.99	<0.0001	-	-	-	-0.1757	2.29	0.1476	-0.0658	46.41	<0.0001
P^2	-0.1213	14.72	0.0008	-0.0449	50.18	<0.0001	-0.4332	13.93	0.0015	-	-	-
Zn^2	-	-	-	-	-	-	-	-	-	0.0138	3.49	0.0746
Mo^2	-0.0763	5.83	0.0237	-	-	-	-0.2732	0.3323	0.0302	-0.0137	3.45	0.076
Model	**Reduced quadratic**			**Reduced quadratic**			**Reduced quadratic**			**Reduced quadratic**		
	R^2	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**
	0.8312	0.796	<0.0001	0.7094	0.6698	<0.0001	0.8050	0.6966	0.0001	0.9725	0.9665	<0.0001

A.8

A8 - Parameters of each model obtained for the four responses analysed for the mutant 7Y of *Chlorella vulgaris*: biomass productivity, specific growth rate, colour and protein productivity.

7Y												
Factors	**Biomass productivity**			**Specific growth rate**			**Colour (b*)**			**Protein productivity**		
	Coef. E.	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**
N	0.0629	4.97	0.0354	0.0057	4.09	0.055	0.5804	7.34	0.0139	0.1539	169.35	<0.0001
P	-	-	-	-	-	-	-0.3296	2.37	0.1403	-	-	-
Cu	-	-	-	-	-	-	-0.2946	1.89	0.185	-	-	-
Zn	0.0548	4.59	0.0425	-	-	-	-	-	-	0.0215	3.31	0.0799
N*P	-	-	-	-	-	-	-0.4631	3.12	0.0935	-	-	-
N*Cu	-	-	-	-	-	-	0.4456	2.89	0.1057	-	-	-
N*Zn	-	-	-	-0.0055	3.08	0.0923	-	-		-	-	-
P*Cu	-	-	-	-	-	-	-	-	-	-	-	-
P*Zn	-	-	-	-	-	-	-	-	-	-	-	-
Cu*Zn	-	-	-	-	-	-	-	-	-	-	-	-
N^2	-	-	-	--	-	-	-0.274	1.81	0.1941	-	-	-
P^2	-	-	-	-	-	-	0.2797	1.89	0.1854	-	-	-
Cu^2	-0.1021	18.09	0.0003	-0.0098	17.61	0.0003	-	-	-	-	-	-
Zn^2	-	-	-	-	-	-	1.09	18.92	0.0003	-	-	-
Model	**Reduced quadratic**			**Reduced quadratic**			**Reduced quadratic**			**Reduced linear**		
	R^2	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**	**R^2**	**Adjusted R^2**	***p*-value**
	0.5486	0.4922	0.0002	0.5367	0.4762	0.0004	0.6838	0.5507	0.0016	0.8648	0.8548	<0.0001

A.9

A9 - Parameters of each model obtained for the four responses analysed for the mutant 31W of *Chlorella vulgaris*: biomass productivity, specific growth rate, colour and protein productivity.

	31W											
Factors	**Biomass productivity**			**Specific growth rate**			**Colour (a*)**			**Protein productivity**		
	Coef. E.	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**	**Coef. E.**	***F*-value**	***p*-value**
N	0.4132	106.5	<0.0001	0.0612	35.68	<0.0001	-1.1	38.83	<0.0001	0.3555	154.31	<0.0001
P	-	-	-	-	-	-	-0.4954	7.93	0.0094	-	-	-
Ca	-	-	-	-	-	-	0.1729	0.966	0.3351	-	-	-
Mg	-	-	-	-	-	-	-	-	-	-	-	-
N*P	0.1417	8.16	0.0087	-	-	-	-	-	-	-	-	-
N*Ca	-	-	-	0.036	8.25	0.0082	-	-	-	-	-	-
N*Mg	-	-	-	-	-	-	-	-		-	-	-
P*Ca	-	-	-	0.0336	7.18	0.0128	-	-	-	-	-	-
P*Mg	-	-	-	-	-	-	-	-	-	-	-	-
Ca*Mg	-	-	-	-	-	-	-	-	-	-	-	-
N²	-0.3013	70.22	<0.0001	-0.0457	23.89	<0.0001	-	-		-	-	-
P²	-0.1025	8.28	0.0083	-	-	-	-	-	-	-	-	-
Ca²	-	-	-	-	-	-	-	-	-	-	-	-
Mg²	-	-	-	-	-	-	-	-	-	-	-	-
Model	**Reduced quadratic**			**Reduced quadratic**			**Reduced linear**			**Reduced linear**		
	R²	**Adjusted R²**	***p*-value**	**R²**	**Adjusted R²**	***p*-value**	**R²**	**Adjusted R²**	***p*-value**	**R²**	**Adjusted R²**	***p*-value**
	0.8869	0.8681	<0.0001	0.75	0.71	<0.0001	0.6562	0.615	<0.0001	0.8464	0.8409	<0.0001

A.10

A10 - Colours obtained in the optimisation of three mutant strains of *Chlorella vulgaris*, 8G, 7Y and 31W. Colour was measured with a Chroma Meter through CIELAB colour space, in which L* corresponds to the brightness, a* to the green-red coordinate and b* to the yellow-blue coordinate.

	Colour								
Factor	**L***	**a***	**b***	**L***	**a***	**b***	**L***	**a***	**b***
Strain		**8G**			**7Y**			**31W**	
Run									
1	37.6	-5.7	14.2	49.4	3.5	30.2	49.1	-9.0	13.7
2	39.0	-6.6	16.5	48.8	1.7	29.4	57.8	-7.2	16.2
3	37.4	-7.4	13.8	52.1	4.1	31.6	53.1	-4.8	13.4
4	36.7	-5.4	12.2	49.8	5.1	29.2	50.1	-5.1	11.6
5	34.9	-5.4	10.5	47.6	2.0	27.7	48.3	-7.6	13.2
6	35.1	-5.7	10.9	47.2	6.1	28.3	52.1	-7.8	14.4
7	37.7	-6.2	14.6	50.4	2.8	30.5	55.7	-9.8	15.6
8	36.9	-5.7	13.0	50.3	4.3	29.8	53.7	-5.1	14.7
9	35.6	-5.9	11.7	50.5	1.2	29.5	52.7	-8.8	15.1
10	35.3	-4.4	11.1	46.0	2.3	26.2	53.1	-9.3	15.4
11	35.8	-5.3	12.1	48.4	2.9	28.9	56.8	-4.9	15.8
12	37.0	-7.0	13.7	50.0	4.4	29.2	53.2	-9.3	14.7
13	35.8	-6.4	11.8	48.1	2.5	29.4	53.6	-8.5	15.0
14	35.9	-6.2	11.6	51.3	4.7	32.7	51.0	-7.7	14.0
15	36.1	-6.6	12.1	48.9	4.1	28.5	55.4	-6.3	14.7
16	35.0	-4.8	10.5	49.1	2.9	29.4	56.7	-9.7	15.4
17	35.2	-4.6	11.4	49.5	3.0	28.9	54.6	-6.5	15.4
18	37.8	-6.9	14.2	51.2	0.7	31.8	50.5	-7.6	13.7
19	36.0	-5.8	12.0	49.3	2.2	29.1	55.8	-8.7	15.0
20	38.0	-7.4	15.1	46.1	2.4	26.1	49.7	-7.1	13.4
21	36.5	-6.8	12.7	47.7	1.8	28.5	58.3	-8.5	16.4
22	36.3	-5.4	12.9	47.9	1.8	28.7	55.3	-6.8	14.5
23	36.7	-6.2	13.1	51.9	3.5	32.1	56.9	-8.5	15.6
24	36.2	-5.1	12.6	49.1	3.4	29.8	55.6	-8.2	15.5
25	36.6	-6.5	13.0	48.0	1.9	27.9	52.7	-7.2	14.0
26	38.1	-6.2	14.7	49.7	3.7	29.8	56.1	-9.7	15.3
27	35.6	-4.9	11.4	50.3	3.8	30.6	52.1	-7.2	14.3
28	35.0	-3.8	10.5	46.5	5.1	26.6	51.2	-7.1	13.9
29	34.9	-5.7	10.8	49.0	4.0	29.7	53.4	-7.5	13.9
30	32.0	-1.2	4.3	47.9	2.8	28.5	54.3	-6.8	15.2

APPENDIX

CHAPTER 6

A.11

A11 - Sidestreams reported in literature that have been used as carbon sources to cultivate *Chlorella* spp. in heterotrophic conditions. Maximum biomass concentration attained (g L^{-1}) – X_{max}; n.a. - not applicable and/or information not available.

Sidestream	Species	C source	Other nutrients	Pre-treatments examples	X_{max}	References	DOI
Sugarcane molasses	*C. protothecoides*	Sucrose converted to glucose	n.a.	Enzymatic hydrolysis	70.9	Yan et al. 2011	10.1016/j.biortech.2011.03.036
Sugarcane molasses	*C. zofingiensis*	Sucrose, glucose, fructose	Crude protein, crude fat, ash, salt	Centrifugation + cation exchange resin (remove metal ions)	6.2	Liu et al. 2012	10.1016/j.biortech.2011.12.047
Sugarcane molasses	*Chlorella* sp.	Sucrose converted to glucose and fructose	Lipid-extracted microalgal biomass residues	Enzymatic hydrolysis + H2SO4	5.58	Zheng et al. 2015	10.1007/s12010-015-1770-4
Crude Glycerol	*C. protothecoides*	Glycerol	Nitrogen (brewer fermentation waste)	Methanol removal (70 ºC, 15 min); Biodiesel and soap removal (H2SO4 + centrifugation)	14.07	Feng et al. 2014	10.1016/j.biortech.2014.03.120
Crude glycerol	*C. vulgaris*	Gycerol	Municipal wastewater (N source)	HCl treatment + centrifugation	2.92	Ma et al. 2016	10.1016/j.biortech.2016.02.013
Crude Glycerol	*C. protothecoides*	Glycerol (+ glucose)	n.a.	Methanol + NaOH catalysis; HCl wash + centrifugation; KOH neutralization	~14	O'Grady and Morgan 2011	10.1007/s00449-010-0474-y
Sugarcane molasses	*C. vulgaris*	Glucose, fructose, sucrose, other sugars	N source, ash, lactic acid (Corn steep liquor)	Centrifugation	n.a.	Mirzaie et al. 2016	10.1080/10826068.2014.995812
Whey permeate	*C. protothecoides*	Lactose converted to Glucose and galactose	n.a.	Hydrolysis (KOH, 30 ºC)	17.2	3. Espinosa-Gonzalez et al. 2014	10.1016/j.biortech.2013.12.028
Corn dry-grind ethanol thin stillage + soy whey	*C. vulgaris*	Glycerol, lactic and acetic acid, sucrose, stachyose, galactose, glucose	N and protein source (soy whey)	n.a.	9.8	Mitra et al. 2012	10.1016/j.algal.2012.03.002
Sweet sorghum stalks and rice straw	*C. vulgaris*	Glucose, fructose, sucrose	N source	Milling + enzymatic hydrolysis	4.8	Sibi et al. 2015	10.3923/jest.2015.113.121
Food waste hydrolysate	*C. pyrenoidosa*	Glucose	Free amino nitorgen and phosphate	Fungal hydrolysis	~20	Pleissner et al. 2013	10.1016/j.biortech.2013.03.088
Swine wastewater	*C. sorokiniana*	Organic carbon	N (ammonia) and P source	n.a.	n.a.	Lee et al. 2021	10.1016/j.chemosphere.2020.127934
Swine wastewater	*C. vulgaris*	Organic carbon	N (ammonia), P source	Centrifugation + filtration	2.35	Wang et al. 2015	10.1016/j.biortech.2015.09.067

A.12

A12 - Examples of waste-based cultivation reports with *Chlorella vulgaris*, where the cultivation mode (CM) (heterotrophic - H; mixotrophic - M; autotrophic - A), sidestream and the respective carbon (C) and nitrogen (N) sources are mentioned as well as the best outcome, namely regarding biomass productivity (r_p), maximum dry weight (X_{max}) and growth rate (μ). (n.a. - not applicable and/or information not available). WW – wastewater; Am – ammonium; Ni – nitrate.

CM	Sidestream	C	N	Best outcome	Reference	DOI	Year
A	Dairy WW	n.a.	Am + Ni	r_p**:** 0.214 g L^{-1} d^{-1}; **μ_{max}:** 0.331 d^{-1}; **X_{max}: 1.843** g L^{-1};	Asadi et al.	10.1007/s11356-019-06051-8	2019
A	Food waste compost	n.a.	Organic N + Ni	**μ_{max}:** 0.35 d^{-1}; **X_{max}:** 1.7 g L^{-1};	Chew et al.	10.1016/j.biortech.2018.07.069	2018
A	Food waste digestate	n.a.	Am	**μmax:** 0.62 d^{-1}; **X_{max}:** 0.86 g L^{-1};	Fernandes et al.	10.1016/j.wasman.2020.08.037	2020
A	Swine WW + nejayote	n.a.	n.a.	**μ_{max}:** 0.7 d^{-1};	López-Pacheco et al.	10.1016/j.scitotenv.2019.04.278	2019
H	Broken rice hydrolysate	Sodium acetate	Am + Ni	r_p**:** 1.65 g L^{-1} d^{-1}; **X_{max}:** 5.04 g L^{-1};	Cai et al.	10.1016/j.biortech.2022.127965	2022
H	Food waste	Glucose	n.a.	r_p**:** 3.22 x 10^7 cells mL^{-1} d^{-1}; **μ_{max}:** 0.82 d^{-1};	Marques et al.	10.3390/foods13071018	2024
H	Food waste hydrolysate	Glucose	Ni	**μ_{max}:** 0.8 d^{-1}; **X_{max}:** 4 g L^{-1};	Lau et al.	10.1016/j.biortech.2014.07.096	2014
H	Municipal WW	Waste glycerol (biodiesel)	Am + organic N	r_p**:** 0.421 g L^{-1} d^{-1}; **X_{max}: 2.92** g L^{-1};	Ma et al.	10.1016/j.biortech.2016.02.013	2016
H	Municipal WW	Na-acetate or glucose	Am + Ni + nitrite	**μ_{max}:** 0.498 d^{-1};	Perez-Garcia et al.	10.1111/j.1529-8817.2010.00934.x	2011
H	Saline WW (cheese whey)	Glucose	Soy peptone	r_p**:** 7.56 g L^{-1} d^{-1}; **X_{max}:** 18 g L^{-1};	Ghobrini et al.	10.1007/s10529-019-02770-7	2020
H	Sweet sorghum hydrolysate + rice straw	Glucose, fructose, sucrose	n.a.	**X_{max}: 4.8** g L^{-1};	Sibi et al.	10.3923/jest.2015.113.121	2015
H & M	Corn dry-grind ethanol thin stillage + soy whey	n.a.	n.a.	**H → Xmax:** 5.5 g L^{-1}; **M → Xmax:** 9.8 g L^{-1};	Mitra et al.	10.1016/j.algal.2012.03.002	2012
H & M	Swine WW	n.a.	Am + Ni	**H →** r_p**:** 1.46 g L^{-1} d^{-1}; **μ_{max}:** 1.21 d^{-1}; **X_{max}:** 2.35 g L^{-1}; **M →** r_p**:** 1.74 g L^{-1} d^{-1}; **μ_{max}:** 1.73 d^{-1}; **X_{max}:** 3.96 g L^{-1};	Wang et al.	10.1016/j.biortech.2015.09.067	2015
M	Centrate WW	Crude glycerol	Am	r_p**:** 0.460 g L^{-1} d^{-1};	Ren et al.	10.1016/j.biortech.2017.09.040	2017
M	Corn steep liquor (also tested cheese whey and vinasse)	n.a.	Am + Ni + organic N	r_p**:** 0.20 g L^{-1} d^{-1}; **μ_{max}:** 0.38 d^{-1}; **X_{max}:** 2.10 g L^{-1};	de Melo et al.	10.1016/j.chemosphere.2018.04.039	2018
M	Municipal WW	Glucose	Am	r_p**:** 0.13 g L^{-1} d^{-1}; **X_{max}: 1.39** g L^{-1};	Gao et al.	10.1016/j.scitotenv.2018.05.380	2018
M	Vinasse	n.a.	Am + Ni + nitrite	**μ_{max}:** 1.41 d^{-1};	Candido and Lombardi	10.1007/s11274-020-2802-y	2020
M & A	Dairy waste (cheese whey)	Glucose or galactose	n.a.	**M →** r_p**:** 0.75 g L^{-1} d^{-1}; **μ_{max}:** 0.47 d^{-1}; **X_{max}: 3.58** g L^{-1}; **A →** r_p**:** 0.10 g L^{-1} d^{-1}; **μ_{max}:** 0.13 d^{-1}; **X_{max}:** 1.22 g L^{-1};	Abreu et al.	10.1016/j.biortech.2012.05.055	2012

2024

MAFALDA TROVÃO DOS SANTOS

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