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Lutein production and extraction improvements from a heterotrophic culture of *scenedesmus almeriensis*

Cristobal Camarena

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Lutein production and extraction
improvements from a heterotrophic
culture of *Scenedesmus almeriensis*
Production de lutéine et amélioration de l'extraction à
partir d'une culture hétérotrophe de *Scenedesmus*
almeriensis

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Lutein production and extraction improvements from a heterotrophic culture of *Scenedesmus almeriensis*

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Under the direction of
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*In memory of my mother,
whose adventurous spirit,
along with my father's ongoing guidance,
has inspired me to pursue new challenges.*

*En memoria de mi madre,
cuyo espíritu aventurero,
junto con la constante orientación de mi padre,
me ha inspirado a perseguir nuevos retos.*

*À la mémoire de ma mère,
dont l'esprit d'aventure,
ainsi que les conseils permanents de mon père,
m'ont incité à relever de nouveaux défis.*

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In particular, to my family, who shared this adventure from beginning to end.

Abstract

Lutein production and extraction improvements from a heterotrophic culture of *Scenedesmus almeriensis*

Abstract:

Microalgae are positioned as a biotechnological solution to major problems of this century, offering answers and alternatives in areas like malnutrition, food shortages, climate change, and pollution. As photosynthetic organisms, microalgae synthesize numerous compounds to harness sunlight for their metabolic functions, including lutein, a yellow carotenoid crucial for the human diet.

Although lutein is a compound associated with the photosynthetic activity of microalgae, some species maintain the capacity to synthesize this important pigment even under heterotrophic conditions, allowing increased productivity of both biomass and lutein in cultures supplemented with an organic carbon source. *Scenedesmus almeriensis* has been reported as a good lutein producer under phototrophic conditions, but its low growth rate compared to other microalgae species has hindered its potential.

This thesis presents results of the lutein productivity obtained from a heterotrophic culture of *Scenedesmus almeriensis*, both at laboratory and pre-pilot scale. In addition to achieving high lutein productivity values, its larger cell size and fragility simplify biomass harvesting and pigment extraction. A simplified method for lutein extraction adapted to this species is also presented. This process uses ethanol as an extraction solvent and requires less time and energy, which represents environmental and product acceptance advantages.

Keywords: Biotechnology, *Scenedesmus almeriensis*, Lutein, Heterotrophic culture, Extraction

Production de lutéine et amélioration de l'extraction à partir d'une culture hétérotrophe de *Scenedesmus almeriensis*

Résumé :

Les microalgues sont considérées comme une solution biotechnologique aux problèmes majeurs de ce siècle, offrant des réponses et des alternatives dans des domaines tels que la malnutrition, les pénuries alimentaires, le changement climatique et la pollution. En tant qu'organismes photosynthétiques, les microalgues synthétisent de nombreux composés afin d'exploiter la lumière du soleil pour leurs fonctions métaboliques, notamment la lutéine, un caroténoïde jaune essentiel pour l'alimentation humaine.

Bien que la lutéine soit un composé associé à l'activité photosynthétique des microalgues, certaines espèces conservent la capacité de synthétiser cet important pigment même dans des conditions de culture hétérotrophe, ce qui permet d'augmenter la productivité de la biomasse et de la lutéine. *Scenedesmus almeriensis* a été rapportée comme une bonne productrice de lutéine dans des conditions phototrophiques, mais son faible taux de croissance par rapport à d'autres espèces de microalgues a entravé son potentiel.

Cette thèse présente les résultats de la productivité en lutéine obtenue à partir d'une culture hétérotrophe de *Scenedesmus almeriensis*, à la fois à l'échelle du laboratoire et à l'échelle pré-pilote. En plus d'atteindre des valeurs élevées de productivité en lutéine, sa taille cellulaire plus grande et sa fragilité simplifient la récolte de la biomasse et l'extraction des pigments. Une méthode simplifiée d'extraction de la lutéine adaptée à cette espèce est également présentée. Ce procédé utilise l'éthanol comme solvant d'extraction et nécessite moins de temps et d'énergie, ce qui représente des avantages pour l'environnement et l'acceptation dans l'industrie.

Mots-clés: Biotechnologie, *Scenedesmus almeriensis*, Lutéine, Culture hétérotrophe, Extraction

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Introduction

Introduction

The appearance of the food products we consume has always been a concern. Since before the industrialization of food, the aspect of fresh foods such as fruits, vegetables and meats indicated whether we could trust their quality or not. For example, the bright colors of vegetables are nature's recommendations regarding taste, freshness, vitamin content and other nutrient richness. In contrast, gray or pale color on raw meat can be strong indicators that something is wrong and it is best to discard it.

On rare occasions, we have the opportunity to taste a product before buying it. Thus, our decisions are based on the signals the other senses give the brain. Among them, sight stands out, and we tend to put most of our trust in the information it gives us to make our own choices.

Since the industrialization of food, commercial and aesthetic incentives have driven an innovation race to keep the color of processed foods as close as possible to their fresh raw materials. However, most natural pigments commonly used for centuries are not sufficiently stable for the complex food transformation processes. At the end of the 19th century, this led to the use of coloring products that were already available, such as paints and dyes used to stain clothes and other items, without ever having been questioned as to their harmful effects on health. Fortunately, despite limited data on their health impacts, many of these additives were quickly banned in food products by newly established regulatory agencies [1, 2].

The toxic dyes based on salts of metals such as arsenic, mercury, lead, chromium and copper were the first to be regulated. These gave rise to dyes derived from aniline, obtained from benzene extracted from petroleum, known as coal tar dyes. These were less toxic, cheaper, more stable and more efficient in their dyeing function, so less quantity was needed. By the mid-20th century, growing concern about the increase in cancer prevalence put the spotlight on processed foods, particularly additives. Among them are coal tar-derived colorants. Many dyes used at that time were banned in several European countries and the United States when substantial evidence of carcinogenicity was found. Others, however, proved to be safe and continue to be used in the food industry despite occasional controversies suggesting allergenic or behavioral effects [3].

In general, regulatory agencies approve synthetic food dyes based on the absence of adverse effects up to a specific concentration in relation to body weight (BW). Subsequently, the levels of dyes allowed in food are regulated based on the Acceptable Daily Intake (ADI), which is the amount of a chemical substance that can be ingested daily without appreciable health risk [4]. According to Bastaki *et al.* [5], the estimated daily intakes of products

with known concentrations of synthetic pigments in the U.S. sample do not exceed the recommendations for any pigments. However, even though most manufacturers keep the concentration of these pigments below the limits in each product, the increasing tendency to consume processed foods, mainly by infants, may cause an accumulated amount over the years that exceeds the suggested limits [6].

As a result, there has been a correlation observed between higher and prolonged intake of synthetic pigments, such as tartrazine, quinoline yellow WS, sunset yellow FCF, carmoisine, ponceau 4R, and allura red AC, and an elevated occurrence of various diseases and conditions. These include gastrointestinal issues, anemia, and allergic reactions such as asthma and urticaria. Additionally, pathological lesions have been reported in organs such as the brain, kidneys, spleen, and liver, along with instances of tumors, developmental delays, abnormalities in offspring, growth impediments, ocular damage potentially leading to blindness and hyperactivity (Attention Deficit Hyperactivity Disorder (ADHD)) in some children [2, 4, 6].

In response to these findings, pigments of natural origin, such as carotenoids, are gaining popularity again. In addition to having been declared non-allergenic by the Codex Alimentarius Commission of the World Health Organization [7], they have benefits in preventing or correcting health problems thanks to their light filtration capacity and antioxidant potential.

Carotenoids are a class of pigments that naturally occur in plants, algae, and some bacteria. They are responsible for the vibrant colors seen in many fruits, vegetables, and other organisms. Carotenoids play essential roles in photosynthesis, where they help absorb light energy. Additionally, carotenoids also have important functions as antioxidants, helping to neutralize harmful free radicals and protect cells from damage. Around 600 carotenoids have been described so far; however, the most well-known ones include beta-carotene (which gives carrots their orange color), lycopene (found in tomatoes and watermelon), lutein (found in leafy greens like spinach and kale), and zeaxanthin (found in corn and peppers) [8].

Carotenoids are considered beneficial for human health, and consuming a diet rich in fruits and vegetables containing these compounds is associated with various health benefits, including reduced risk of chronic diseases such as heart disease and certain types of cancer [9]. Moreover, carotenoids can be used as food and feed pigments as an alternative to synthetic dyes, contributing to reducing reliance on artificial additives while taking advantage of their health benefits.

Although there are currently known and reliable natural sources for obtaining carotenoids industrially, they have only been able to displace synthetic dyes in some very specific cases [10]. Synthetic dyes are still cheaper to produce, more stable, have better solubility, and perform better when consumers look for vibrant and consistent colors in their products. Examples of carotenes extracted from natural sources and currently industrially produced and widely commercialized are β -carotene, lycopene, astaxanthin, lutein and zeaxanthin [11]. However, the high demand for natural pigments, both to displace synthetic ones and to be used as nutritional supplements to take advantage of their health benefits, is driving the search for new and improved ways to produce them.

Lutein, belonging to the xanthophylls subgroup of the carotenoids, is a yellow-colored pigment naturally found in various fruits, vegetables, and microalgae. It is particularly abundant in leafy greens such as spinach, kale, and collard greens, as well as in egg yolks and certain fruits like kiwi and orange peppers.

Beyond its role as a pigment, lutein is highly valued for its beneficial effects on human

health. It is classified as a dietary antioxidant, meaning it helps protect cells from damage caused by harmful molecules known as free radicals. In addition to its antioxidant activity, lutein is also recognized for its role in promoting eye health. It is selectively accumulated in the macula of the retina, where it acts as a natural filter to absorb harmful blue light and protect against photodamage. This helps maintain the macula's structure and function, which is crucial for central vision and visual acuity [12].

Research has also suggested potential benefits of lutein beyond eye health. Studies have linked higher dietary intake of lutein with a reduced risk of chronic diseases such as cardiovascular disease and certain types of cancer. Additionally, lutein may have anti-inflammatory properties and contribute to cognitive health and skin protection.

Due to its health-promoting properties, lutein is already for sale worldwide and is often included as a dietary supplement in the form of capsules, tablets, or soft gels [13]. It is also added to various food products, such as fortified beverages, to enhance their nutritional value and provide additional health benefits. Additionally, incorporating lutein, along with other carotenoids, into poultry feed to enrich the color of chicken skin and egg yolks is a common and widely accepted practice in the poultry industry. It helps meet consumer preferences for visually appealing products while potentially providing added nutritional benefits.

Currently, lutein is produced by extracting it from marigold flowers on an agro-industrial scale in countries such as Mexico, India and China. When the flowers reach maturity, they are harvested and processed to extract the lutein pigment by solvent extraction methods. However, the sustainability of lutein extracted from marigold flowers is questioned due to environmental concerns stemming from large-scale cultivation practices, which may lead to habitat loss and pollution, as well as social issues related to poor working conditions of laborers, often from economically disadvantaged backgrounds in developing countries [13].

In this context, microalgae represent a promising and sustainable alternative for lutein production. They can be cultivated in controlled environments, such as photobioreactors, allowing year-round production independent of seasonal variations. This controlled cultivation also enables the optimization of growth conditions to maximize lutein content and productivity [14].

Additionally, microalgae have higher growth rates than plants and can accumulate higher lutein content, leading to efficient production yields. Furthermore, microalgae cultivation can be optimized to require minimal land and freshwater resources and can be integrated with wastewater treatment processes, reducing environmental impact and contributing to circular economy principles.

As a result, an intense effort has been carried out in the last 25 years by numerous research groups to find microalgae species with the ability to produce and accumulate lutein in attractive quantities to develop mass-production processes. This has led to a much better understanding of the cellular processes that trigger lutein synthesis in microalgae and how different species react to changes in culture and stress. However, the effects of these extensive tests have failed to substantially increase the total lutein content, at least without genetic modifications [15].

The hopes of industrially producing lutein from microalgae are stalled due to metabolic limitations for lutein accumulation pathways inside each cell. However, in recent years, different cultivation strategies have compelled researchers to change its perspective. The scientific community faces a critical juncture and must address a pressing question: should we continue to explore phototrophy as a method for effectively producing significant quantities of microalgal lutein, or is it time to explore alternative avenues? This is the

first scientific question this work intend to contribute to answer. In the first chapter, I present the result of a state-of-the-art analysis that shows the need for a shift in research to focus on increasing overall lutein productivity and not only the accumulated lutein content in biomass. This shift is not only evident when looking at the results reported in recent years, but also necessary to revive the potential of using microalgae as lutein producers at the industrial level.

This increase in lutein productivity is achieved thanks to the implementation of cultivation strategies that maximize the growth rate while preserving a certain level of lutein synthesis. Mixotrophic and heterotrophic cultivation of some microalgae species reduces lutein production capacity but allows for the reaching of high cell concentrations in a short time, resulting in productivity comparable to that of other commercially produced carotenoids.

Once the state of the art has been exposed, and important conclusions have resulted from its interpretation, the obvious question arises about the comparison of the different species of microalgae to produce lutein. Among the great diversity of species, not only do we find differences in size and shape, but we also observe a great diversity in how they adapt to changes in their environment, be it a natural ecosystem or a controlled bioreactor. *Chlorella* group species such as *C. vulgaris*, *C. sorokiniana*, *Parachlorella kessleri* and *Auxenochlorella protothecoides*, have shown the highest concentrations of lutein (up to 13 mg g⁻¹DW) at the cellular level and are also capable of producing high amounts of biomass in a short time when cultured in heterotrophic mode. However, scaling up their downstream process presents challenges due to their small cell size and tough cell wall, which makes harvesting and lutein extraction difficult.

The second and third chapter explores the potential of the species *Scenedesmus almeriensis* to emerge as a candidate for heterotrophic lutein production. *S. almeriensis* is acknowledged for its high lutein content in phototrophic cultivation (up to 8 mg g⁻¹DW); however, its potential for heterotrophic growth had not been investigated. In addition to achieving a lutein productivity comparable to that reported for some *Chlorella* species, the presented results show that *S. almeriensis* exhibits larger cell size and greater susceptibility to cell rupture, which contributes to facilitating biomass harvesting and lutein extraction. This reflection leads to the generation of novel insights in this study (second research question) concerning *Scenedesmus almeriensis*, and by extension, the *Scenedesmus* genus in general.

Based on these findings, a third research question emerged. The batch culture of *S. almeriensis* in heterotrophic mode using glucose as an organic carbon source showed the possibility of achieving up to tenfold greater biomass concentrations than those attained in phototrophic mode. However, under this batch strategy, the maximum biomass density attainable is limited by the maximum initial concentration of glucose that the culture can withstand.

To overcome this issue, a feeding strategy based on growth rate and substrate consumption was designed to maintain the glucose concentration below the inhibitory level. The fed-batch culture performance regarding biomass and lutein production is presented in Chapter Four, along with the new research avenues emerging from these experiments.

State of the art - Focus on lutein productivity

Lutein is a vital nutrient, and exploring new and improved methods for its production is crucial, especially in the context of a global crisis concerning the availability of quality nutrients and the need for sustainable production practices. This chapter explains the relevance of lutein and the importance of finding better production methods. This section starts by explaining the structure and function of lutein in photosynthetic organisms that produce it, but also the need for humans to introduce it into our diet to fulfill vital functions in our body. Finally, from the production point of view, a review is made of the last 25 years of study of lutein produced by microalgae. This section addresses the relentless search for ways to induce lutein hyperaccumulation in different species of microalgae based on the optimal culture conditions and the metabolic limits presented by the cells to achieve it. After this careful review of the literature, a paradigm shift in research is proposed to the scientific community to focus efforts on increasing the overall productivity of lutein, taking into account not only the amount of lutein per cell, but also the maximum amount of biomass in the shortest possible time.

This chapter is largely based on a previously published review article, which was written during the PhD: Camarena-Bernard, C. and Pozzobon, V., 2024, Evolving perspectives on lutein production from microalgae - A focus on productivity and heterotrophic culture. *Biotechnology Advances*, 73:108375. For this manuscript, the chapter has been expanded with greater detail in the sections on the function of lutein in the chloroplast of plants and microalgae, as well as the benefits of lutein in human health.

1.1 Introduction

Lutein is a yellow-coloured carotenoid pigment produced naturally by some plants and microorganisms. Due to its antioxidant activity, there have been longstanding claims about its health benefits [13, 16]. Their recent demonstrations for eye vision [17, 18], brain health, and cognitive functions [19, 20], especially in the context of aging, are driving the currently increasing interest for this specific molecule (from 249.7 millions (USD) in 2016 to a projected 491 million by 2029 [21]). Indeed, one of the major difference between lutein (and zeaxanthin) and the other carotenoids is its ability to cross the blood-brain barrier.

It can therefore access and accumulate in otherwise unreachable tissues such as the retina and the brain [19]. In addition to its antioxidant capabilities, which induces health benefits by fighting off reactive oxygen species, lutein has a light-filtering mechanism for violet-blue color, which contributes to the protection and visual performance of the eye [19]. In this regard too, compared to other carotenoids, lutein shows greater filtering effects for short wavelengths, probably due to the polarity of the rings in context with the orientation within the lipid membranes [20, 22].

Humans, and animals, do not synthesize lutein and need to acquire it through their diets. In the case of a human diet, lutein can be found in dark green leafy foods, such as broccoli, lettuce, cilantro, spinach and kale, as well as in yellow-orange fruits and roots, like guava, cashews, sweet potato, corn, peppers, pumpkin and eggs [23]. However, currently the average dietary intake of lutein in Europeans and North Americans stands at a mere 1.7 mg day⁻¹, while studies show that between 6 and 14 mg day⁻¹ would be needed to reduce the risk of age-related diseases [24]. There is therefore a need to increase the daily lutein dose either by diet modification, or, more surely, by diet supplementation.

While not as renowned as fish oil or magnesium supplementation, lutein-rich diet supplementation are currently available to the general public. To date, all the commercial lutein is extracted from marigold flowers, mainly Mexican/African marigold (*Tagetes erecta* L.) and French marigold (*Tagetes patula* L.) cultured in China, India and Mexico [23, 25]. The *Tagetes* genus is a group of plants native to America, from southern United States to South America and different cultivars have been developed for various uses. Its high content of red-yellow pigments, among which lutein stands out (3% of dry petals weight), has led to its cultivation mainly for the production of this value-added compound [23, 25]. From a technical point of view, marigolds are cultured seasonally and flowers are harvested from July to October. The harvest is followed by drying and chemical processing of the petals to obtain a lutein rich oleoresin with a final 10.6 kg hectare⁻¹ year⁻¹ productivity [26]. Nevertheless, this process suffers some drawback.

First, its cultivation requires large amounts of land and water for irrigation. Second, although efforts have been made to develop machinery for harvesting [27] and processing flowers [28], there is no evidence that these efforts have materialized in commercial agricultural equipment, which means that the work continues to be done manually, with the consequent risks and labor costs. Third, the environmental resources required for this method are substantial, with estimates of 60 m³ of water, 8.2 kg of fertilizers, 556 L of hexane, 11.1 L of ethanol, 1.1 kg of KOH, and 121 MJ of energy needed for every 1 kg of non-esterified lutein produced [25, 29]. Finally, by shifting the focus from technical to financial consideration, one could state that being a seasonal production with only one harvest per year, lutein intrinsically bears an economical risk. Therefore, marigold's growing conditions, requirements, and the increasing demand for lutein worldwide are encouraging the search for new sources of production.

Consequently, alternatives emerge, namely, a chemical sourcing and a biotechnological sourcing. Although pigments obtained from chemical synthesis are becoming widely rejected and alternatives are being sought, some efforts have been made to synthesize lutein by chemical processes. However, the process involves numerous steps and the yield is very low (between 1 and 5%) [30, 31]. For this reason, strategies to produce biologically synthesized lutein are still the most studied. Among them, synthetic biology tools have recently been proposed for the production of lutein from bacteria and yeast, microorganisms that do not produce this carotenoid naturally [32]. Microbial fermentation exhibits fast growth rates, making its combination with genetic engineering a promising

substitution pathway for the production of value-added compounds [33, 34]. A lutein titer of 11 mg L^{-1} in its free form was obtained from a genetically engineered *Escherichia coli* [32]. On the other hand, engineered *Saccharomyces cerevisiae* was developed to enable lutein biosynthesis and reached a maximal cell concentration of 19.92 mg L^{-1} [33]. Despite the general scientific consensus that products derived from genetically modified organisms are safe for consumption, concerns about their negative effects and low social acceptability hinder its market.

In this landscape, microalgae represent an additional alternative. Indeed, microalgae are postulated as a rich source of carotenoids, offering more favorable cultivation conditions and higher lutein productivity compared to traditional plant crops. They require less water and land, involve less labor intensity, can be cultivated in non-agricultural land, and boast better yield per unit of area, allowing for year-round cultivation [13, 25]. With a wider focus, microalgae are also an attractive source of biomass, natural colourants, and chemical compounds with applications in the food and feed industry, as additives in cosmetics, medicines and nutritional supplements, and as a source of by-products for the formulation of bio-plastics and bio-fuels [35, 36]. Therefore, microalgal lutein production would not be restricted to a single output product but could enter a more diverse and robust valorization scheme through the concept of biorefinery [37].

The content of carotenoids in microalgae, including lutein, has been studied since the 1960s [38]. But it is only in the 1980s and 1990s, together with the intensification of research on microalgae cultivation at an industrial level, that the first works on the optimization of carotenoid biosynthesis in microalgae began to appear [39, 40]. Since then, the only carotenoid pigments produced industrially from microalgae are astaxanthin and β -carotene. This has been possible due to the capacity of certain microalgae strains to store secondary carotenoids as a survival mechanism. *Haematococcus pluvialis* and *Dunaliella salina*, can accumulated up to 4% and 10% (Dry Weight, DW) of astaxanthin and β -caroten, respectively [41]. These species can accumulate such a large amount of pigments due to cellular mechanisms that respond to stress conditions [42].

However, lutein content among studied microalgal species varies considerably between 0.19 and 0.72% DW [43] and only some strains stand out as lutein producers under certain conditions, such as *C. vulgaris* CS-41 (0.94% DW) [44], *D. salina* (0.88% DW) [45] or *Parachlorella* sp. JD-076 (1.18% DW) [46].

As a primary carotenoid, lutein synthesis is linked to biomass growth and there are no known metabolic pathways in microalgae that can lead to lutein sequestration and accumulation in lipid bodies in the chloroplast or cytoplasm, as there are for the accumulation of astaxanthin and β -carotene in some microalgal species [47].

In recent years, numerous studies have focused on studying lutein content in microalgae, mainly from the genus *Chlorella* (*C. vulgaris* [44], *C. pyrenoidosa* [48], *C. protothecoides* [49–52], *C. sorokiniana* [53, 54], *C. zofingiensis* [55]) and *Scenedesmus* (*S. obliquus* [43, 56], *S. almeriensis* [57], *S. incrassatulus* [58]), but also on *Chlamydomonas reinhardtii* [59], *Muriellopsis* sp. [60] *Coccomyxa onubensis* [61–63], *Dunalliella salina* [45]. The majority of these studies focus on understanding how such species respond to changes in culture parameters and how lutein content is affected. The parameters most studied to understand how microalgae adjust the amount of lutein to environmental changes are light (intensity, quality and light-dark cycles), nutrients (mainly nitrogen and carbon), temperature and salinity. Unlike what happens with the accumulation of astaxanthin and β -carotene, the induction of stress by the lack or excess of any of these parameters does not substantially increase the amount of lutein. In fact, in many cases, it reduces it. These stress factors

also reduce the capacity to generate microalgal biomass, ultimately affecting overall lutein productivity.

In an economic feasibility comparison between marigold and microalgae lutein production, Lin *et al.* [25] suggest that potential microalgal strains must have a lutein content of at least 1% DW to be economically feasible. Furthermore, Xie *et al.* [47] calculated the maximal theoretical content a microalgae cell can accumulate, reporting a similar value of 1% DW and presents several limiting factors that must be addressed to achieve higher contents and promote commercial production. Genetic improvement of microalgae has been suggested to increase lutein synthesis and accumulation. The primary improvement mechanism used in microalgae is random mutagenesis. This process selects individuals with desirable characteristics after being subjected to chemical or physical treatments that alter parts of their DNA. While, in certain cases, there is documentation of increased lutein levels, the enhancements achieved fall short of the targeted 1% threshold [53, 64]). Additional methods characterized by targeted modifications, such as knockout of repressor genes or heterologous expression of genes that control the synthesis and cyclization of carotenoid precursors, such as phytoene and lycopene, have yielded interesting results in terms of increase, but still below the 1% [65]. For example, Rathod *et al.* [66] reported a lutein percentage increase of 83% on *Chlamydomonas reinhardtii*, after an heterologous expression of the phytoene- β

-carotene synthase gene from red yeast *Xanthophyllomyces dendrorhous*. However, the total lutein content was 8.9 mg g⁻¹, still below the target. Additionally, it has then been suggested that future studies should focus on precise targeted DNA modifications using editing techniques, such as CRISPR-Cas9 [67]. These modifications could focus on increasing the enzymatic activity for esterification of lutein, increasing its resistance to light and ROS damage and providing a first step towards the potential formation of lutein-sequestering lipid bodies [47]. Although some critical genes for these processes have been identified in plants and microalgae in recent years, further advances in microalgae genomics and proteomics are needed to achieve substantial progress.

In the pursuit of harnessing lutein from alternative sources, previous reviews have predominantly focussed on assessing lutein content within various microalgal species [47, 68–72]. These reviews delved into exhaustive experiments, meticulously dissecting culture conditions to enhance the lutein content of microalgae. While these efforts have undeniably contributed valuable insights into the biochemical pathways and environmental factors influencing lutein accumulation, the quest for elevating lutein content alone may have reached a plateau.

Despite elaborated adjustments on the culture protocols, the increments in lutein content often remain negligible (Figure 1.1), raising a pivotal question: should we continue focusing primarily on content, or is it time to shift our collective attention toward enhancing lutein productivity? The subtle distinction between content and productivity holds immense significance. The former accentuates the quantitative aspect of lutein within the organism, while the latter emphasizes the capacity to produce lutein efficiently on a larger scale.

In the field of microbial compound production, the distinction between productivity and content is not just a matter of semantics, and lutein production is no exception. While the amount of lutein per unit biomass quantifies the amount of this pigment contained in the cells, productivity encompasses a broader concept. It comprises not only the amount of lutein in the cells but also the capacity of the system to produce these cells efficiently. And efficiency in a process always has one critical aspect: time. Productivity integrates

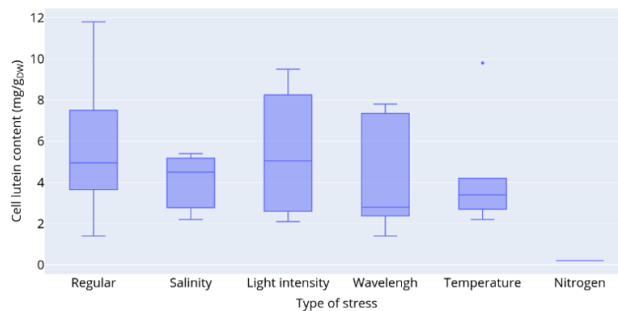


FIGURE 1.1: The boxplot illustrates the variation in lutein content in microalgae resulting from different stress factors, including light intensity, light wavelength, nitrogen quantity, temperature and salinity. Each box represents the interquartile range (IQR) of lutein content for a specific stress condition, with the whiskers indicating the full range of data points. The median value is depicted as a horizontal line within each box. These data were compiled from published articles, and the graphical representation clearly visualizes the impact of various stress conditions on lutein content in microalgae cultures.

time into the equation, and this aspect is crucial in the context of industrial production, as it directly influences the economic viability of a project for commercial purposes. As discussed below, when considering the three factors for determining productivity (lutein content, density of biomass obtained and the time required to produce it), we understand that it is not always the species with the highest lutein content that yields the highest productivity. The same is true if we take any one (or even two) of these parameters individually. We could have a microalgae species with the capacity to produce a large amount of lutein-rich biomass. Still, if it takes two months to produce it, its productivity will be lower than that of species with lower capacity but more efficient in time.

In exploring lutein from microalgae, this review endeavors to redirect the spotlight towards lutein productivity. Our rationale stems from a critical observation: elevating lutein productivity might require a preliminary emphasis on biomass production. By magnifying the biomass output of lutein-producing organisms, we can inherently enhance lutein yield, making the process economically viable.

This perspective shift is both timely and practical, especially in light of the increasing global demand for lutein. As we navigate the landscape of alternative lutein sources, understanding and optimizing lutein productivity could hold the key to unlocking the full potential of microalgae as a sustainable and economically feasible source of this essential nutrient.

1.2 Chemical structure and synthesis

From a chemical standpoint, lutein is a yellow carotenoid of the xanthophylls group containing two cyclic groups at the end of its C40 chain, which is common to all carotenoids (Figure 1.2). This chain can be presented in *cis* or *trans* conformation, the *trans* form being the most predominant in nature. The *trans* conformation can change to *cis* under the influence of high light intensity, presence of oxygen or pH shifts [25]. Since lutein found in human tissues is mainly *trans*, commercial production of this carotenoid is sought in this conformation [25].

The oxygenated groups at the ends of the carbon chain, characteristic of the xanthophyll group, react in the presence of fatty acids to form lutein mono- and di-esters (mainly lutein dipalmitate - 56% -, followed by lutein dimyristate - 36% and monomyristate - 8% - [73]).

However, these can be converted back to free lutein through saponification with alkali. Lutein is more stable in its esterified form and maintains its color longer if exposed to heat, light or other agents [25]. However, there is still debate about the different benefits and levels of bioavailability of consuming free lutein or lutein esters in nutritional supplements [25].

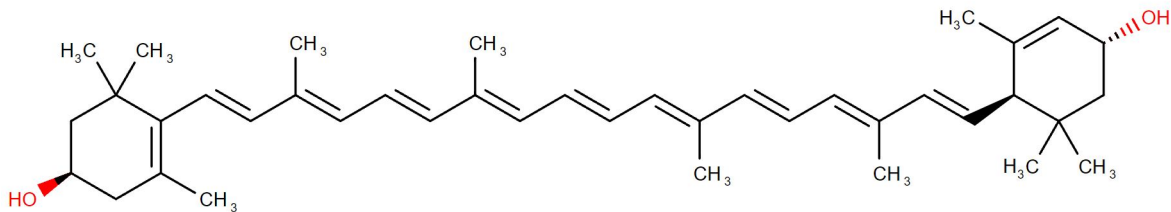


FIGURE 1.2: Lutein structure in trans configuration

From a biological standpoint, lutein is associated with photosystem proteins and participates in the light-harvesting complex, where it plays three main roles: (i) lutein has been found to play an important role in the correct folding of photosystem II proteins [74, 75]; (ii) it absorbs blue-green light (maximal absorption at 446 and 476 nm in acetone [42]) to optimize photosynthetic capacity under low light conditions, transmitting the energy it receives to the chlorophyll [45, 76]; (iii) it plays an essential role as an antioxidant. Its structure and position in the light-collecting complex allow it to quench harmful oxidative species and excited chlorophyll [76, 77]. Indeed, under conditions of high light intensity, the reaction centers cannot process all the energy they receive. Consequently, triplet state chlorophylls are formed, which in turn react with oxygen, forming Reactive Oxygen Species (ROS)[77]. These ROS can damage lipids, proteins and nucleic acids, which are essential for photosynthetic apparatus and, with a broader focus, cell integrity. To prevent this damage from happening, lutein can absorb the energy of triplet-state chlorophyll and singlet oxygen, forming a triplet-state lutein that can return to the stable ground state by safely releasing the excess energy as heat [68]. Moreover, lutein can scavenge ROS that may be formed otherwise [78].

In addition to its biological role, it is also interesting to introduce its biological origin as it helps to contextualize cultivation procedures introduced by different authors. Carotenoids, like lutein, are synthesized in the plastids of plants and microalgae, mainly in chloroplasts (chromoplast in plants also play an essential role in the biosynthesis of carotenoids), from the condensation of two geranylgeranyl pyrophosphate molecules [42]. Photosynthetic proteins and carotenoid-associated enzymes are encoded in the nucleus DNA, but transcription and translation are controlled, at least in part, by mitochondria and chloroplast [68, 79].

The main metabolic pathways for synthesizing the major xanthophylls in green algae and plants are detailed in Figure 1.3. The synthesis of carotenoids starts with the formation of phytoene from a 20-carbon chain molecule named geranylgeranyl pyrophosphate, assisted by the enzyme phytoene synthase. This enzyme has been reported to be a rate-limiting enzyme of the carotenoid biosynthesis pathway in microalgae and plants [8, 79]. Phytoene is the precursor molecule for all carotenoids and experiences a series of desaturation to form phytofluene, ξ -carotene, neurosporene and finally lycopene [61].

The cyclization of lycopene at one or both ends of the chain is an important branching point in the pathway. Each branch leads to either α - or β -carotenes and their derivatives, like lutein, zeaxanthin or astaxanthin. β -cyclase and ϵ -cyclase are responsible for the

formation of β - and ϵ -rings, resulting in α -carotenes, while the formation of β -carotenes depends on β -cyclase alone, to catalyse two β -rings [80].

Finally, xanthophylls are formed through enzymatic hydroxylation of the rings. On one side, lutein is formed from α -carotene due to hydroxylation at the C-3 and C-3' positions by β - and ϵ -carotene hydroxylase enzymes, while zeaxanthin is hydroxylated only by β -carotene hydroxylase. In some algae, additional pathways lead to the synthesis of other xanthophylls from β -carotene, such as astaxanthin.

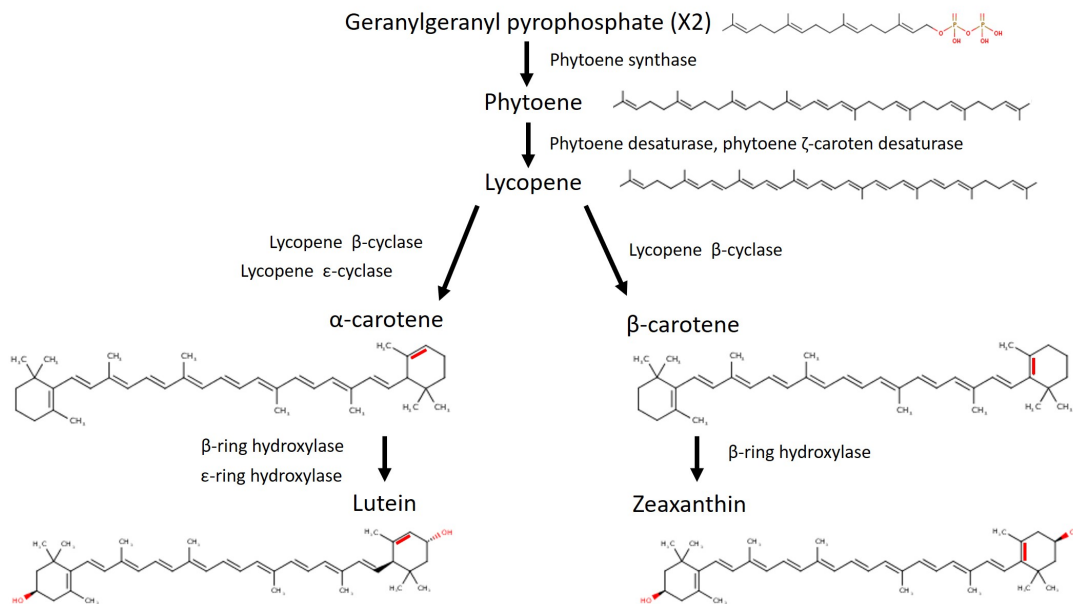


FIGURE 1.3: Schematic diagram of lutein and zeaxanthin biosynthetic pathway (adapted from Esteban *et al.* [42]). Lutein and zeaxanthin are isomers but not stereoisomers; the only difference is the location of the double bond in one of the rings (marked in red).

In microalgae, carotenoids in the photosynthetic apparatus that participate in light harvesting and photoprotection are termed primary carotenoids. At the same time, the secondary are carotenoids synthesized and accumulated under stress conditions, such as high light stress, nutrient deprivation or salinity stress [81]. Secondary carotenoids accumulate in lipid bodies within the chloroplast or in the cytoplasm [41, 68]. A good example of this is the accumulation of the carotenoids astaxanthin and β -carotene in *Haematococcus pluvialis* and *Dunaliella salina*, respectively [41, 82]. When they face unfavorable environmental or harsh culture conditions, like excess light, nutritional stress, high salinity, extreme temperatures and UV-B irradiation, *H. pluvialis* and *Chlorella zofingiensis* can accumulate astaxanthin in lipid bodies outside the chloroplast, while *Dunaliella salina* has the capacity of accumulating β -carotene in lipid droplets inside the chloroplast [41, 55, 83]. The accumulation of secondary carotenoids allows them to store significant amounts of energy and carbon to reactivate cell metabolism once there are less stressful conditions [84]. In addition, these large amounts of stored carotenoids offer great protection against oxidative stress during harsh conditions [83, 85]. To date, no report has shown that lutein can accumulate in lipid bodies.

1.3 Lutein health effects

Thanks to its chemical composition, lutein possesses antioxidant activity and light-filtering effects that have been demonstrated to affect health positively. While its most striking benefits are associated with its ability to cross the blood-brain barrier, others are worth mentioning. Finally, as with any molecule, the context in which its effects are evidenced matters (cell lines experiments, cohort studies, etc.) as they do not bear the strength. Care was therefore taken in specifying the context of the subsequently reported findings.

1.3.1 Eye and vision performance

In the eye, lutein is found in the macula (Figure 1.4) and is thought to be implicated, along with zeaxanthin, in two eye functions: acting as a filter of light to protect foveal photoreceptors from short-wavelength visible light (blue and violet light); and as an antioxidant protective agent, quenching toxic agents, like free radicals and singlet oxygen from the visual cycle [17, 18].

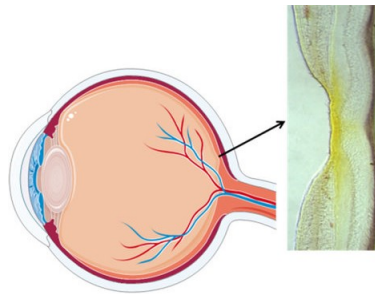


FIGURE 1.4: Vertical section of a monkey fovea showing the macular xanthophylls in yellow color [86] (adapted from Snodderly [87]).

These two eye carotenoids are referred to as the Macular Pigments (MP) and are measured *in-vivo* through psychophysical methods, heterochromatic flicker photometry being the most commonly used [17]. This method gives an Optical Density value of the Macular Pigment (MPOD). Values of MPOD can range among humans from 0 to values as high as 1.6, which has been found to correlate with visual performance [88]. MPOD values close to 1.6 are associated with better visual performance, like tolerance to extreme light intensity (reduced glare and shorter glare recovery), a faster speed of visual processing and better image contrast sensitivity [18, 19].

Age-related Macular Degeneration (AMD) is the most frequent source of blindness in developed countries [89, 90]. Izumi-Nagai *et al.* [90] demonstrated that lutein supplementation in mice led to the suppression of choroidal neovascularization, a critical factor in AMD pathogenesis, owing to lutein's antioxidant activity.

Although some studies have found that supplemental lutein intake does not increase MPOD levels [91], a meta-analysis conducted by Feng *et al.* [92] in 2019 concluded that, with the available evidence, it is possible to state that intake of 10-20 mg day⁻¹ for more than six months significantly increases MPOD and improves vision in patients with AMD.

On the other hand, since short wavelength light has a greater potential to generate ROS and cell damage, lutein is essential in developing eye tissue in children, who have a

blue light filtration rate half that of a healthy 60-year-old adult. This aspect is currently more relevant due to the increasing exposure to blue LED light from electronic device displays [20].

1.3.2 Brain and cognitive function

The function at the brain level is proposed because lutein has polar groups at each end of its molecule, so it is believed to be embedded in the cell membrane in a perpendicular position in brain cells, thereby blocking the oxidation processes of vulnerable lipids in the brain cells [19, 20]. In addition, this chemical feature contributes to the fact that lutein, along with its isomer zeaxanthin, are the predominant carotenoids found in brain tissue. Their capacity to traverse the blood-brain barrier results in the highest concentration of these carotenoids being detected at various developmental stages and persisting into later life [88].

Recent evidence indicates that lutein improves several functions of the brain, like the processing of visual and auditory signals, cognition processes, decision-making and motor coordination [18, 20]. Moreover, lower Alzheimer's mortality has been reported in individuals with higher serum levels of lycopene and lutein+zeaxanthin [93].

Investigations into lutein's impact on the brain have been carried out at various levels of complexity, spanning from *in vitro* studies on brain cell lines to *in vivo* experiments in animal models and, most notably, clinical trials involving human subjects.

Dhas and Mehta [94] demonstrated the capacity of lutein to cross the blood-brain barrier in a cell co-culture model using epithelial and brain cells. Additionally, the authors showed that free lutein, as well as nanoencapsulated lutein, significantly reduced oxidative stress in brain cell lines. On the same line, Alonso-Garrido *et al.* [1] showed the benefits of dietary carotenoids on mitochondrial function of *in vitro* brain cells after they were exposed to different mycotoxins.

In animal models, do Prado Silva *et al.* [95] significantly increased mice's object recognition index by supplementing lutein nanoparticles at 1.5 mg kg^{-1} . In a similar way, Gunal *et al.* [96] showed that lutein/zeaxanthin isomers administered to mice decreased infarct volume and bloodbrain barrier permeability after a traumatic brain injury. Lutein also significantly reduced proinflammatory cytokines and improved mitochondrial function. Due to its antioxidant and neuroprotective properties, Alzheimer's model rats improved their learning and memory, evidenced by different maze tests [97]. The findings from this animal model align with the notion of lutein holding promise as a viable therapeutic agent for addressing and potentially preventing Alzheimer's disease.

In humans, evaluating brain lutein levels is not straightforward. Nevertheless, its accumulation in the eye and the brain relies on the fact that it can cross the blood-brain barrier. Therefore, it is suggested that macular concentration of lutein may correlate with the concentration in the brain [19], allowing for a noninvasive evaluation of brain lutein level.

Cognitive decline may stem from damage, malfunction, and loss of brain cells, with neural connectivity being a key factor. Since the structural integrity and proper function of brain membranes significantly impact overall brain health, the presence of lutein within these membranes might potentially impact cognitive function by preserving cell viability through the prevention of these detrimental processes [98]. In a double-blinded, placebo-controlled trial spanning four months involving elderly women, it was observed that individuals receiving supplementation of lutein (12 mg day^{-1}), DHA (800 mg day^{-1}), or

a combination of both exhibited enhanced verbal fluency scores when compared to the placebo group [99].

For a more in-depth exploration and a comprehensive review of the literature on this topic, as well as a complementary perspective supported by their experiments, it is recommended to refer to the thorough analysis conducted by Erdman *et al.* [98] in their insightful review article.

1.3.3 Other benefits

Based on the hypothesis that the consumption of antioxidants, such as lutein, could reduce inflammation caused by excess reactive species in the body, numerous studies have focused on the benefits of carotenoid-based treatments to reduce inflammation [18]. In addition to the scavenging of reactive species, it has also been proposed that lutein acts as an inhibitor of inflammatory cytokine cascade in the body [100]. Low-level chronic inflammation is beginning to be associated with various chronic diseases (*e.g.*, cardiovascular disease, diabetes, some types of cancer, autoimmune diseases) and disorders (anxiety, depression, bipolar disorders, schizophrenia and post-traumatic stress disorder) [18].

Lutein and zeaxanthin dietary intake were associated with a lower risk of colorectal cancer in a hospital-based case-control study with Korean patients [101]. Lutein extracted from alfalfa plants (*Medicago sativa*) showed a significant antiproliferative role in breast and liver cancer cell lines compared to standard drug doxorubicin [102]. In a mice study, Park *et al.* [103] reported an inhibitory effect of lutein on mammary tumor development, even at very low amounts of dietary lutein (0.002%). All these authors agree that the effect of lutein must be related to its antioxidant activity.

In contrast, some authors suggest that lutein anticancer properties are independent of the antioxidant activity and are more likely to be related to the modulation of key protein transcription process [104, 105]. Lutein treatments have shown that its apoptotic effects only act on tumor cells and not on normal cells [104]. In addition, lutein could protect against cell death when normal cells undergo treatment with chemotherapy agents but does not interfere when the treatment is applied to cancer cell lines [104]. To get a better approach to the molecular effect of lutein in reducing cancer cell growth, Kavalappa *et al.* [105] measured ROS levels, cell viability, antioxidant defense and apoptosis proteins expression after lutein treatment on breast cancer cells. Lutein blocked the expression of key intracellular proteins that regulate delicate ROS balance. Their results confirm a significant role of lutein as an effective inhibitor of breast cancer cell growth.

Another benefit of lutein that is not only related to its antioxidant properties is in the prevention of cardiovascular diseases. In a systematic meta-analysis reviewed by Hajizadeh-Sharafabad *et al.* [24] it was found that *in vivo*, *ex vivo* and *in vitro* lutein treatments reduced the risk of atherosclerosis by reducing Low-Density-Lipoproteins (LDL) and monocyte migration; decreased inflammatory responses by lowering pro-inflammatory cytokines and increasing gene expression antioxidant enzymes; and improving endothelial function by changing blood lipid profile.

Lutein supplementation is also recommended to enhance liver protection, lung function, bone formation, skin solar protection and pregnancy overall health. These recommendations are based on a comprehensive review by Buscemi *et al.* [100].

1.3.4 Bioavailability

Lutein is absorbed by the mucosa of the small intestine via passive or facilitated diffusion and optimal absorption can be determined by external and host factors [20]. Bioavailability is the quantity of nutrients that can be incorporated and absorbed in the body, while bioaccessibility refers to the amount of nutrients released from the food matrix, otherwise called "digestibility". Lutein bioavailability and bioaccessibility have been evaluated in different studies to determine the best way to ingest it, both in the diet and in the form of supplements [16, 23, 106].

Many types of foods are natural sources of lutein. For example, it is well known that egg yolk is an essential source of lutein [106]. Although its content is lower ($15 \mu\text{g g}^{-1}$ of yolk) than that of some vegetables (between 20 and $30 \mu\text{g g}^{-1}$ in green leafy vegetables), its bioavailability is higher [23]. The mechanism behind the high bioavailability of egg lutein is unknown. However, it is presumed to be due to the other components in the egg, such as cholesterol and other lipids [106], suggesting that lutein ingested along with lipidic compounds has better intestinal absorption. This was confirmed by Granado-Lorencio *et al.* [16], who found that lutein extracts mixed with olive oil have 90% higher incorporation value compared to crude lutein extracts. Although the mechanism by which lutein is absorbed is not entirely clear, it has been proposed that lutein is emulsified into small lipid droplets before being absorbed by enterocytes at the intestine level [107], explaining the relation between lipids and lutein bioavailability.

Another factor that affects bioavailability is the structural form of lutein. Depending on its function, lutein can be found in a structural form (free lutein), involved in photosynthetic functions in green leafy vegetables and microalgae. In the second form, an esterified form of lutein accumulates in lipid vessels of fruits and flowers with one or two fatty acids at the end of the chain [47]. According to Bowen *et al.* [73] and Hedrén *et al.* [108], the esterified form of lutein has better bioavailability than the free form. In contrast, Chung *et al.* [106] stated that no differences were found between free and esterified lutein. This would confirm the speculation of Breithaupt *et al.* [109] that humans have a very efficient hydrolysis system for xanthophyll esters and, therefore, there is no limitation to the uptake of lutein esters. In terms of bioaccessibility, the type of food and the function lutein plays in that source is an important factor that determines how easy it is for the digestive system to extract the lutein from the matrix that contains it [16]. Raw yellow-orange fruits and roots, which accumulate esterified lutein in chromoplasts [110], show overall higher bioaccessibility of lutein (yellow potatoe 70%; tomato paste 92%; red pepper 49%) compared to raw green vegetables and microalgae (broccoli 7%; spinach 5%; microalgae 1%), where lutein is associated with photosynthetic proteins [16, 23, 111]. The extremely low value of microalgal lutein bioaccessibility supports the idea whole cell direct inclusion of microalgae in a diet would not result in an efficient supplementation. Cells would therefore have to be weakened before ingestion or go through an extraction procedure to recover lutein before delivering via other means.

Do Prado Silva *et al.* [95] increased lutein solubility in water by nanoencapsulating it in polyvinylpyrrolidone (PVP). When supplemented to mice in a memory test, the lutein-PVP nanoparticles exhibited similar effects to free lutein but at lower doses, suggesting increased bioavailability and greater absorption by the body, consequently enhancing its biological effect. Likewise, various studies have showcased the efficacy of lutein emulsions and nanoencapsulation within diverse matrices to enhance bioavailability. Consequently, they surpass the plasma and tissue levels attained through the administration of free lutein. These matrices include bovine and caprine casein emulsions [112], cationic liposomal

carriers [113], chitosan polylactic-glycolic acid nanoparticles [94], graphene oxide-titanium dioxide nanoparticles [114]. Additionally, these approaches contribute to enhancing lutein's photo and thermal stability.

1.3.5 Adverse effects

The growing interest in the consumption of lutein to prevent disease has raised questions about the safety and long-term effects of supplementation; however, several studies have analyzed its toxicity and adverse effects in animals, including humans [20]. The results of these studies have been considered by authorities to determine the maximum recommended daily doses. The Food and Agricultural Organization (FAO) [115] determines an Accepted Daily Intake (ADI) of 2 mg of lutein kg^{-1} of body weight day^{-1} . In Europe, the European Food Safety Agency (EFSA) [116] established an ADI of 1 mg kg^{-1} body weight day^{-1} for adults and children and considers it a traditional ingredient for use in food, beverages and food supplements. Furthermore, lutein is a compound Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) of the United States, which authorized its inclusion in food products and infant formulas [117].

1.4 Culture parameters affecting microalgal lutein productivity

Microalgae requires four essential components for their growth and metabolic processes: firstly, a source of energy, which may either be as solar or artificial light in the case of phototrophic regimes or organic carbon compounds in the context of heterotrophic regimes; secondly, a source of carbon either organic or inorganic; thirdly, access to major mineral nutrients such as nitrogen, phosphorus, and potassium, and also minor elements, like zinc, magnesium, copper, boron, manganese, which play pivotal roles in cellular processes; and fourth, a set of physicochemical conditions encompassing temperature, salinity, pH, and other factors, all of which must be carefully maintained within suitable ranges to ensure optimal growth and productivity of microalgae.

Considering these requirements, research efforts have driven microalgal biomass production to different scales and volumes. However, synthesizing by-products, such as carotenoids, requires extensive research to optimize their production and establish themselves as economically viable productions. As explained above, the synthesis and accumulation of astaxanthin and β -carotene by some microalgae species is a cellular response to adverse conditions in its environment [41]. In culture, stress induction is usually imposed by increasing light intensity, reducing nitrogen in the medium or increasing salinity.

Since these adverse conditions for stressing cells to produce carotenoids inhibit cell growth, productivity is usually low in one-stage cultures. Two-stage culture strategies are used to overcome this problem that affects the commercial viability of the process. In the first stage, optimal conditions are maintained for biomass accumulation, and then stress is induced to promote astaxanthin or β -carotene synthesis [10, 55]. Astaxanthin productivity of up to 17 mg L^{-1} d^{-1} can be achieved in *Haematococcus pluvialis* grown in tubular photobioreactors outdoors. For this, it is necessary to consider that *H. pluvialis* has the capacity to accumulate up to 3.8% astaxanthin in its biomass (DW) [118].

Following these successful examples, lutein synthesis by microalgae has been studied under optimal conditions for cell growth and under stress conditions to increase cell lutein

content. However, the diverse functions of lutein in microalgae suggest contradictory growth conditions for increasing its cellular content. On the one hand, its function as an antioxidant and photoprotectant suggests that lutein synthesis is enhanced under adverse culture conditions, such as high light intensity or high ROS concentrations. On the other hand, its participation in photosynthetic efficiency by acting as a light harvester suggests that low light intensity could promote the synthesis of light-harvesting complexes and their antennae along with the corresponding lutein molecules. Both scenarios have been tested for different species, indicating that the outcome is species-dependent. However, the consensus is that stress conditions increase lutein content marginally and, in addition, decrease biomass concentration so that the total balance tends to be negative [45].

Several studies showed that different stress conditions in microalgae culture increase the amount of reactive oxygen species (ROS) in the cells, including hydrogen peroxide (H_2O_2), radicals and singlet oxygen [82, 119]. ROS act as signaling molecules at appropriate levels that regulate cellular processes. However, when these levels exceed a certain limit, they oxidize proteins, nucleic acids and lipids, generating oxidative damage in the cells [82]. To deal with the oxidative damage that this represents for macromolecules, microalgae employ a variety of antioxidant compounds, like carotenoids [81]. However, the type of carotenoid synthesized depends mainly on the type of stress and microalgae species.

Some results will be presented below, showing how the different factors that affect the metabolism of microalgae modify not only the lutein content but also the biomass production, affecting the overall lutein productivity. First, the light factor is discussed as one of the main factors that require the participation of lutein in phototrophic cultures, both for its photoprotective and antioxidant activity, as well as for its role in the harvesting of light energy. Secondly, stress conditions during the culture of microalgae, such as the role played by the amount of nitrogen in the medium and other stress factors such as high temperature, salinity and pH will be addressed, focusing on the effect these factors have on lutein productivity. Thirdly, results obtained using organic compounds as energy sources are presented, taking advantage of the capacity of some microalgae species to thrive in heterotrophic and mixotrophic regimes. In addition, results of studies combining different two-stage cultivation strategies are presented, showing that in order to achieve higher yields than conventional ones, it is necessary to integrate more than one approach to prioritize both biomass generation and lutein synthesis.

1.4.1 Light

Sunlight is the most cost-effective energy source for the production of photosynthetic organisms and the most widely used for large-scale microalgae production in open ponds; however, it presents serious obstacles when seeking to optimize a culture by regulating the intensity and wavelength [120]. Electric light offers better control for precise illumination in photobioreactors. Different types of lamps provide light with different characteristics and advantages. For example, fluorescent lamps are commonly used because they give a wide range of wavelengths. Over the recent years, light-emitting diodes (LEDs) have positioned themselves as a cost-effective option because they have a longer life time, are more compact, produce less heat and are more electrically efficient [120].

As aforementioned, light is of peculiar interest in the scope of lutein production as microalgae adapt to it in a phenomenon known as photoacclimation. More specifically, light intensity, along with wavelength and light/dark periods, are major driving factors

in promoting growth, biomass productivity and biochemical synthesis in photosynthetic organisms [121].

1.4.1.1 Light intensity

Because outdoor microalgae production scale-up is generally carried out at intensities given by the sun (up to 2000 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ at midday in summer at some locations), studies on lutein production usually include the effect that light intensity has on its synthesis. However, not all photosynthetic organisms respond in the same way and metabolic pathways still require deeper understanding. For instance, plants grown under low light intensity tend to upregulate ε -cyclases, favoring the accumulation of α -carotene, while at high intensities, there is a higher expression of β -cyclases. Both enzymes are necessary for the synthesis of lutein [42]. In a general context, it has been argued that the lutein content in plants tends to increase under conditions of intense illumination [79].

In microalgae, it has been found that low and moderate light intensities (50 - 400 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$) generally promote relatively high lutein content [122]. The reason for this may be that the cells, in their quest to enhance light collection, increase the amount of light-harvesting systems along with the pigments associated with light capture [122, 123]. Yet, the photoprotective role of lutein would also indicate an increase in cell content when microalgae are subjected to high light intensities [78].

However, from a perspective where lutein productivity is considered, cultures exposed to low light intensity rapidly reduce their specific growth rate due to the reduction of the average light irradiance caused by the self-shading effect. The higher the biomass concentration, the more light-limited the culture becomes. On the contrary, saturation by light inhibits the proper functioning of photosystem II and reduces the cell's ability to grow. The light intensity level that inhibits biomass growth, either by limitation or saturation, depends on each species.

In general, microalgae strains collected from high-light intensity environments tend to develop cellular mechanisms to protect themselves, usually by increasing carotenoid content [42]. *Scenedesmus almeriensis* is a microalgae isolated from southern Spain that was reported to have high tolerance to high light intensity [124]. When cultured under controlled conditions, this strain showed a maximum lutein content of 0.43% under 1700 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$. Additionally, biomass productivity was also higher under this light intensity, giving a total lutein productivity of 3.8 $\text{mg L}^{-1} \text{d}^{-1}$.

Total carotenoid content in the marine microalgae *Tetraselmis* sp. CTP4 was 1.5-fold higher under a light intensity of 33 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ compared to 170 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ after a 5-day incubation period, suggesting the importance of carotenoids in general for a more efficient light utilization. However, lutein content was 1.5-fold higher under light intensity of 170 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ compared to 33 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$, possibly due to the photoprotecting role of this pigment for this species collected in the south of Portugal [123]. In this study, biomass concentration was also higher at 170 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$, resulting in lutein productivity of 1.83 $\text{mg L}^{-1} \text{d}^{-1}$, compared to 0.35 $\text{mg L}^{-1} \text{d}^{-1}$ obtained at 33 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$.

Parachlorella sp. JD-076 has been reported as a species tolerant to high light intensities [46]. When cultured in a tubular photobioreactor at 1000 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ achieved a biomass concentration of 8.45 g L^{-1} and a lutein content of 11.8 $\text{mg g}^{-1} \text{DW}$, which led to a productivity of 25 $\text{mg L}^{-1} \text{d}^{-1}$. Although this is the highest lutein productivity ever reported for a microalgae culture, more studies are needed to verify the repeatability of the results.

This photoprotective role of lutein was confirmed in *Chlamydomonas reinhardtii*, where lutein synthesis increased (+116%) under light stress of 800 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ compared to 100 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ [125]. When the culture was exposed to high light intensity, there was also an increase in the transcription of hydroxylase enzymes associated with carotenoid synthesis. When lutein and zeaxanthin synthesis was chemically and genetically inhibited, high susceptibility to light stress was observed in *C. reinhardtii* culture, suggesting that the role of these carotenoids is fundamental in the photoprotection of *C. reinhardtii* cells.

Nevertheless, not all microalgae species respond the same way to increase in light intensity. *Dunaliella salina* is a well-known strain for accumulating carotenoids when subjected to high light intensity stress; however, when subjected to a change in light intensity from 200 to 1400 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$, the carotenoid that increased was β -carotene, while the lutein content decreased [85].

At the other extreme is the phenomenon related to the function of lutein as a primary carotenoid: at low light intensity, lutein is synthesized in order to capture more light and increase photosynthetic capacity. Kona *et al.* [126] reported almost 4-fold higher lutein content in *Scenedesmus* sp. SVMIICT1 under 50 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ compared to 250 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$.

On the same line, Gong and Bassi [127] cultured *Chlorella vulgaris* UTEX265 in a coiled tubular photobioreactor and found that at low light intensity of 25 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ the lutein content was 22.9% higher than at 85 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$. However, as the growth rate is higher at 85 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$, the specific lutein production is also higher (11.98 $\text{mg g}^{-1} \text{d}^{-1}$).

Ho *et al.* [43] found that light intensity has opposite effects between lutein accumulation and biomass productivity in *Scenedesmus obliquus* FSP-3. While an increase from 30 to 300 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ led to an increase in biomass productivity and growth rate (+858% and +266%, respectively), lutein content was higher between 30 and 75 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ (0.54-0.55% of DW). Accordingly, the highest lutein productivity was found at 300 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$, with 4.08 $\text{mg L}^{-1} \text{d}^{-1}$.

McClure *et al.* [44] reported that the increase in light intensity (from 160 to 440 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$) in *Chlorella vulgaris* culture is proportional to the specific growth rate and biomass production (+124% and +219% increase, respectively), but is inversely proportional to the specific lutein concentration (-41% decrease). However, the highest lutein productivity was at 440 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ (0.58 $\text{mg L}^{-1} \text{d}^{-1}$).

Dineshkumar *et al.* [128] found that lutein concentration is affected in the same way as biomass productivity when different light intensities are used in *Chlorella minutissima* cultures. However, lutein productivity was 29% higher when increasing light in a linear mode compared with constant intensity, even though biomass productivity was slightly lower (4%), suggesting a need for higher light intensity as the culture grows to upregulate carotenoid synthesis genes.

These studies allow us to observe how different microalgae species modulate the amount of lutein depending on light intensity. However, although this parameter is essential in determining the amount of lutein, it has surprisingly little effect on increasing its overall productivity. Indeed, as anticipated, in most cases, increasing light intensity increases biomass production while lowering its lutein content, resulting in stable productivity.

1.4.1.2 Light wavelength

It has been proposed that different wavelengths can produce diverse impacts on the metabolism of microalgae [129]. The need for a strict energy balance between the two photosystems of microalgae requires that these organisms have a diversity of light-absorbing pigments to respond to energy at different wavelengths [120]. The diversity of these pigments is essential for capturing light throughout the visible spectrum, as each pigment has a specific affinity for certain wavelengths of light. Chlorophyll a, for example, absorbs light mainly in the red and blue regions of the spectrum. Other photosynthetic pigments, such as chlorophyll b and carotenoids, extend the light absorption range. Carotenoids absorb light in the blue and green regions of the spectrum [130]. Additionally, carotenoids exhibit photoprotective functions against wavelengths that can be particularly damaging to the photosynthetic system [131]. These characteristics have led to the study of light quality as a factor that can stimulate lutein synthesis, either by increasing light energy uptake or counteracting the damaging effects of high energy wavelengths.

As the scientific community has deepened its investigations in this area, divergent results have emerged, raising questions about the best light wavelength to optimize growth and lutein content in microalgae. As a consensus, blue light (420-490 nm) is considered to stimulate carotenoid synthesis, while white and red light (610-700 nm) increase biomass productivity [131]. However, there is evidence that this is not always the case. Atta *et al.* [121] cultured *C. vulgaris* under blue light and found an increase of 133% in cell density and 5% in specific growth rate compared to white light at $200 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$. Additionally, cultivation time was reduced by two days. In contrast, Fu *et al.* [132] increased by 25% the average growth rate and β -carotene and lutein content of *D. salina* when cultured under a combination of blue and red light (1:3) at $170 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$ compared to red light alone at $128 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$, suggesting that the outcome is a result of the interaction between the two wavelengths.

In accordance with the general consensus, Li *et al.* [133] found that lutein content in *Chlorella sp.* AE10 under high-intensity blue light ($850 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$, peak at 457 nm) was 1.63 times higher to that of white light. However, lutein productivity ($4.44 \text{ mg L}^{-1} \text{d}^{-1}$) was higher using red light (peak at 640 nm), caused by a higher biomass productivity. In the same line, Gatamaneni Loganathan *et al.* [120] reported a 25% decrease in biomass yields using low-intensity blue light ($40 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$) compared with cool white light at the same intensity in a consortium culture that included *Chlorella variabilis* and *Scenedesmus obliquus*. However, in this case, the lutein content was reduced by 75% under blue light but increased under white light. It should be noted that the culture medium for this study contained diluted dairy effluent, which may result in different adaptations to respond to light. It has been previously reported that the presence of glucose in the culture medium under phototrophic conditions can inhibit carotenoid synthesis [51].

In summary, research on the influence of light wavelength on lutein production in microalgae has yielded diverse results, with some studies emphasizing the benefits of blue light and others advocating for white light. However, in light of the gathered evidence and the referenced studies, selecting a single wavelength may not be the decisive factor in substantially increasing lutein productivity. Instead, the convergence of research suggests that a two-stage cultivation strategy, capitalizing on the capabilities of different wavelengths in specific phases, could be the key to optimizing both biomass production and lutein synthesis. This conclusion aligns with the reports from Zhao *et al.* [129], who did not find differences in lutein productivity between white light alone and a mixture of white and blue LED light in a *Chlamydomonas sp.* JSC4 culture. Nonetheless, the implementation of

a two-stage approach, involving white light in the initial phase followed by the application of blue light and temperature reduction in the subsequent stage, resulted in a remarkable 61% enhancement in lutein productivity, yielding $3.25 \text{ mg L}^{-1} \text{ d}^{-1}$.

Beyond the influence of different wavelengths in the Photosynthetically Active Radiation (PAR) spectrum, it is imperative to address the role of ultraviolet (UV) light as a stress factor in microalgae cultivation. UV light, especially in high doses, has been recognized as a potential stressor affecting the carotenoid levels in microalgae [131]. However, as with other stress factors, the induction of carotenoid synthesis by UV light is coupled with a reduction in cell growth as a consequence of metabolic dysfunction resulting from oxidative damage. In some cases, however, there is evidence that low UV-A intensities (320-400 nm) can not only stimulate carotenoid accumulation but also maintain sufficient cell viability to observe biomass accumulation. Bermejo *et al.* [62] found that supplementing UV-A light (8.7 W m^{-2}) to cultures of *Coccomyxa onubensis* induced a 34% increase in lutein content and also increased growth rate from 0.30 to 0.40 d^{-1} , compared to cultures under white light alone ($140 \text{ } \mu\text{molPhoton m}^{-2} \text{ s}^{-1}$). In a similar way, Salguero *et al.* [134] reported that *Dunaliella bardawil* cultured under white light ($100 \text{ } \mu\text{molPhoton m}^{-2} \text{ s}^{-1}$) plus UV-A ($70 \text{ } \mu\text{molPhoton m}^{-2} \text{ s}^{-1}$) increased growth rate by 16% and lutein content by 180%. However, this lutein content increase was after the cells' adaptation period during 84 h.

Although these studies with UV-A at low intensity demonstrate that it is possible to preserve cell viability while increasing the amount of lutein, further studies are needed to determine whether overall lutein productivity is increased.

1.4.1.3 Light and dark cycles

In addition to intensity and wavelength, light/dark cycles can affect microalgae metabolism. Under natural conditions, these cycles are determined by the day/night alternation, meteorological changes, movements in water bodies, and interference from other organisms [135]. These patterns of light intermittency may be a fundamental part of microalgae acclimation to changing aquatic environments. In artificial cultures at high cell density, the effect of cell self-shading, the geometry of the photobioreactor and the efficiency of mixing determine the frequency that the cell moves from illuminated to dark zones [136, 137].

Microalgae rely on photosynthesis to convert light energy into chemical energy, and the presence or absence of light profoundly influences this process [131]. During the light period, photosynthetic efficiency peaks as microalgae capture and utilize photons to fix carbon dioxide and produce organic compounds. Photosynthesis ceases in the absence of light during the dark period, but respiration continues. Microalgae undergo dark respiration, consuming some of the stored photosynthates and releasing carbon dioxide. In addition, microalgae exposed to high light intensities benefit from dark periods to recover from photodamage [136].

Faced with variations in the amount of light derived from these photoperiods, microalgae adapt pigment concentration, including lutein, to improve photosynthetic efficiency. However, although several studies show variations in culture growth and lutein concentration at different light/dark periods rather than continuous light, this parameter does not seem to influence the increase in lutein productivity.

Gong and Bassi [127] and Zheng *et al.* [138] reported an increase in lutein concentration in *Chlorella vulgaris* and *Chlorella sorokiniana*, respectively, when reducing the hours of light in a 24-hour photoperiod, however, the results are not comparable. [138] used a culture medium with corn starch, suggesting that their cultures were under mixotrophic conditions, while Gong and Bassi [127] conducted purely phototrophic cultures. However,

both concur that even though lutein concentration increases when reducing the hours of light, the maximum biomass concentration is achieved under continuous light.

Gayathri *et al.* [139] cultured *Chlorella salina* under light/dark periods of 24h:0h, 16h:8h and 12h:12h. Although they report a 1.5-fold increase in productivity at the 16h:8h photoperiod, it is not clear whether the result is due to this factor or to the combination of the other parameters tested (light intensity and airflow).

It seems that the influence of photoperiod is a function of light intensity. While continuous light is ideal for low light intensities, photoperiods with a few hours of darkness are necessary when the intensity is high. This may be because, at high intensities, cells require a period of darkness to recover the full functionality of their photosystems.

On the contrary, light intermittency at higher frequencies is differentiated from photoperiods, which measure the light:dark interval in hours. In flashing light treatments, the intervals are commonly measured in Hz, and it has been suggested that applying light/dark treatments in periods of seconds may vary microalgal culture growth and biochemical composition.

Lima *et al.* [140] reported a moderate lutein increase of 2.3 times under flashing lights at 5 Hz in three microalgae species. However, all three species greatly reduced their biomass productivity compared to continuous light. Similarly, Schüller *et al.* [141] reported higher biomass concentrations in continuous light cultures compared to flashing light at 0.5, 5 and 50 Hz in *Diacronema lutheri* and *Tetraselmis striata*. However, *Tetraselmis striata* showed higher lutein productivity at 5 Hz (1.3 times higher), mainly due to the increase in lutein concentration and to the fact that the reduction in growth was not so severe compared to continuous light.

In contrast, Pozzobon [142] reports an increase of 39% on lutein content in a *Chlorella vulgaris* culture under flashing lights at 7000 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$ and a similar growth rate than the continuous light at 200 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$. The author suggests that, among the three functions of lutein, including ensuring the folding of antenna proteins, transferring energy to chlorophyll and quenching the triple state of chlorophyll, it is the latter that triggers the hyperaccumulation of lutein under this high light intensity condition, despite the flashes.

1.4.2 Nitrogen

Since microalgae have been positioned as alternatives for biofuel production, one of the most studied treatments for cellular lipid overproduction is nitrogen starvation [143]. Subsequently, several studies focused on the effects of nitrogen deficiency stress not only on the content and profile of lipids, but also on protein, carbohydrate and pigment content [143]. Even though nutrient limitation, particularly nitrogen, generates stress in the cells and promotes the accumulation of specific carotenoids such as β -carotene and astaxanthin, most microalgae under nitrogen deficiency do not lead to high carotenoid content. This is probably due to decreased protein synthesis necessary for photosynthetic functions [123]. On the other hand, a sufficient supply of nitrogen causes high growth rates and biomass accumulation, thus obtaining higher carotenoid contents [44].

Different nitrogen availability levels and light quality did not affect increasing lutein production in the marine microalgae *Dunaliella salina*, as the highest productivity and content (3.68 $\text{mg L}^{-1} \text{d}^{-1}$ and 8.87 $\text{mg g}^{-1} \text{DW}$) were obtained at values established as optimal also for biomass growth [45]. *Scenedesmus obliquus* FSP-3, cultured in 1 L photobioreactor under batch mode, showed a sharp decrease (from 4.57 mg g^{-1} to

2.5 mg g⁻¹ approximately) in lutein content when nitrogen depletion occurred from day 5 of cultivation [43]. This was confirmed by Pozzobon *et al.* [144], who cultured *Desmodesmus pleiomorphus* under moderate light (150 $\mu\text{molPhoton m}^{-2} \text{s}^{-1}$) and detected how chlorophyll a, chlorophyll b and lutein content decreased when the nitrogen source was consumed (87%, 81% and 41% decrease, respectively). This suggests that nitrogen starvation leads to chlorophyll and lutein breakdown for nitrogen reuse and to be used to accumulate energy-rich compounds, such as lipids and carbohydrates [43].

On the other hand, culture medium with high nitrogen (NaNO₃) content or medium renewal strategies increased lutein content and productivity in *Chlorella vulgaris* (2.44 and 4.21 fold, respectively) [44]. In the same direction, cells of *Tetraselmis* sp. CTP4 showed a 2.5-fold higher carotenoid content under nitrogen repletion conditions compared to nitrogen depletion cultures [123]. Similarly, Xie *et al.* [145] achieved a lutein productivity of 5.22 mg L⁻¹ d⁻¹ on a *Desmodesmus* sp. F51 culture by increasing the ammonium-N concentration from 30 to 150 mg L⁻¹. This increase in nitrogen concentration not only enhanced the biomass density but also boosted the amount of lutein in the cells by 91%.

While nitrogen depletion conditions in microalgae culture promote the synthesis of some compounds of interest, such as lipids and secondary carotenoids, biomass and lutein production are reduced by nutrient limitation or adverse environmental conditions [81].

1.4.3 Other stress factors: temperature, salinity, pH, oxidative compounds

Abiotic stressors like high temperature and salinity, alkaline or acidic medium or the presence of oxidative compounds are responsible for the generation and accumulation of ROS, which can be responsible for triggering the cellular metabolic pathways for the synthesis of some carotenoids in microalgae [81, 119].

It has been observed in plants that lutein synthesis generally decreases under low temperatures [42]. However, in microalgae, the response to changes in temperature is strain dependant: some species can increase lutein content under high (30-40 °C) temperature while others increase it at lower (4-10 °C) temperatures [57].

In the marine microalgae *Tetraselmis* sp. CTP4, all pigments content decreased 2-fold when the temperature changed from 20 to 10 °C. However, when raised from 20 to 30 °C, all carotenoid content increased except for lutein, which did not change significantly [123], suggesting that lutein synthesis is linked to optimal culture conditions for biomass accumulation on this strain. In a similar way, Del Campo *et al.* [60] found that the optimal conditions for cell growth also apply to lutein accumulation and productivity in *Muriellopsis* sp. However, they propose a two-step culture strategy, as they found that lutein accumulation increases in the early stages of the stationary phase and is induced by cell growth stress factors, such as temperature. Similar conclusions were proposed by Ma *et al.* [146] in cultures with *C. sorokiniana*.

In contrast, Gong and Bassi [127] studied the lutein and growth rate response of *Chlorella vulgaris* at low temperatures and reported a 55% higher lutein content at 4 °C compared to a 10 °C cultures. However, at 4 °C, the specific growth rate is 48% lower, resulting in 25% higher productivity at 10 °C.

On the other hand, Zhao *et al.* [129] report that the best temperature for growth rate (35 °C) is not the best for lutein content (25 °C) in *Chlamydomonas* sp. JSC4, suggesting the need to implement a two-stage culture system to increase lutein productivity on this strain. In the same line, Ma *et al.* [59] reported higher lutein productivity in

Chlamydomonas sp. JSC4 grown at 35 °C (3.27 mg L⁻¹ d⁻¹), even though the highest content was obtained at 20 °C (3.82 mg g⁻¹). The authors suggest that lutein plays an important role at low temperatures by providing greater fluidity to membranes.

Finally, Sánchez *et al.* [57], working with the high temperature and high light irradiance resistant strain *Scenedesmus almeriensis*, reported that the highest biomass and lutein productivity was found between 35 and 40 °C, which is considered extreme temperatures for microalgae cultivation. This shows the great diversity of microalgae responses to adapt to temperature changes, and it is suggested that this variable should be analyzed to find the best temperature for each proposed species. However, it is evident that stress caused by temperature changes in microalgal cultures, at least in single-stage, does not increase lutein productivity. Therefore, it is necessary to look for different alternatives to increase it.

Salinity is another stress factor that can influence carotenoid synthesis in certain microalgae. Bermejo *et al.* [62] found that cultures of the acidophilic microalgae *Coccomyxa onubensis* can produce 47% higher lutein content when increasing NaCl from 0 to 500 mM. However, the highest lutein productivity is found at a salinity of 100 mM, which happens to be the same salinity that yields the highest biomass productivity. In the same lane, Ali *et al.* [147] reported a 6-fold increase in carotenoids in *C. vulgaris* when adding 10 g L⁻¹ of NaCl to the culture, but this salinity resulted in the lowest biomass productivity. In contrast, Sánchez *et al.* [124] report only a 15% increase in lutein content of *Scenedesmus almeriensis* when increasing NaCl from 0 to 5 g L⁻¹. McClute *et al.* [44] also reported a slight increase when a concentration of 100 mM of NaCl was added to a *Chlorella vulgaris* culture (1.4 fold for lutein content and 1.9 fold for lutein productivity).

Most microalgae species show better growth performance at pH between 6.5 and 7.5 [45, 60]. Alterations in this neutrality generate stress conditions that can be reflected in ROS formation and, therefore, responses from microalgae in the form of antioxidants, such as carotenoids. Sampathkumar and Gothandam [48] obtained a threefold increase in lutein when cultivating *C. pyrenoidosa* at a pH of 9.7 compared to 7.5; however, this scenario did not occur until the 30th day of cultivation, significantly reducing productivity. Similarly, Blanco *et al.* [148] observed that maintaining a pH level of 9.5 resulted in the highest lutein content in a *Muriellopsis* sp. culture within an outdoor pond system; however, higher biomass productivity between 7.5 and 8.5 resulted in similar lutein productivities throughout this range of 7.5-9.5. Only the reduction of pH to 6.5 caused both biomass and lutein productivity to drop by as much as 35%. Nevertheless, from an industrial perspective, the increase in lutein productivity at pH 9.5 offers the potential to control the growth of other species in open cultures.

While the induction of reactive oxygen species (ROS) in microalgae cultures has demonstrated a notable effect in elevating the quantity of lutein per unit of biomass, the predominant outcome remains consistent: an increase in ROS, often attributed to various stressors, accompanies a rise in lutein content per cell. Yet, as exemplified in a study by Wei *et al.* [49], who intentionally increased ROS levels in microalgal cultures through the addition of oxidizing compounds, the boost in lutein content was discernible (13%). However, this increment in lutein was accompanied by a consequential reduction in overall biomass. This pattern accentuates the trade-off between enhancing lutein production and the compromised total productivity resulting from diminished biomass under stress conditions. Thus, while stress-induced mechanisms may augment lutein concentration within cells, the net effect on total productivity invariably involves a compromise due to reduced biomass.

As a conclusion for this section, it can be stated that, although lutein has diverse functions, it seems that the cell synthesizes it so that it contributes to the photosynthetic process as a primary carotenoid. However, as we will see in the next section, it is not clear what triggers its synthesis in microalgae cultures with an organic carbon source, with or without light, i.e., in mixotrophy or heterotrophy. Moreover, it is not known what the main function of lutein is in total darkness, where photosynthesis is not necessary.

1.4.4 Metabolic regimens

Although microalgae are photosynthetic organisms adapted to use light energy to metabolize inorganic carbon sources and produce organic compounds (phototrophy), some species still retain the ability to use sugars and other organic compounds as their sole source of energy (heterotrophy)[149]. In phototrophic cultures, where light is the only source of energy, accessibility to light is inversely proportional to cell concentration due to mutual shading of cells [149]. To overcome this, heterotrophic cultivation strategies have been proposed, using different sources of organic carbon as an energy source, such as glucose, acetate or glycerol [149]. According to a review article by Perez-García and Bashan [149], heterotrophic biomass productivity can reach values over 200 times higher than phototrophic cultures. Jin et al. (in 2020 and 2021) achieved ultra-high cell densities of 271 g L⁻¹ and 286 g L⁻¹ in *Chlorella sorokiniana* [150] and *Scenedesmus acuminatus* [151] in heterotrophic cultures, respectively.

Heterotrophic microalgae cultures have advantages such as (a) higher growth rate and biomass productivity, (b) higher lipid productivity, (c) improved productivity per area of culture, (d) simpler and cheaper bioreactor designs, (e) simpler harvesting processes due to high cell concentration; (f) less risk of contamination by other photosynthetic organisms [69, 149, 152]. However, there are limitations such as the cost of carbon sources, increased risk of contamination by faster-growing organisms like bacteria and yeasts, and most importantly, lower productivity of light-related compounds, like lutein [149, 153].

Although it has been established that lutein acts as a primary carotenoid in microalgae and, therefore, its main function is to contribute to photosynthetic efficiency, it has been observed that many species continue to synthesize this pigment under conditions of complete darkness [69, 154]. This phenomenon has been explained by suggesting that microalgae that continue to synthesize lutein during a heterotrophic regime do so in order to take advantage of the other functions offered by lutein, such as its antioxidant capacity [155]. Furthermore, it is evident that species retaining a certain level of photosynthetic pigments during dark culture would exhibit improved adaptability when transitioning to light conditions [155, 156]. Regarding productivity, the sacrificed lutein content per cell is compensated by the high cell concentration achieved in heterotrophic cultures. As for the time factor, the growth rate under this regime is usually higher [69].

Since each regime (photo- and hetero- trophic) has advantages and disadvantages, it has been suggested that a mixture of both conditions removes the weaknesses and enhances the benefits, i.e., mixotrophic cultivation [69, 149]. The hypothesis generally put forward is that mixotrophic culture takes advantage of the presence of light to stimulate photosynthetic activity and, thus, photosynthetic pigment synthesis, while the presence of organic carbon accelerates the growth rate, yielding higher biomass production.

Chlorella sorokiniana was reported as a microalgae with potential for lutein production since 2000 [157]. Since then, numerous efforts have been carried out to improve the yield of this species and have culminated in several cultivation proposals under different regimes

taking advantage of its potential to change metabolism [53, 146, 152, 153, 158–161]. In phototrophic culture, Do *et al.* [160] reported a maximum lutein productivity of 4.57 mg L⁻¹ d⁻¹. Still, Chen *et al.* [159] obtained 7.14 mg L⁻¹ d⁻¹ in heterotrophic culture while Ma *et al.* [146] under mixotrophic conditions obtained 4.79 mg L⁻¹ d⁻¹. Later, Do *et al.* [152] cultured *C. sorokiniana* under mixotrophic growth and achieved a biomass concentration and lutein content of 26.21 g L⁻¹ and 5.01 mg g⁻¹ respectively using sodium acetate as organic carbon source, 2% CO₂ and 75 μmolPhoton m⁻² s⁻¹ light intensity. Although they later succeeded in increasing lutein content 69% by raising light intensity to 100 μmolPhoton m⁻² s⁻¹ and CO₂ to 3.5%, biomass concentration decreased 77%, confirming that under certain conditions it is possible to increase lutein content, but overall lutein productivity is decreased.

Another *Chlorella* species that has been extensively studied for its ability to accumulate high cell densities in heterotrophic culture is *C. protothecoides*. Shi *et al.* reported the optimal concentrations of glucose [162] and nitrogen [52] in addition to the ideal nitrogen source to increase biomass and lutein production. The authors reported the highest biomass accumulation (19.6 g L⁻¹) and lutein content (4.58 mg g⁻¹) when using urea as a nitrogen source at 1.7 g L⁻¹ and glucose at 40 g L⁻¹. In line with these findings, Xiao *et al.* [51] compared the biomass and lutein content under phototrophic, mixotrophic and heterotrophic modes of *Auxenochlorella protothecoides* (formerly known as *Chlorella protothecoides*). In agreement with the other references, the highest amount of lutein was obtained in the phototrophic mode culture (2.69 mg g⁻¹), while in the mixotrophic and heterotrophic modes, it is less than 1 mg g⁻¹. However, the authors highlighted the heterotrophic mode as the best way to produce lutein with this strain due to the high cell density obtained.

Chromochloris zofingiensis is recognized for its potential in astaxanthin production, even under heterotrophic conditions. Chen *et al.* [64] inhibited by selective mutagenesis the synthesis of astaxanthin in this species and demonstrated that the metabolic pathway was diverted to synthesize other carotenoids in heterotrophic culture. The low amount of lutein obtained (1.9 mg g⁻¹) was compensated by the high biomass concentration (13.7 g L⁻¹), yielding a final productivity of 6.5 mg L⁻¹ d⁻¹.

Similarly, Correia *et al.* [163] compared biomass and lutein production in the microalgae *Chlorococcum amblyostomatis* under heterotrophic and phototrophic conditions. Although they found 3.3 times less lutein content in the heterotrophic culture, the biomass concentration was 5.5 times higher than in the phototrophic culture. Unfortunately, the authors do not provide the number of days of culture or lutein productivity, but their data contribute to understanding the particularities of each culture regime.

On the same lane, Koh *et al.* [164] found 42% lower lutein content in *Scenedesmus obliquus* under heterotrophic conditions compared to phototrophic; however, the biomass content under heterotrophic conditions was three times higher, resulting in lutein productivity also three times higher with 6.5 mg L⁻¹ d⁻¹.

Although microalgal biomass productivity tends to be higher when providing an organic carbon source, it is necessary to consider the increased cost of production. Yun *et al.* [153] reported a 12- and 9-fold increase in biomass productivity under mixotrophy and heterotrophy, respectively, compared to phototrophic conditions. However, the authors highlight the high dependence on glucose to achieve these values, compared to phototrophic cultivation, which only uses sunlight and CO₂.

Since heterotrophic cultivation entails the extra cost of adding organic compounds, emerging efforts are being made to find alternatives to costly and traditional classical

substrates, such as glucose and acetate. Wang *et al.* [165] obtained a lutein content and lutein productivity of 7.27 mg g^{-1} and $7.34 \text{ mg L}^{-1} \text{ d}^{-1}$ by culturing *C. protothecoides* using waste *Monascus* fermentation broth. This represented an increase of 42 and 54% compared to the Basal Medium with 30 g L^{-1} of glucose. Similarly, Zheng *et al.* [138] were able to grow *C. sorokiniana* using hydrolyzed corn starch wastewater and obtained a maximum biomass concentration of 1.36 g L^{-1} with a lutein amount of 8.29 mg g^{-1} .

1.4.5 Culture process strategies

While numerous strategies have been explored to augment lutein production in microalgae, the focus has predominantly centered on batch-mode cultivation techniques. These methods have inherent limitations in sustaining consistent and optimized lutein productivity over extended periods. Fed-batch, continuous, pulse-feeding medium, and two-stage cultures emerge as innovative methodologies offering promising avenues to enhance lutein productivity in microalgae.

On one hand, fed-batch, continuous cultures, and pulse-feeding nutrients offer controlled nutrient supplementation, steady-state conditions, and intermittent nutrient supply, respectively. On the other hand, multi-stage cultures consist of varying culture conditions to promote different metabolic pathways that usually favor high growth rates in the first instance and subsequently induce compound synthesis under other conditions [166].

Using a feeding strategy, Wang *et al.* [165] increased lutein productivity by 45% in a heterotrophic culture of *C. protothecoides* compared to a batch culture. Similarly, Chen *et al.* [167] reported a lutein productivity of $5.67 \text{ mg L}^{-1} \text{ d}^{-1}$ in *C. sorokiniana* under heterotrophic conditions by adding sodium acetate and sodium nitrate in a semi-batch mode, an increase of 85% compared to batch culture. On the same line, Xie *et al.* [168] increased lutein productivity by 16% using a fed-batch cultivation strategy with pulse-feeding of nitrate on a phototrophic culture of *Desmodesmus* sp. Furthermore, Chen *et al.* [169] reported a lutein productivity of $4.96 \text{ mg L}^{-1} \text{ d}^{-1}$ in a mixotrophic culture of *S. obliquus* CWL-1, an 11-fold increase compared to the batch system.

Regarding two-stage cultivation, Zhao *et al.* [129] cultured *Chlamydomonas* sp. JSC4 and increased lutein productivity by 60% under a two-stage process, where they shifted from white to blue light after three days of culture.

Xiao *et al.* [51] were able to produce up to 6.3 mg g^{-1} of lutein in *Auxenochlorella protothecoides* by taking advantage of the ability of this species to switch metabolic pathway between heterotrophic and autotrophic and vice-versa. A high biomass concentration (100.5 g L^{-1}) was obtained during cultivation without light in a culture medium enriched with glucose. After switching to autotrophic mode, lutein productivity of $12.36 \text{ mg L}^{-1} \text{ d}^{-1}$ was achieved under light and a nitrogen (glycine) enriched medium.

Chen *et al.* [159] obtained an increase in lutein productivity in *C. sorokiniana* when they tested fed-batch and semi-batch strategies compared to the initial batch culture. However, the greatest increase was obtained when they integrated these two strategies in a two-stage culture, starting with a fed-batch to maximize biomass concentration and replacing 75% of the culture medium for the second stage, favoring lutein accumulation. This strategy resulted in 150% higher lutein productivity ($7.14 \text{ mg L}^{-1} \text{ d}^{-1}$) than the batch culture and 56% higher than the semi- and fed-batch strategies separately.

Similarly, Ma *et al.* [146] obtained better lutein productivity results ($8.25 \text{ mg L}^{-1} \text{ d}^{-1}$) with *C. sorokiniana* FZU60 integrating a first fed-batch stage in mixotrophic mode and a second purely phototrophic stage once the culture consumed all the acetate, suggesting

that lutein inhibition by acetate can be reversed when acetate has been consumed, and there is a light source. Moreover, Xie *et al.* [170] proposed integrating all these ways to increase biomass concentration, using a fed-batch mixotrophic culture of *C. sorokiniana* as the first step and a photoinduction process for the second step, obtaining a lutein productivity of $11.57 \text{ mg L}^{-1} \text{ d}^{-1}$, one of the highest values reported.

On the same line, Florez-Miranda *et al.* [58] cultured *Scenedesmus incrassatulus* on a two-stage strategy, beginning with a heterotrophic stage reaching 17.9 g L^{-1} of biomass, followed by a photoinduction stage to promote lutein synthesis. The photoinduction process increased seven times the lutein content, resulting in a lutein productivity of $3.1 \text{ mg L}^{-1} \text{ d}^{-1}$, which improved 1.6 times compared to autotrophic fed-batch culture with this microalgae. This approach was also used by Koh *et al.* [164] to increase lutein productivity in a culture of *S. obliquus*. After the heterotrophic culture, the photoinduction stage increased the lutein content by 34%. Furthermore, Fan *et al.* [171] suggested the need to dilute the microalgal culture obtained in heterotrophic mode before photoinduction, arguing that even photoinducing at high light doses (up to $600 \mu\text{mol Photon m}^{-2} \text{ s}^{-1}$) the high cell density of the first stage does not allow light penetration to the whole culture. With this same strategy, Camarena-Bernard *et al.* [172] obtained a productivity of $11.68 \text{ mg L}^{-1} \text{ d}^{-1}$ in a two-stage culture of *Scenedesmus almeriensis*. The highest reported for the *Scenedesmus* genus and comparable with the highest reported for the *Chlorella*. This is particularly attractive from an industrial point of view, as *Scenedesmus* species present advantages at the time of harvesting due to their larger cell size.

An unconventional cultivation strategy was reported by Sansawa and Endo [173] to improve carotenoid content in *Chlorella regularis* S-50. They described the methodology for obtaining synchronized heterotrophic cultures by regulating glucose supply. Once the life cycle of the cells is synchronized, the authors report a decrease in carotenoid content during the first 6 hours with glucose, while starch reserves increase. Once glucose is depleted, an increase in cell division and a threefold increase in the lutein content is reported. Although no productivity values are reported, this strategy could be further explored to understand the dynamics of the synthesis of carotenoids and other compounds of interest during the life cycle of other microalgae species. This phenomenon had already been mentioned in 1965 by Theriault [174] in *Chlorella pyrenoidosa*, but had not been proposed as a cultivation strategy for lutein production.

Although strategies offer higher biomass and lutein productivity advantages, adaptability to change between metabolic modes is strain-dependent. The number of strains that can grow under heterotrophic or mixotrophic conditions is still limited, and further studies are required to maximize biomass and lutein productivity.

1.5 Perspectives

Given the need to increase lutein productivity to make large-scale microalgae cultivation for this carotenoid production attractive, the evidence shows a clear tendency to use alternative cultivation modes to the purely phototrophic one (Table 2.1).

Although the metabolism of lutein synthesis is mainly linked to photosynthetic activity, its productivity is more affected by the biomass concentration than by the carotenoid content per cell. Therefore, the scientific community is realizing that exceeding the theoretical limit of 10 mg of lutein per gram of biomass on microalgae suggested by Xie *et al.* [47] is not possible by optimizing culture parameters (such as medium components, pH

and temperature) nor by changing the culture conditions to induce stress (such as high light intensity, extreme salinity and nitrogen depletion).

In some cases, the marginal increase in lutein derived from these stress conditions is due to a change in the ratio of lutein to total carotenoid content [51]. Although this strategy has not been able to increase lutein productivity substantially, continuing to prioritize research into the metabolic mechanisms behind this ratio shift is crucial [154], especially considering its potential integration with other strategies aimed at boosting biomass production.

For example, as mentioned above in the light wavelength section, low intensities of UV-A light have promoted lutein synthesis without affecting biomass production. In this same regard, the use of chemical inhibitors has been shown to have positive effects on lutein synthesis. These inhibitors act on key enzymes for carotenoid synthesis, such as lycopene β - and ϵ -cyclases that result in two different pathways for the synthesis of α -carotenes, such as lutein, or β -carotenes, such as zeaxanthin and astaxanthin [65]. Yildirim *et al.* [175] showed that the addition of imidazole in a *Dunaliella salina* culture increased the lutein content (1.7 fold), changing the ratio of β -carotene and lutein. They suggested this inhibitor might be more effective in reducing the lycopene β -cyclase activity, favoring lutein synthesis. Although this strategy did not result in extraordinary productivity, it shows that the complexities of lutein metabolism are far from being fully understood. On the other hand, the role of lutein in the early growth phases of a microalgal culture is little explored. Unlike astaxanthin, lutein synthesis is not favored by the induction of stress in the culture, which could indicate that its regulation is more closely linked to growth stimulating factors. It is generally agreed that the highest lutein accumulation is found when the cultures reach the stationary phase, coinciding with nitrogen depletion. However, the early stages of the culture may contain insights that could help to externally regulate its metabolism to stimulate lutein synthesis. The potential of these strategies to increase lutein content, integrated with processes to increase biomass production, could result in scenarios with industrially attractive yields.

Moreover, taking advantage of the ability of some microalgae species to synthesize lutein even in lightless culture conditions [156], the productivities shown by these strains under heterotrophic conditions give hope for commercial cultivation in the near future. The different strategies in the cultivation process, which allow taking advantage of different metabolic regimes and cultivation modes that optimize biomass production, strengthen this vision of the future of lutein production from microalgae [69].

Heterotrophic culture of microalgae has demonstrated that biomass yields can be high enough to increase lutein productivity indirectly. The utilization of glucose by microalgae strains that have been tested under this mode of cultivation is very similar to that of bacteria and yeasts, converting nearly 50% of the glucose into biomass. Furthermore, besides the aforementioned heterotrophic advantages, biomass production in industrial fermenters represents a field with substantial expertise. The knowledge acquired from the large-scale growth of bacteria and yeast readily translates and adapts to microalgae culture with organic carbon source media.

So far, the lutein productivities reported from high cell density of heterotrophic and mixotrophic cultures are similar to those of commercial production of astaxanthin and β -carotene from *Haematococcus pluvialis* and *Dunaliella salina*. However, as production costs differ due to the use of glucose for heterotrophic and mixotrophic cultures, further analysis of techno-economic factors is needed to compare properly. The current industrial-scale cultures for producing astaxanthin and β -carotene from these microalgae species

are carried out in open ponds. The cultivation mode is phototrophic in two stages, using sunlight as an energy source, CO₂ as a source of inorganic carbon and a stress trigger to induce the accumulation of the carotenoid in a second stage. Although the cell density achieved by these cultures is low, the moderate investment in energy and carbon source compared to the productivity of the pigments make these projects viable at the industrial level.

While the technical feasibility of heterotrophic and mixotrophic cultivation of microalgae has been evidenced in numerous studies, the high cost associated with glucose and other conventional organic carbon sources poses challenges for its scalability. Consequently, studies proposing alternative organic carbon sources have emerged, yielding performances akin to those achieved with glucose [72]. These alternate sources can be found in agro-industrial residues, food processing waste or food-grade wastewater, such as dairy manufacturing waste, brewery waste, and residues from higher cell culture media.

Additionally, it is necessary to consider the impact of metabolic regimes other than phototrophic in all process steps. Although the use of glucose and other organic carbon sources increases upstream costs, it has been reported that the size of the microalgal cells tends to be larger under this modality [153, 156, 176], which would reduce the costs of the downstream processes of harvesting and concentrating the biomass. Moreover, since downstream processes generally carry the highest costs in obtaining compounds from microalgae [177], it is necessary to continue efforts to simplify and optimize each step of lutein recovery.

Initial investigations indicate that, under specific conditions, the necessity for biomass drying is unnecessary, as lutein extraction from wet biomass yields comparable results [127, 178]. Similarly, exploring the utilization of less hazardous solvents with reduced ecological footprints presents a viable alternative for mitigating adverse environmental effects during the process [179]. The poor molecular stability of lutein must be taken into account for extraction processes, as well as for its stabilization and formulation into the final product to ensure it reaches the consumer with all its characteristics intact. Lutein is sensitive to heat and light, causing degradation and reducing its effectiveness in the health treatments described above. Efforts for its encapsulation are underway with promising results [94, 95]; however, it is necessary to focus efforts on finding alternatives that can be industrially scalable.

TABLE 1.1: Overview of lutein content and lutein productivity for different microalgae species in different culture modes, under different metabolic regimens and on one or two stages. Note 1: Interpretation of the productivity values requires careful consideration as various authors may employ different methodologies for calculating productivity. When the productivity value is not reported it was calculated manually by multiplying the lutein content by the biomass content, and then divided by the number of days the culture required to reach those values. Note 2: *Value reported in $\text{mg g}^{-1} \text{d}^{-1}$ **Strain obtained by mutation of the wild type.

Species	Culture mode	Metabolic regime	# of stages	Lutein content (%DW)	Lutein productivity ($\text{mg L}^{-1} \text{d}^{-1}$)	Ref.
<i>Chamydomonas</i> sp. JSC4	Batch	Phototrophic	One-stage	0.23	3.27	[59]
<i>Chlamydomonas</i> sp.	Batch	Phototrophic	Two-stage	0.42	3.25	[129]
<i>Chlorella minutissima</i>	Semi- continuous	Phototrophic	One-stage	0.80	5.35	[128]
<i>Chlorella sorokiniana</i> TH01	Batch	Phototrophic	One-stage	0.98	4.57	[160]
<i>Chlorella</i> sp. AE10	Batch	Phototrophic	One-stage	0.9	4.44	[133]
<i>Chlorella vulgaris</i> UTEX266	Batch	Phototrophic	One-stage	0.79	11.98*	[127]
<i>Desmodesmus</i> sp. F51	Batch	Phototrophic	One-stage	0.55	5.22	[145]
<i>Dunaliella salina</i>	Batch	Phototrophic	One-stage	0.70	3.68	[45]
<i>Parachlorella</i> sp. JD-076	Batch	Phototrophic	One-stage	1.18	25.03	[46]
<i>Scenedesmus almeriensis</i>	Continuous	Phototrophic	One-stage	0.54	4.77	[57]
<i>Scenedesmus obliquus</i> FSP-3	Batch	Phototrophic	One-stage	0.48	4.08	[43]
<i>Scenedesmus</i> sp. FSP3	Batch	Phototrophic	Two-stage	0.64	2.30	[180]
<i>Terastelmis</i> sp. CTP6	Batch	Phototrophic	One-stage	0.31	1.83	[123]
<i>Chlorella protothecoides</i>	Fed-batch	Heterotrophic	One-stage	0.91	10.57	[165]
<i>Chlorella sorokiniana</i> MB-1	Semi-batch	Heterotrophic	Two-stage	-	5.67	[167]
<i>Chlorella sorokiniana</i> MB-1-M12	Fed-batch	Heterotrophic	Two-stage	0.49	7.14	[159]
<i>Chromochloris zofingiensis</i>	Fed-batch	Heterotrophic	Two-stage	0.13	19.68**	[64]
<i>Auxenochlorella protothecoides</i>	Batch	Heterotrophic/photoinduction	Two-stage	0.49	12.36	[51]
<i>Scenedesmus incrassatulus</i>	Batch	Heterotrophic/photoinduction	Two-stage	0.14	3.10	[58]
<i>Scenedesmus obliquus</i>	Batch	Heterotrophic/photoinduction	Two-stage	0.15	6.50	[164]
<i>Scenedesmus almeriensis</i>	Batch	Heterotrophic/photoinduction	Two-stage	0.16	11.68	[172]
<i>Chlorella sorokiniana</i>	Batch	Mixotrophic	One-stage	0.38	3.97	[54]
<i>Chlorella sorokiniana</i>	Batch	Mixotrophic	One-stage	0.58	2.39	[158]
<i>Chlorella sorokiniana</i> Kh12	Batch	Mixotrophic	One-stage	1.73	0.45	[161]
<i>Scenedesmus obliquus</i> CWL-1	Fed-batch	Mixotrophic	One-stage	0.10	4.96	[169]
<i>Chlorella sorokiniana</i> FZU60	Fed-batch	Mixotrophic/photoinduction	Two-stage	9.57	11.57	[170]
<i>Chlorella sorokiniana</i> FZU63	Fed-batch	Mixotrophic/photoinduction	Two-stage	1.12	8.25	[146]
<i>Chlorella sorokiniana</i> C16	Batch	Mixotrophic	One-stage	1.74	9.04	[181]

Furthermore, integrating a biorefinery framework presents an opportunity in this context [37]. By adopting the principles of a biorefinery, this process can be optimized to extract maximum value from microalgae biomass and its associated by-products, including protein production [182], lipids and carbohydrates for biofuel generation [183] and the formulation of biostimulants adapted to agricultural applications [184].

Integrated biorefineries aim to efficiently convert diverse industrial biomass feedstocks into biofuels, energy, and various chemicals and materials, thereby achieving economic viability and positive energy balances. Microalgae, particularly heterotrophic and mixotrophic strains, offer potential for producing biofuels and high-value chemicals [149]. Prioritizing the isolation of proteins and lipids from microalgae biomass is crucial initially, as these constitute the major fractions, while carbohydrates and pigments contribute significant value when separated [15, 185]. Overcoming bottlenecks in fraction separation is imperative, requiring the development of gentle, cost-effective, and energy-efficient techniques applicable to diverse end products of sufficient quality and quantity. For example, Nobre *et al.* [186] coupled the production of lipids, carotenoid pigments and hydrogen from the processing of *Nannochloropsis* sp. biomass. Using supercritical CO₂ extraction and 20% ethanol the authors were able to extract 4.5 g_{lipids} g⁻¹DW of lipids and recover 70% of the pigments, while the remaining biomass was fermented by *Enterobacter aerogenes* for hydrogen production.

When incorporated into this process, the biorefinery concept not only improves resource efficiency but also diversifies its results, allowing a more sustainable and versatile approach to harness the potential of microalgae in various industries.

Although there are economic feasibility studies on the production of different microalgae species, it is still premature to compare cost and resource consumption against production from marigold flowers. Different authors base their case studies on various factors and consider different outputs. For example, Acién *et al.* [187] estimated a production cost of 12.6 euros per kg of *Scenedesmus almeriensis* and energy consumption of 42 MJ per ton, grown in phototrophy mode, while Jin *et al.* [150] consider a cost of \$1.60 per kg of *Chlorella sorokiniana* in heterotrophic mode, but the latter does not include the cost of biomass harvesting. Furthermore, valuable examples can be found on microalgae production cost [188, 189]; nevertheless, nothing has been explicitly reported on lutein production. Additionally, the cost of extraction will depend on the lutein yield, which varies from one species to another and from one cultivation mode to another. Also, water consumption and nutrient costs present significant variability that must be considered. This demonstrates the urgent need to conduct techno-economic studies for lutein production, starting from high productivity values and considering all steps of a pilot-scale process.

1.6 Conclusions

The pursuit of optimal lutein production from microalgae necessitates a shift in focus toward overall productivity rather than solely emphasizing lutein content. Throughout this review, it became evident that optimal culture parameters for phototrophic biomass accumulation generally induces the highest lutein synthesis. This optimization involves fine-tuning variables such as light quality and quantity, nitrogen levels, and temperature. However, pushing these parameters to their extremes can induce stress in the cells, resulting in diminished lutein productivity. Although initially promising, phototrophic batch cultures exhibit limitations as they reach a plateau concerning both lutein content and productivity. In contrast, heterotrophic and mixotrophic cultures, particularly those

employing photoinduction in two-stage processes and incorporating varied culture medium feeding strategies, have shown remarkable potential and achieved the highest reported lutein productivities. Nonetheless, it becomes imperative to delve deeper into these strategies' techno-economic feasibility to pave the way for commercial viability. Further studies are essential to validate these approaches' scalability and economic viability, ultimately propelling microalgae-derived lutein production towards commercial realization.

Heterotrophic culture of *Scenedesmus almeriensis*

Following the conclusions drawn from the previous section, this chapter presents the results of lutein productivity obtained in heterotrophic culture from a microalgae species previously identified as promising in a phototrophic regime. The kinetic parameters derived from the optimal substrate concentrations are presented, providing a new microalgae alternative for high-yield lutein production.

The results in this chapter were significantly enhanced by the valuable experimental contributions of Théo Julien.

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2.1 Introduction

Lutein is a primary carotenoid that belongs to the xanthophyll group. It is found in photosynthetic organisms, specifically in leaves and fruits of higher plants and in vegetables, including kale, spinach, and corn, but also in microalgae [25]. Since animals cannot synthesize lutein, it must be obtained directly from the diet. Once acquired, it is accumulated primarily in the eye, where it has protective functions against retinal oxidation [18]. In addition to its benefits for eye health, lutein has also been studied for its potential to improve skin health and reduce the risk of certain types of cancer [100]. Furthermore, lutein has been shown to cross the blood-brain barrier, a semi-permeable barrier that separates the brain from the circulatory system [190]. This ability to cross the blood-brain barrier may be important for the benefits of lutein for brain health. Some studies have suggested that lutein may have a protective effect on the synapses by mitigating Reactive Oxygen Species (ROS), potentially reducing the risk of age-related cognitive decline and improving cognitive function [19]. The current daily dietary intake of lutein among Europeans and North Americans stands at a mere 1.7 mg, which represents approximately 12% of the recommended 6-14 mg daily intake shown to be beneficial in reducing the risk of age-related diseases [24].

Currently, the commercial production of natural lutein is made from cultivating

Marigold flowers (genus *Tagetes*). After a laborious flower harvest, a drying process of the petals is required to obtain a lutein-rich oleoresin with a final lutein productivity of 10.6 kg hectare⁻¹ year⁻¹ [25, 26]. The rising demand for lutein, projected to reach a global market value of USD 491 million by 2029 [21], is causing concerns about the sustainability of this mode of production. The shortage of arable land and irrigation water, excessive use of pesticides and chemical fertilizers, and the need for specific climate conditions for growth are factors that are driving the exploration of alternative sources of production [25, 29].

Alternatives to Marigold exist to produce lutein. Artificial synthesis of lutein has been possible and synthetic biology tools have recently been proposed for the production of lutein from bacteria and yeast, microorganisms that do not produce this carotenoid naturally [32, 33]. However, chemical synthesis of lutein and its precursors can only be obtained at very low yields (1-5%) and involving numerous steps [30, 31]. On the other hand, despite the great potential for increasing lutein production using genetic engineering tools, low social acceptability and concerns about their adverse effects hinder its market.

Alternatively, microalgae are photosynthetic organisms naturally producing lutein at concentrations ranging from 0.1 to 1% of their dry weight [46]. Their cultivation cycles are short and they can be grown biotechnologically in continuous mode all year long [36]. Traditionally, microalgae are cultivated outdoors to take advantage of the sunlight they require for photosynthesis or are provided with artificial light under controlled conditions. Under this photoautotrophic mode of cultivation, Gong and Bassi [127] obtained 0.91% lutein in a Dry Weight basis (DW) culture of *Chlorella vulgaris*. Similar results were obtained with *Chlorella sorokiniana* (0.58% DW) [158], *Dunaliella salina* (0.7% DW) [132], *Parachlorella* sp. (1.18% DW) [46] and *Muriellopsis* sp. (0.54% DW) [60]. However, in phototrophic cultures, where light is the only source of energy, accessibility to light is inversely proportional to cell concentration due to mutual shading of cells [149]. Ultimately, limited light availability constrains the overall process productivity. On the one hand, it reduces the attainable microalgal density, and on the other, as light intensity increases and cell density rises, it lowers the cell pigment content.

To overcome this limitation, heterotrophic, mixotrophic and two-stage cultivation strategies have been proposed, using different sources of organic carbon as energy and carbon source [51, 58, 129, 152, 166]. Chem *et al.* [54] achieved a 42% enhancement in lutein productivity using 3 g L⁻¹ of acetate as carbon source for a mixotrophic culture of *Chlorella sorokiniana* Mb-1. Zhao *et al.* [129] cultured *Chlamydomonas* sp. JSC4 and increased lutein productivity by 60% under a two-stage process. Do *et al.* [152] cultured *C. sorokiniana* under mixotrophic growth and achieved a biomass concentration and lutein content of 26.21 g L⁻¹ and 5.01 mg gDW⁻¹, respectively, using sodium acetate as an organic carbon source.

Among the microalgal candidates for industrial lutein production, the genus *Scenedesmus* has been extensively studied for its potential to generate high biomass and pigment yield [57, 191, 192]. Compared to species in the *Chlorella* group, *Scenedesmus* species have a weaker cell wall, according to Spain and Funk [193], who reported half the concentration of rhamnose in the cell wall of *Scenedesmus* sp. compared to a strain of *Chlorella vulgaris*. The rhamnose concentration in the cell wall is directly related to wall rigidity in microalgae [194]. This characteristic makes cell disruption and pigment extraction more accessible and cost-effective. Additionally, *Scenedesmus* cells are larger than *Chlorella*'s, which eases the harvesting process.

Within the *Scenedesmus* genus, the strain *Scenedesmus almeriensis* has been found to

have high lutein content, with levels reaching up to 8.5 mg gDW^{-1} under phototrophic conditions [195]. Several authors have positioned *S. almeriensis* as a species with potential for industrial cultivation for various reasons [57, 191, 192, 196, 197]. In addition to containing one of the highest contents of lutein among microalgae, it is a species that withstands high cultivation temperatures (up to $35 \text{ }^{\circ}\text{C}$), allowing its cultivation in areas with high solar incidence and high temperatures [124].

Still, while achieving a high cellular lutein content, a *S. almeriensis* phototrophic bioprocess would suffer from the above-mentioned light limitation. This article explores a new approach for lutein production with this strain: the heterotrophic regime. To the best of our knowledge, this is the first report of *S. almeriensis* being cultured under heterotrophic conditions. Although other microalgae species with the potential to produce lutein have been studied under heterotrophic conditions, the characteristics of *S. almeriensis* make it a potential candidate for exploring this mode of cultivation. Additionally, *S. almeriensis* has been proposed as an attractive candidate for nutrient removal during wastewater treatment [198]. Hence, the behavior of this species under heterotrophic conditions contributes to a better understanding of the biology of the species with prospects for industrial use.

While this study primarily investigates lutein, the expression of other carotenoids has also been monitored. In fact, within the context of a biorefinery approach, the traditional single-product culture has been replaced by a multi-output biotechnological process. As a result, the levels of VAZ (Violaxanthin + Antheraxanthin + Zeaxanthin) cycle pigments, particularly zeaxanthin, are also reported in this study. Zeaxanthin is a natural carotenoid recognized for its importance in various aspects of human health, serving as a potent antioxidant and light filter. As with lutein, its market has increased in recent years. Zeaxanthin is generally used with other carotenoids, particularly lutein, so the simultaneous increase of its production presents advantages from the industrial point of view [37]. Additionally, other fractions of biomass content are reported to provide a spectrum of opportunities within a biorefinery concept.

In this study, our initial focus was examining the growth and lutein production of *S. almeriensis* under heterotrophic conditions. Subsequently, we explored a two-stage culture approach to maximize lutein productivity. The first stage involved utilizing a glucose-rich medium to obtain a concentrated biomass culture, while the second stage involved introducing a light source to drive the cell to express their photosynthetic apparatus, hence lutein content, once the glucose was depleted from the medium. The findings showcased in this study indicate this species' prospective suitability for expanding industrial-level cultivation. This assertion stems from the significant biomass concentration achieved through heterotrophic culture, coupled with the augmentation of lutein synthesis during the photoinduction phase. As a result of these factors, the attained lutein productivities stand among the most remarkable reported to date. Although this species was recently renamed by Turiel *et al.* [199] as *Tetradesmus almeriensis*, throughout this paper, reference will be made to the former name *Scenedesmus almeriensis* to facilitate comparison with previous work on this same species.

2.2 Materials and Methods

2.2.1 Strain and phototrophic culture conditions

A strain of *Scenedesmus almeriensis* was received as a kind donation from Prof. Francisco Gabriel Acien from the University of Almeria. The strain was grown on agar with nutrient

medium and glucose as a routine procedure to check for bacterial contamination. During the process, it was found that *Scenedesmus almeriensis* grew in the absence of light, so the strain was preserved under two cultivation methods, phototrophic in B3N culture medium under artificial white light and heterotrophic in B3N culture medium added with 10 g L⁻¹ of glucose and without a light source. The phototrophic culture was carried out in 250 mL flasks with 50 mL of B3N culture medium containing (per liter): NaNO₃ (750 mg), MgSO₄ 7H₂O (75 mg), NaCl (25 mg), K₂HPO₄ (75 mg), KH₂PO₄ (175 mg), CaCl₂ 2H₂O (25 mg), ZnSO₄ 7H₂O (8.82 mg), MnCl₂ 4H₂O (1.44 mg), MoO₃ (0.71 mg) CuSO₄ 5H₂O (1.57 mg), CO(NO₃)₂ 6H₂O (0.49 mg), H₃BO₃ (11.42 mg), EDTA (50.0 mg), KOH (31 mg) and FeSO₄ 7H₂O (4.98 mg) [200]. Cultures were incubated on a shaker at 100 rpm, at 30 °C, under 60 μmolPhoton m⁻² s⁻¹ illumination provided by cool white LED lamps. Cultures were performed in biological triplicates.

2.2.2 Determination of glucose and nitrogen concentration for heterotrophic growth

In order to establish the strain affinity concerning glucose and nitrogen in the culture medium, tests were carried out at 100 rpm, 30 °C, without a light supply. To determine the maximum glucose concentration, B3N medium (containing 6 g L⁻¹ sodium nitrate) was used and the amount of glucose was varied (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 g L⁻¹). To determine the noninhibitory nitrogen concentration, B3N medium (with 10 g L⁻¹ of glucose) was used and the amount of sodium nitrate added was varied (0, 5, 7.5, 10, 15, and 20 g L⁻¹). Cultures were duplicated biologically.

2.2.3 Heterotrophic and two-stage culture conditions

Following the substrate affinity test, cultures were led on a maximal noninhibitory medium to increase biomass productivity before inducing the photosynthetic apparatus. For this, 25 mL of B3N culture medium at 4X concentration supplemented with 40 g L⁻¹ of glucose was inoculated with 1 mL of *Scenedesmus almeriensis* previously subcultured every week under a heterotrophic regime. Given that the oxygen demand in the heterotrophic regime is high, the culture volume was low to ensure no limitation due to lack of aeration. Therefore, the sampling was limited to a minimal volume in order to measure only the cell concentration. Under these circumstances, the heterotrophic stage was maintained for seven days to guarantee the total consumption of glucose from the medium before starting the photoinduction stage, referred to as Late Photoinduction further on in the text.

Subsequently, the biomass was recovered, washed and resuspended in 50 mL of fresh B3N culture medium, which meant a dilution by half of the final heterotrophic concentration. This dilution was done for four reasons: i) to wash out any remaining glucose in the medium that could inhibit carotenoid synthesis [58]; ii) to provide fresh nutrients, mainly nitrogen, to allow the formation of new proteins needed for the photosynthetic complex that hosts the carotenoids; iii) to allow daily sampling and monitoring of the evolution of the biomass and its pigments without exhausting the culture volume; and iv) to enable a higher average light irradiance in the dense culture. Then, the diluted samples were divided into two groups. The first group was exposed to a light intensity of 60 μmolPhoton m⁻² s⁻¹ to induce lutein synthesis and the rest of the culture conditions were maintained in the dark, as the previous heterotrophic culture.

Once the growth kinetics during the heterotrophic phase were determined, a second test

was conducted (referred to as Immediate Photoinduction further on in the text). On this test, the photoinduction stage started immediately after the heterotrophic culture entered the stationary phase, only three days after the start of the heterotrophic cultivation. The results section compares the lutein content and productivity of the two tests, and the significance of the cells' metabolic state in responding to photoinduction is discussed. These tests were performed in biological triplicates.

2.2.4 Microalgal growth assessment

For all the cultures, flasks were sampled twice daily for growth monitoring. Additionally, during the photoinduction stage, the sampling included biomass recovery for pigment profiling. For this, each sample was centrifuged and the pellet was washed with distilled water and centrifuged again. The resulting biomass was stored at -20 °C in dark conditions until processing. The growth of *S. almeriensis* cells were tracked by measuring the absorption of samples at 750 nm using a UV/Visible spectrophotometer (Shimadzu UV-1800 spectrophotometer)[201]. Samples were diluted to an optical density of 0.4 or less before recording the value. To determine the dry weight of the biomass, absorption values were recorded at various biomass concentrations, which were subsequently filtered and dried for 24 h at 100 °C and a calibration curve was developed linking absorbance to dry weight (10 points, ranging from 0.03 to 0.7 g L⁻¹, R²=0.995). This curve yielded the equation (Eq. 2.1):

$$DW = 0.796 \times A_{750nm} - 0.0247 \quad (2.1)$$

where DW represents the dry weight biomass concentration in grams per liter (g L⁻¹), and A represents the total absorbance measured at 750 nm, using distilled water as blank.

The growth rate of the cultures under different glucose and sodium nitrate concentration were calculated in the exponential growth phase using the classical equation (Eq. 2.2):

$$\mu = \frac{\ln(C_{t_2}) - \ln(C_{t_1})}{t_2 - t_1} \quad (2.2)$$

2.2.5 Extraction of carotenoids from microalgae biomass

Lutein was extracted from biomass by adapting the method described by Ahmad *et al.* [179]. In general, a wet pellet of known biomass (4-8 mgDW) was mixed with 1 mL of laboratory-grade inert sand (Fisher Scientific Code: 10132590) and 10 mL of 100% ethanol. Cell disruption was carried out in a high-speed benchtop homogenizer (MP Biomedicals FastPrep-24 5G Instrument, Fisherbrand, Waltham, MA, USA) at 6.5 m s⁻¹ in two cycles of 30-second with a 60-second pause. The samples were then allowed to rest at room temperature in a rotator (Stuart Rotator SB3) at 10 rpm for 60 minutes. Finally, the samples were filtered through a 0.22 µm pore filter to separate particles before passing them through a High-Pressure Liquid Chromatograph (HPLC). All this protocol was conducted with light protection around the samples.

2.2.6 Pigment quantification

Pigments were quantified on an Ultima 3000 HPLC (Thermo Fisher Scientific) coupled with a UV detector. Separation was achieved on an Acclaim Polar Advantage II C18 column

(4.6 x 150 mm, 3 μm , 120 \AA) from Thermo Fisher Scientific. The column temperature was maintained at 30 $^{\circ}\text{C}$. Pure methanol was the mobile phase. The flow rate was 0.5 mL min^{-1} , and the elution was set in isocratic mode. The injection volume was 5 μL , and the total run analysis was 40 min. Compounds were identified by comparing their retention time and UV-Vis spectra with standard solutions. UV-Vis spectra were recorded from 200 nm to 700 nm. Absorbance was recorded at 400, 450, 500, and 650 nm. Pigment quantifications were led using the area of the peaks in external calibration for the most sensible of the recorded wavelength. External calibration concentrations ranged from 0.25 to 5 mg L^{-1} . Pigment standards and methanol were purchased from Sigma-Aldrich. Standards had a purity greater than 97%. The three pigments of interest (lutein, violaxanthin, and zeaxanthin) were reported systematically for each sample. "N.A." was used whenever a sample could not be detected or quantified.

2.2.7 Lipid, protein and carbohydrate quantification

Lipids were quantified gravimetrically. First, 100 mg of freeze-dried microalgae powder was resuspended in 10 ml of milliQ. Then, cells were homogenized using MP Biomedicals FastPrep42 bead miller. Lipids were extracted from lyzed cells following Bligh and Dyer protocol [202]. The chloroform phase containing the lipids was then left to evaporate, and the solid residues were weighed.

For determination of the cells' protein content, 1.95 mg of freeze-dried microalgae was resuspended into 20 ml of water which was then analyzed by TOC-L CSH analyzer (Shimadzu) for total nitrogen quantification. Protein content was calculated from total nitrogen content using a correction factor derived for *Chlorella* sp., yielding the following equation (Eq.2.3) [203]:

$$Protein_{mg/l} = 4.78 \times TN_{mg/l} \quad (2.3)$$

5 mg of freeze-dried cells were homogenized before total carbohydrates determination using the anthrone blue method [204] (calibration curve realized twice at 630 nm using glucose, linearity range 0.05 g/L to 0.5 g/L, 5 points, $R^2 = 0.999$, with control to nullify potential chlorophyll contribution at 630 nm).

2.2.8 Statistical analysis

Statistical significance was assessed using the ANOVA test. When the null hypothesis was rejected ($p < 0.05$), data were further analyzed using Tukey's Honestly Significant Difference (HSD) test. The following results are presented as the average of the replicate ($n = 3$ unless stated otherwise), while the error bars account for the standard deviation.

2.3 Results

This study aimed to maximize lutein productivity from *S. almeriensis* cultured under heterotrophic conditions, compared to phototrophic conditions. This work generated data on cell density and the quantity of lutein, violaxanthin and zeaxanthin. The resulting data enabled a comparison of the carotenoid productivity between the two cultivation modes based on the difference in produced biomass. The subsequent sections describe the findings and discuss their implications for lutein production from *S. almeriensis*.

2.3.1 Glucose and nitrogen affinity and inhibition

Two separate tests were conducted independently to determine the carbon and nitrogen concentration to which the microalga *Scenedesmus almeriensis* is best adapted to grow and produce lutein. These experiments were conducted to determine the ideal concentration of glucose (on B3N medium supplemented with 6 g L⁻¹ of sodium nitrate) and sodium nitrate (on B3N medium supplemented with 10 g L⁻¹ of glucose). Figure 2.1a shows the growth rate of cultures at glucose concentrations ranging from 10 to 100 g L⁻¹ in increments of 10 g L⁻¹. The results show that concentrations from 10 to 50 g L⁻¹ belong to the same statistical group ($p < 0.05$), between 1.17 ± 0.02 and 1.42 ± 0.03 day⁻¹ with a probably marginal inclusion of the culture under 50 g L⁻¹ as discussed below. Concentrations higher than 50 g L⁻¹ of glucose were detrimental to the growth rate, most probably due to an inhibitory effect of the substrate.

Since the objective was to obtain the highest biomass concentration, the 40 g L⁻¹ glucose concentration was chosen, as the cultures at this concentration gave a similar growth rate compared to lower concentrations. Higher amounts of glucose showed increasing inhibition as the concentration was increased. Additionally, we can see in Figure 2.1b that after exponential growth started and up to day 4 of the culture, cultures with glucose concentrations from 10 to 40 g L⁻¹ had similar biomass concentrations ($p = 0.440$). On the contrary, cultivation with 50 g L⁻¹ of glucose exhibited a much shallower onset of the exponential phase, indicating that the substrate concentration is inhibiting faster growth. Ultimately, at higher concentrations, growth is not even observed within the initial 4-day period. Furthermore, Figure 2.1c shows the linear growth, where the trend concerning the glucose concentration is confirmed.

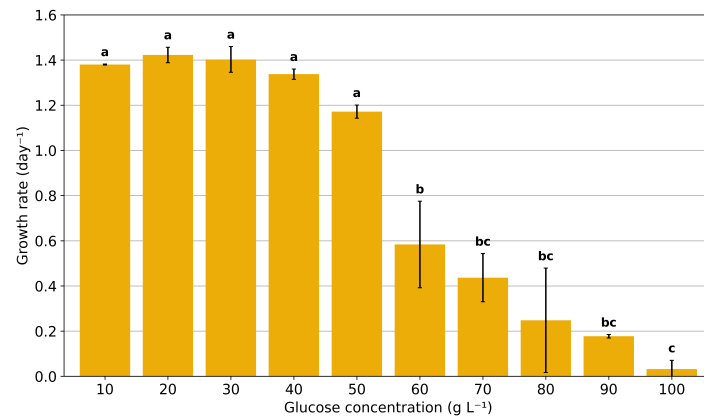
Considering that an increase in growth is expected due to glucose in the medium, an accelerated rate of nitrogen consumption is also anticipated, necessitating the determination of sodium nitrate concentration to prevent potential limitations or inhibitions. To achieve this, a parallel test was conducted with cultures on B3N medium with 10 g L⁻¹ of glucose and the sodium nitrate content was varied at 0.75, 5, 7.5, 10, 15 and 20 g L⁻¹. Figure 2.2 shows the evolution of *S. almeriensis* growth rate with increased sodium nitrate concentrations. Also, one can see a reduction in growth rate at sodium nitrate concentrations above 7.5 g L⁻¹, so a concentration of 3 g L⁻¹ was determined for subsequent experiments to support rapid biomass development.

The combined effect of the glucose concentration at 40 g L⁻¹ and sodium nitrate at 3 g L⁻¹ was tested to confirm no adverse interaction between the two concentrations. The result showed an even higher growth rate (1.45 ± 0.011 day⁻¹) than the individual glucose and sodium nitrate tests.

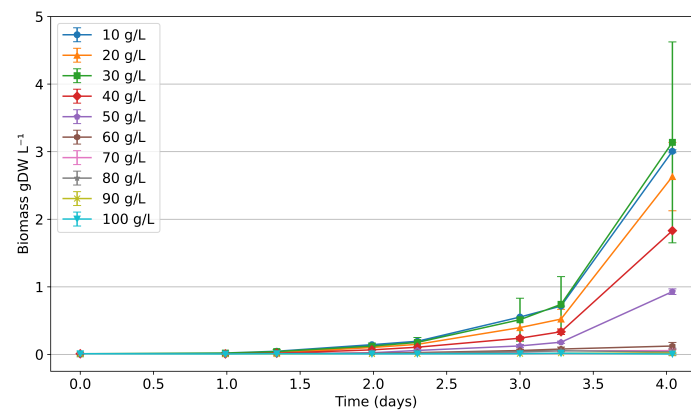
2.3.2 Comparison between phototrophy and heterotrophy

In addition to comparing biotechnological performance, this study also offered the opportunity to compare differences in *S. almeriensis* morphology induced by the different modes of cultivation. Figure 2.3 presents pictures of *Scenedesmus almeriensis* cells in cultures under phototrophy and heterotrophy conditions during the late exponential phase of each culture. As one can see, cells cultured with light ($60 \mu\text{mol Photon m}^{-2} \text{s}^{-1}$) and under nutrient sufficiency exhibited the classical oval shape with pointed tips of this species as well as coenobia made of 2 and 4 cells, as also described by Sánchez *et al.* [124] and Turiel *et al.* [199].

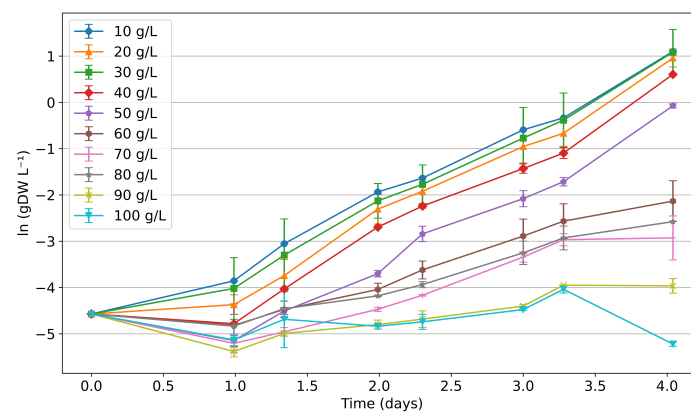
On the contrary, cells cultured under the heterotrophic condition with 10 g L⁻¹ of



(A)



(B)



(C)

FIGURE 2.1: Effect of different glucose concentrations on *S. almeriensis* using 6 g L⁻¹ of sodium nitrate: (a) Growth rate (Different letters indicate differences between growth rates, $p < 0.05$), (b) Growth curves, and (c) Natural logarithm-transformed biomass concentration. Points are the average of duplicates.

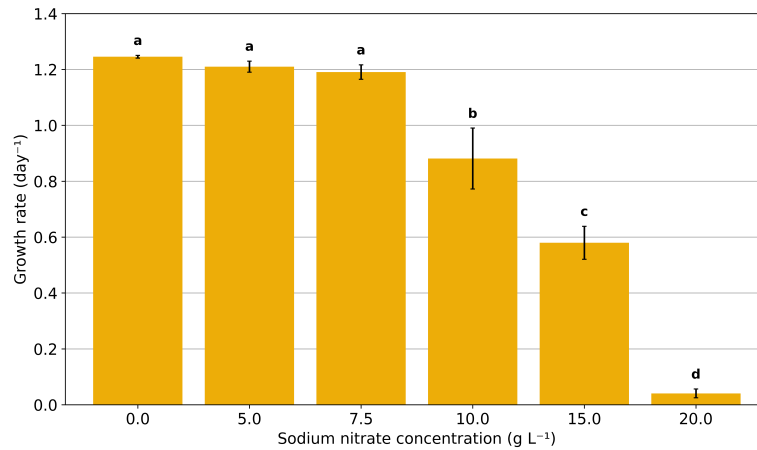


FIGURE 2.2: Growth rate of *S. almeriensis* under different sodium nitrate concentrations. The concentration shown indicates the amount of sodium nitrate that was added to the regular B3N medium. Initial glucose concentration: 10g L⁻¹. Different letters indicate differences between growth rates, $p < 0.05$. $n = 2$

glucose displayed a much rounder shape, with smoother tips and can only be found as single floating cells. The latter are also generally larger in volume compared with the cells grown under light. These characteristics are consistent with cell changes under heterotrophic cultures of species in the *Scenedesmaceae* family, like *Scenedesmus obliquus* ABC-009 [164], *Scenedesmus acuminatus* [151] and *Scenedesmus* sp. [176].

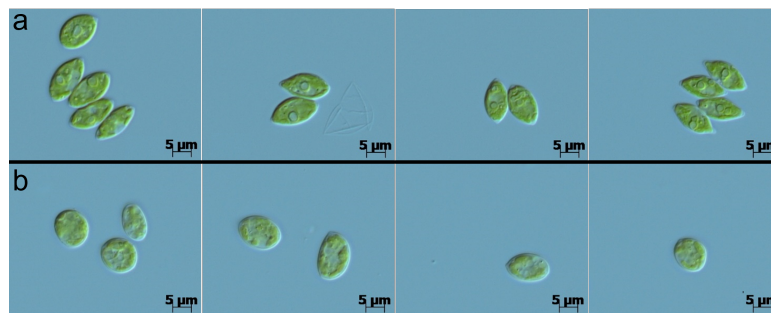


FIGURE 2.3: *S. almeriensis* cells cultured under phototrophic (a) and heterotrophic (b) conditions. Pictures were taken from samples at the exponential growth phase of each cultivation mode. Cells growing in phototrophic culture were at 30 °C with 60 $\mu\text{mol Photon m}^{-2} \text{s}^{-1}$ of light intensity on B3N medium and at 100 rpm. Cells in heterotrophic culture were in the same conditions except that they had no light and the medium contained 10 g L⁻¹ glucose. Both cultures had nutrient availability at the time of sampling.

2.3.3 Growth dynamic and lutein content

2.3.3.1 Phototrophic and heterotrophic comparison

Figure 2.4 shows the difference in cell concentration over time between cultures on phototrophic, heterotrophic and heterotrophic+photoinduction conditions. The culture on phototrophy reached a maximum biomass content of $1.47 \pm 0.18 \text{ g L}^{-1}$ on day eleven after showing linear growth. In comparison, the heterotrophic culture had an accelerated growth during the first two days. On the third day, it reached the stationary phase with $22 \pm 0.8 \text{ g L}^{-1}$ of biomass, a 15-fold increase in biomass content compared to day

eleven on phototrophy mode. On the other hand, it can be observed that, after dilution of the heterotrophic culture by half with fresh B3N medium, the culture that was kept under dark conditions as a control did not show any growth ($p=0.8783$), which was to be expected, as it lacked energy source (neither glucose nor light). However, the culture that was transferred to light conditions to induce lutein synthesis was able to benefit from the low light intensity of $60 \mu\text{molPhoton m}^{-2} \text{s}^{-1}$ to stimulate the growth of the culture ($p=0.0009$).

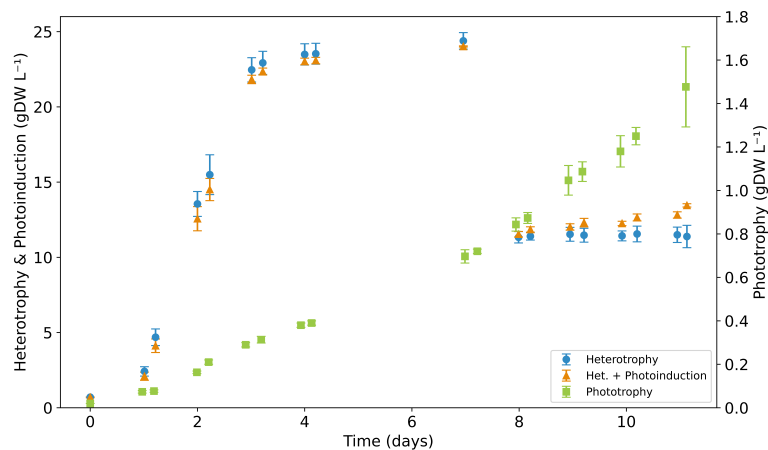


FIGURE 2.4: Time course profile of *S. almeriensis* biomass concentration under different cultivation modes ($n = 3$).

The carotenoid content of the cultures grown under phototrophic and heterotrophic conditions are presented in Figure 2.5. The phototrophically grown cells exhibited maximal lutein and violaxanthin contents after 11 days of culture, with values of 2.65 ± 0.29 and $0.42 \pm 0.06 \text{ mg gDW}^{-1}$, respectively. Comparatively, cells cultured under heterotrophic conditions presented 1.43 ± 0.04 and $0.3 \pm 0.03 \text{ mg gDW}^{-1}$ of lutein and violaxanthin, respectively, at the end of the exponential growth phase (day 3). On the other hand, zeaxanthin content was the other way around, with a cell content 1.8 times higher in the heterotrophic culture ($0.43 \pm 0.09 \text{ mg gDW}^{-1}$ on heterotrophy and $0.25 \pm 0.005 \text{ mg gDW}^{-1}$ on phototrophy, $p = 0.006$).

2.3.3.2 Late and immediate photoinduction comparison

As explained before, a first photoinduction test was made starting the light treatment four days after the heterotrophic culture reached the stationary phase (Late Photoinduction). This delay was born of the conjunction of the will to ensure that glucose had been depleted and staff availability. In this first test, the lutein content reached a maximum value of $1.54 \pm 0.13 \text{ mg gDW}^{-1}$ after 1.2 days of photoinduction, but 5.2 days after the heterotrophic culture reached the stationary phase (Figure 2.6). Despite the increase in lutein content due to photoinduction, the overall productivity is very low due to this long delay. Therefore, a second test was carried out to initiate photoinduction immediately after the heterotrophic culture reached the stationary phase (Immediate Photoinduction). On this second test, the lutein content reached a maximum of $1.62 \pm 0.13 \text{ mg gDW}^{-1}$ after 10 hours of light treatment, an increase of 13.28% compared to the end of the heterotrophic culture.

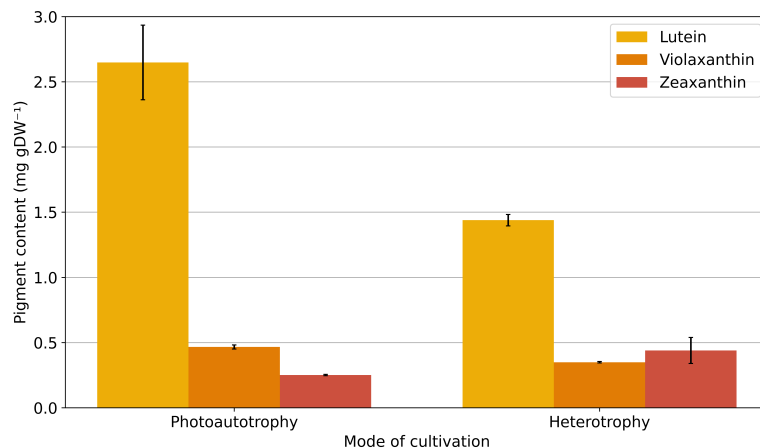


FIGURE 2.5: Lutein, violaxanthin and zeaxanthin content of *S. almeriensis* under different culture regimes. For the phototrophic mode, the highest content was at day 11. For the heterotrophy mode, values were taken on day 3, corresponding to the end of the exponential phase.

2.3.4 Lutein productivity

Despite the phototrophic culture having higher lutein content, the increased biomass productivity achieved through heterotrophic growth mode led to an equivalent increase in lutein productivity, both in heterotrophic and photoinduced cultures (Figure 2.7). Maximum lutein productivity under phototrophic culture was reached on day 11, with $0.34 \pm 0.04 \text{ mg L}^{-1} \text{ d}^{-1}$. In turn, lutein productivity at day 7 of the heterotrophic culture (Long Heterotrophy) was $3.43 \pm 0.3 \text{ mg L}^{-1} \text{ d}^{-1}$. The resulting biomass, diluted by half with fresh B3N medium and subjected to $60 \mu\text{mol Photon m}^{-2} \text{ s}^{-1}$ of light, showed a productivity of $2.09 \pm 0.15 \text{ mg L}^{-1} \text{ d}^{-1}$ after 1.2 days of light (Late Photoinduction).

As stated earlier, the Late Photoinduction stage began four days after reaching the stationary phase of the heterotrophic culture. This long heterotrophic delay caused the calculated productivity to fall short of expectations, as several days went by that could have been avoided. Subsequently, for the Immediate Photoinduction test, the heterotrophic stage was stopped on day three after reaching lutein productivity of $9.79 \pm 0.17 \text{ mg L}^{-1} \text{ d}^{-1}$ (Short Heterotrophy), while the lutein productivity after dilution and 10 hours of photoinduction reached $5.76 \pm 0.51 \text{ mg L}^{-1} \text{ d}^{-1}$.

2.4 Discussion

Different species of the genus *Scenedesmus* have been grown in a heterotrophic manner and have shown higher final biomass concentration than those obtained in illuminated cultures [150, 156, 176]. However, this is the first report of *Scenedesmus almeriensis* grown without a light source. Therefore, the ideal glucose level had to be determined to take advantage of the glucose utilization capacity of this species without exceeding the limits that would induce growth inhibition.

In preliminary tests, *S. almeriensis* under a heterotrophic regime showed a biomass yield of 0.5 gDW per 1 g of glucose feed. This value is quite classical for microbial growth and demonstrates that *Scenedesmus almeriensis* grows well with glucose [162]. These tests were carried out under 10 g L^{-1} of glucose. However, it was imperative to investigate the maximal glucose concentration within the growth medium to achieve maximal biomass

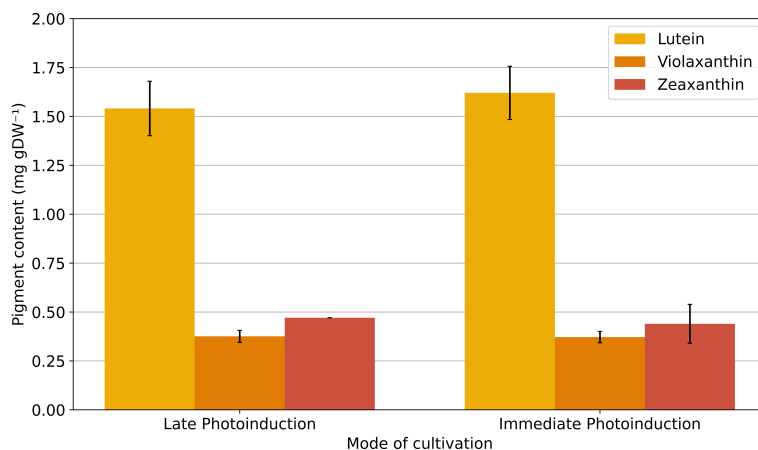


FIGURE 2.6: Lutein, violaxanthin and zeaxanthin content of *S. almeriensis*. In the first test (Late Photoinduction), the photoinduction started four days after the heterotrophic culture reached the stationary phase. For the second test (Immediate Photoinduction), the photoinduction started immediately after the heterotrophic stationary phase. Carotenoid values are taken on day 8.2 for the Late Photoinduction and on day 3.3 for the Immediate Photoinduction, corresponding to the maximum content after each photoinduction started.

productivity within a particular culture system. At the levels defined as optimal (40 g L^{-1} of glucose and 3 g L^{-1} of sodium nitrate), cells could fully utilize the organic substrate and grow at a growth rate of $1.45 \pm 0.011 \text{ day}^{-1}$ while avoiding inhibitory effects. This value is twice as high than the one reported by Florez-Miranda *et al.* [58] (0.74 day^{-1}) with *Scenedesmus incrassatulus* on heterotrophic conditions using 30 g L^{-1} of glucose as the carbon source. On the other hand, the defined concentration gives a C:N ratio of 32, which is higher than other reported studies. Jin *et al.* [151] tested different C:N ratios from 4 to 32 and reported the highest growth rate (1.03 day^{-1}) and maximum biomass concentration (220 g L^{-1}) using a rate of 12 on a culture of *Scenedesmus acuminatus*, however, they used urea as the nitrogen source, which is metabolized by microalgae in a faster way than sodium nitrate [205]. As for the biomass production per unit of substrate (glucose) in other microalgae species, it is similar to the 0.5 yield found in this work. Florez-Miranda *et al.* [58] reported a yield of 0.59 in *S. incrassatulus*, while Koh *et al.* [164] report 0.55 for *S. obliquus*. Similar observations have been made in other studies using different carbon sources ([151, 159, 162]).

Given that the synthesis pathway for lutein in microalgae is closely linked to photoautotrophic biomass production [57], light appears essential to promote high lutein expression. However, low biomass production results from mutual shading between cells under photosynthetic growth, reducing lutein productivity. The biomass content obtained under phototrophic conditions is consistent with other reports for *Scenedesmus* species on phototrophic conditions on conventional culture systems [164, 176]. Nevertheless, the extent of this statement is to be modulated, since short light path photobioreactor can also be a means to achieve dense culture (for example, 20 g L^{-1} in 7-8 days with *Chlorella* genus [206]) at the price of high areal requirement. On the other hand, the high biomass production of 22 g L^{-1} in the heterotrophic culture corresponds to an efficient utilization and conversion of substrate into biomass.

The growth that can be observed in the culture that was transferred to light conditions after having grown in heterotrophy is possibly due to the rapid adaptation to this new source of energy despite the low light intensity of $60 \mu\text{molPhoton m}^{-2} \text{ s}^{-1}$. This light

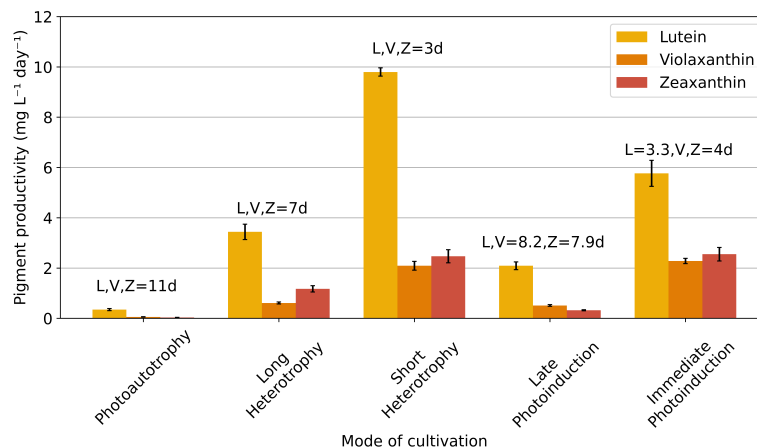


FIGURE 2.7: Productivity content of *S. almeriensis* cultured under different cultivation modes. The culture duration (days) at which the maximal productivity was obtained is indicated for each pigment above every mode of cultivation (Lutein = L; Violaxanthin = V; Zeaxanthin = Z).

intensity was selected since, at low intensity, photosynthetic cells attempt to capture more light, developing their photosynthetic system, including the content of carotenoids that contribute to collecting more light. Additionally, it demonstrates that the photosynthetic system can be reactivated once the energy and carbon source changes. This has been previously reported by Kamalanathan *et al.* [176] in a comparison between phototrophic, heterotrophic and mixotrophic culture of *Scenedesmus* sp.

Although heterotrophic cultivation may suggest that photosynthetic pigments are unnecessary, studies have shown that some microalgae strains produce a certain amount of pigments, even under dark conditions. However, it should be noted that phototrophically grown microalgae typically exhibit substantially higher levels of carotenoid content. Xiao *et al.* [51] reported an almost 5-fold lower amount of lutein in *Auxenochlorella protothecoides* in mixotrophic and heterotrophic cultures compared to a phototrophic culture, arguing that the presence of glucose in the medium may have had inhibitory effects on lutein synthesis. In a similar way, Koh *et al.* [164] reported almost three times more lutein in a *Scenedesmus obliquus* culture grown in phototrophy compared with heterotrophically grown cells. In this regard, the heterotrophic culture was expected to have a lower value than the phototrophic culture. Maximum lutein and violaxanthin content in the phototrophic culture is 2.2 and 1.5 times higher than in the heterotrophic culture ($p = 0.001$ for lutein and $p = 0.058$ for violaxanthin). This has been previously reported by Xiao *et al.* [51], who demonstrated that the major enzyme encoding genes for carotenoid synthesis are downregulated during heterotrophic culture of *Auxenochlorella protothecoides*. On the other hand, zeaxanthin's higher values on the heterotrophic culture may be due to an antioxidant requirement, supplemented in the first instance by this carotenoid. Previous studies on *Chromochloris zofingiensis* showed that zeaxanthin could be overexpressed in response to stress when grown heterotrophically by Chen *et al.* [64], who reported an increase of 44 % in zeaxanthin after a stress stage using 10 mg L^{-1} of gibberellin acid-3 with high C/N ratio and NaCl concentration. These observations may be related to reports suggesting that an increased mitochondrial metabolic activity in yeast [207] and animal cells [208] gives rise to ROS generation, subsequently leading to enhanced antioxidant synthesis. In microalgae, mitochondria and chloroplasts are the main ROS generators [209]. However, in the case of heterotrophic culture supplemented with exogenous glucose, it is

possible that the increased metabolic activity of mitochondria leads to an overproduction of ROS, which will stimulate the onset of zeaxanthin synthesis in response to oxidative stress.

Although the yield of lutein in relation to the amount of glucose is not commonly reported, it is interesting to note that 7 mg of lutein per gram of glucose was obtained in the present work. Meanwhile, Florez-Miranda *et al.* [58] and Koh *et al.* [164] only obtained 1mg of lutein per gram of glucose under similar conditions using *S. incrassatulus* and *S. obliquus*, respectively. These differences may be due to particular strategies of each species to cope with culture under dark conditions by modifying the metabolic pathways for carotenoid synthesis. However, it is an aspect that requires further study to understand the underlying mechanism.

Since lutein content is lower in cells grown in darkness due to the reduction of photosynthetic complexes [166], two-stage culture strategies have been proposed in other *Scenedesmus* species [58, 164]. This is also true for astaxanthin and β -carotene production in other microalgae species; however, the underlying mechanism differs from that of lutein synthesis and accumulation. Both cases aim to increase pigment productivity through two steps [85, 118, 166]. In the first step, optimal culture conditions for biomass generation are favored, followed by a second stage that induces pigment synthesis and accumulation, resulting in high productivity. Nevertheless, in the case of astaxanthin and β -carotene, the second stage consists of stressing the cells to induce the synthesis of these carotenoids. In contrast, for lutein, the second stage consists of reactivating the photosynthetic capacity of the cells through the application of light. In this study, the 13.28 % increase in lutein content after 10 hours of photoinduction is an essential step toward increasing the overall lutein productivity. A similar approach is reported by Florez-Miranda *et al.* [58], who increased total carotenoids by 40 % after 24 hours of photoinduction of a *Scenedesmus incrassatulus* heterotrophic culture.

From an industrial point of view, lutein content per unit of biomass is not the only indicator to evaluate the viability of a project. Although the lutein content must be taken into account to determine the energy input of the extraction process, overall lutein productivity is a critical value that includes time, which is fundamental in a feasibility analysis. Therefore, although the lutein content on the phototrophic culture was higher, the increased biomass productivity obtained under the heterotrophic mode resulted in an equivalent increase in lutein productivity. In this sense, the two schemes presented for high biomass production (Long and Short Heterotrophy) and the subsequent photoinduction (Late and Immediate Photoinduction) offered higher lutein productivity than those obtained under phototrophic conditions. The accumulation of biomass and lutein during three days in heterotrophy yielded a productivity 28 times higher than that obtained in phototrophy.

Comparing the two photoinduction strategies, there is an increase of 175.5 % in the Short Photoinduction compared to the Late Photoinduction. This increase in productivity, apart from the delay preceding the photoinduction, may be due to the fact that the metabolic state of *S. almeriensis* cells are more favorable to lutein synthesis when the light phase is induced immediately after the end of the heterotrophic growth phase. On the other hand, since the biomass obtained from the heterotrophic culture was diluted for the photoinduction stage, the productivity calculation only considers half of the biomass produced. Therefore, the total productivity could be considered 11.68 mg L⁻¹ d⁻¹ in relation to the initial biomass per liter. The productivity values (9.79 and 11.68 mg L⁻¹ d⁻¹) are higher than other reports on the *Scenedesmus* genus and is comparable to results from *Chlorella* strains (see Table 2.1).

TABLE 2.1: Overview of maximum biomass concentration, lutein content and lutein productivity for different microalgae species under phototrophic, heterotrophic and photoinduced cultures

Algal species	Cultivation condition	Max. biomass concentration (g L ⁻¹)	Max. lutein content (mg gDW ⁻¹)	Max. lutein productivity (mg L ⁻¹ d ⁻¹)	Ref.
<i>Auxenochlorella protothecoides</i>	Heterotrophic/photoinduction	6.9	4.9	12.36	[51]
<i>Chlorella minutissima</i>	Phototrophic		8	5.35	[128]
<i>Chlorella prototecoides</i>	Heterotrophic	20	9.1	10.57	[165]
<i>Chlorella sorokiniana</i>	Mixotrophic/phototrophic	6	11.2	8.25	[146]
<i>Desmodesmus</i> sp.	Phototrophic	3.9	5.05	3.56	[168]
<i>Scenedesmus obliquus</i>	Phototrophic	3.38	4.52	4.15	[43]
<i>Scenedesmus obliquus</i>	Phototrophic	2	3.5	0.8	[164]
<i>Scenedesmus almeriensis</i>	Phototrophic		5.4	4.77	[57]
<i>Scenedesmus incrassatulus</i>	Heterotrophic/photoinduction	5.4	1.4	3.1	[58]
<i>Scenedesmus obliquus</i>	Mixotrophic	12.8	1	4.96	[169]
<i>Scenedesmus</i> sp.	Phototrophic	2.8	6.4	2.3	[180]
<i>Scenedesmus almeriensis</i>	Heterotrophic/photoinduction	22	1.62	11.68	This study

Moreover, it is essential to highlight that *Scenedesmus almeriensis* exhibits resilience to elevated irradiance and temperature levels and is characterized by weaker cell walls and larger cell sizes, which not only facilitate the harvesting process but also simplify the pigment extraction. These characteristics make this species an even more appealing choice for industrial applications. Additionally, the high cell productivity represents not only a way to increase lutein productivity but also delivers enough biomass to envision the valorization of the other fractions. The analysis of *S. almeriensis* biomass obtained from a heterotrophic culture has revealed a rich composition, showcasing its viability within the biorefinery paradigm. The combined constituents of carbohydrates (30 %), proteins (47 %), and lipids (17 %) offer a myriad of opportunities for extraction, processing, and utilization beyond a singular component such as lutein. This comprehensive composition does not differ much from that obtained by Sánchez *et al.* [124] in a phototrophic culture of this same species but reinforces the feasibility of biorefinery utilization and highlights the versatility and richness of microalgae biomass as a valuable resource in various industries and applications.

2.5 Conclusions

Our results demonstrate that culturing *Scenedesmus almeriensis* under heterotrophic conditions can lead to high biomass concentrations and increased biomass productivity. Although the lutein content in the phototrophic culture was higher, during the heterotrophic conditions the pigment content was not negligible and resulted in a higher lutein productivity than in the phototrophic culture due to the rapid increase in biomass content. Additionally, even though the photoinduction stage increased lutein productivity, the growth is minimal and further techno-economical analysis is needed to justify this phase. Future research should focus on optimizing the cultivation process under heterotrophic conditions to enhance lutein production by increasing biomass concentrations. Exploring novel nutrient supplementation strategies and fine-tuning light intensity during the photoinduction stage could potentially lead to even greater lutein yields. A comprehensive exploration of these avenues, coupled with rigorous techno-economic analyses, will be crucial in unlocking the full potential of *Scenedesmus almeriensis* as a valuable source of lutein.

Lutein extraction optimization

The lutein production capacity of *S. almeriensis* places the strain as a promising candidate for industrial use from an upstream perspective. However, the biomass harvesting and compound extraction processes represent a significant portion of the production cost and should be included in the overall process analysis. This chapter emphasizes the importance of incorporating downstream processes into the feasibility analysis of *S. almeriensis* for lutein production.

The results shown in this chapter demonstrate the possibility of using wet biomass and ethanol as solvent for lutein extraction without compromising lutein recovery capacity. This method avoids the excessive use of energy for biomass drying. In addition, ethanol is a solvent with lower environmental impact and greater public and regulatory acceptance in processes aimed at obtaining food or nutritional supplements.

3.1 Introduction

Lutein, a carotenoid found in various foods such as green leafy vegetables, carrots, and eggs, has attracted considerable interest due to its numerous health benefits for humans [23]. Among its functions, its antioxidant capacity and its role in protecting ocular health, particularly in preventing diseases such as age-related macular degeneration, stand out [19]. In addition, its capability to cross the blood-brain barrier positions it as a supplement for cognitive reinforcement and neurodegenerative conditions [98]. Currently, commercial production of lutein primarily relies on marigold flowers. However, in light of agroecological considerations, microalgae have emerged as a promising alternative. Despite its potential, the commercial production of lutein from microalgae faces significant challenges, with downstream processing being a prominent bottleneck. Efficient extraction of lutein and other carotenoids from microalgae, such as *Scenedesmus almeriensis*, has become a crucial research area to improve the commercial viability of these compounds. In addition to optimizing lutein production, there is a growing urgency to transition to more sustainable and environmentally friendly extraction processes. Pressure to use solvents accepted for human use and consumption and reduce the environmental footprint of industrial processes has driven the search for more efficient and less polluting alternatives [210].

Since drying constitutes the primary energy and time consumption in the process of carotenoid extraction from biomass [189], it is necessary to optimize the conditions of

these factors for wet extraction in order to reduce the impact of this process as much as possible and make it economically feasible [211].

Lutein recovery generally consists of biomass pretreatment and solvent extraction. Additionally, certain studies incorporate saponification and/or thermal treatment steps [212], yet there remains no consensus regarding the necessity of these two steps. The pretreatment is important to break the cells and allow direct contact of the solvent with the lutein, which is located inside the chloroplast. Numerous pretreatments, both mechanical and chemical have been proposed, such as bead beating homogenization [212], ultrasonication [56, 213], microwave [178, 179], French press [167], ball milling [191] and alkaline treatment [162, 167]. The solvent extraction is performed with organic solvents of different polarity, such as methanol, ethanol, acetone, dichloromethane, diethyl ether and hexane, but also with supercritical fluids [197], like CO₂.

Lutein is a molecule composed of a chain of 40 carbons and two hydroxyl functional groups at its ends. This configuration defines it as a polar compound. However, it is not soluble in water due to the hydrophobicity of the carbon chain. On the other hand, it is soluble in polar solvents, such as acetone and ethanol, and poorly soluble in non-polar solvents, such as hexane and diethyl ether [177]. Despite this, several studies propose using non-polar solvents for lutein extraction [196]. The reason for this may lie in the form (free or esterified) in which lutein is found in the biomass and the biomass condition (wet or dry) [15].

The treatment of biomass with alkaline solutions during the extraction of lutein has been approached with three objectives. Firstly, as a chemical mechanism of cell disruption by its action on cell membrane lipids, allowing the solvent to penetrate and extract lutein [159, 214]. Secondly, as a saponification process to obtain free lutein, in the case of esterified lutein [196]. Finally, the saponification step serves as a way to remove chlorophyll from the extract, avoiding the spectral overlap with the species found within the carotenoid family [191]. While in plants lutein is generally esterified, such as in marigold flower petals [215], in microalgae it is generally found in free form [15]. However, there is no general consensus, and it appears that various ratios of free/esterified lutein can be found in microalgae depending on the species and culture conditions [216].

The utilization of heat in the extraction procedure is a topic of discussion. While it is recognized that lutein and other carotenoids are heat-sensitive, with degradation starting at temperatures between 40 °C and 60 °C, there is a notion that elevated temperatures enhance the efficacy of solvents and, if applicable, saponification, thereby accelerating the reactions [177, 217, 218].

S. almeriensis is a promising microalga for lutein production. When cultivated under phototrophic conditions, it can produce up to 8.5 mg gDW⁻¹ (Dry Weight basis) of lutein [195]. Even though in heterotrophy the amount of lutein from *S. almeriensis* is lower than in phototrophy, the higher amount of biomass produced shows an increase in productivity of up to 11.68 mg L⁻¹ d⁻¹, one of the highest reported productivities to date [172].

The objectives of this work are multiple and focus on developing a more efficient and sustainable extraction process for lutein and other carotenoids. Firstly, we aimed to determine the differences in lutein extraction between dry and wet biomass. Additionally, we aimed to evaluate the effectiveness of saponification using aqueous or ethanolic KOH as saponifying agents. Furthermore, a comprehensive comparison of different solvents was conducted. Finally, a comparison was conducted to assess the cell wall resistance of *S. almeriensis* cultivated in heterotrophic conditions against those grown under light conditions, aiming to illustrate variances in susceptibility to cell disruption. Similarly,

comparisons were made using *Chlorella vulgaris* cells cultivated under both metabolic regimes, providing insights into interspecies distinctions and possible generalization of the proposed process.

3.2 Materials and Methods

3.2.1 Microorganism and culture conditions

A strain of *Scenedesmus almeriensis* was generously donated by Prof. Francisco Gabriel Acien of the University of Almeria. The strain has been cultivated under heterotrophic conditions in our laboratory using 4x B3N medium supplemented with 40 g/L glucose. B3N culture medium contained (per liter): NaNO₃ (750 mg), MgSO₄ 7H₂O (75 mg), NaCl (25 mg), K₂HPO₄ (75 mg), KH₂PO₄ (175 mg), CaCl₂ 2H₂O (25 mg), ZnSO₄ 7H₂O (8.82 mg), MnCl₂ 4H₂O (1.44 mg), MoO₃ (0.71 mg) CuSO₄ 5H₂O (1.57 mg), CO(NO₃)₂ 6H₂O (0.49 mg), H₃BO₃ (11.42 mg), EDTA (50.0 mg), KOH (31 mg) and FeSO₄ 7H₂O (4.98 mg) [200]. The culture was continuously agitated (100 rpm) in a dark incubator at 30 °C and subcultured weekly to replace nutrients. For every trial, the culture was harvested during the exponential phase and the samples were centrifuged (4 °C, 11000 rpm, 10 minutes), the supernatant was discarded and the pellet was washed with distilled water, divided into 1 mL tubes and then centrifuged again. For the wet biomass trial, the tubes were divided in three; one part was stored at 4 °C, another one at -20 °C and the last part was freeze-dried (1-day primary drying, 1-day secondary drying, Christ alpha 1-2 LD +) before being stored at -20 °C. All samples were protected from light.

3.2.2 Biomass conditioning and storage test

An extraction of carotenoids from wet biomass stored at 4 °C and -20 °C was compared with the extraction obtained from biomass dried by freeze-drying. For this, all samples were resuspended in 1 mL of distilled water and pigments were extracted using a modified protocol described by Pozzobon and Camarena-Bernard [219]. Cell disruption was performed by mixing 1 mL of laboratory-grade inert sand (Fisher Scientific Code: 10132590) and 9 mL of methanol. Cell disruption was carried out in a high-speed benchtop homogenizer (MP Biomedicals FastPrep-24 5G Instrument, Fisherbrand, Waltham, MA, USA) at 6.5 m s⁻¹ in two cycles of 30-second with a 60-second pause. The samples were then macerated at room temperature in a rotator (Stuart Rotator SB3) at 10 rpm for 60 minutes. Finally, the samples were filtered through a 0.22 µm pore filter to separate particles before passing them through a high-pressure liquid chromatograph. All this protocol was conducted with light protection around the samples.

3.2.3 Saponification treatment

In the first instance, the effect of saponification was compared using aqueous KOH solutions at concentrations of 30 and 60% (w/v). For this, the same disruption procedure described above was followed using wet biomass, except that acetone was used instead of methanol. After cell disruption, 1 mL of an aqueous KOH solution was added to the mixture, and the tubes were allowed to macerate in the rotator for 1 hour at room temperature at 10 rpm. The KOH solution was replaced with 1 mL of distilled water for the control group. In the second stage, since carotenoids like lutein are not soluble in water, the extraction

of pigments was compared using a 2M ethanolic KOH solution (*i.e.*, 11%) instead of an aqueous one. This was performed this way to increase the solvent-to-biomass ratio while reducing the volume of water in the mixture. Additionally, a test was included replacing acetone with ethanol as an extraction solvent. This was done as a control to discard any interaction between acetone and the ethanol in the saponification solution. The rest of the extraction steps remained as stated before.

3.2.4 Solvent evaluation

To evaluate the solvent to extract lutein and other carotenoids in *S. almeriensis*, five different solvents were tested: methanol, acetone, ethanol, isopropanol and 2-Methyltetrahydrofuran (2-MeTHF) added with 50 ppm BTH as an antioxidant.

To avoid the effect of cell disruption in the solvent test, disruption was done by employing an ultrasound probe with the cells suspended only in water, and then the solvent was added. An ultrasonic probe (VC 50 1/4" Microtip, Vibra-Cell Sonics VCX130, Newtown, CT, USA) was used. Previous tests showed similar recovery yields of carotenoids from this strain compared to the high-speed benchtop homogenizer (results not shown). Sonication was performed at 130W, 20KHz for 60 seconds, followed by a 60-second pause. This was repeated twice. After cell disruption, 9 mL of solvent and 1 mL of 2M ethanolic KOH were used for saponification.

3.2.5 Membrane integrity comparison

In a step back in terms of pigment extraction, a test was performed to determine the cell wall fragility to bead beating. *S. almeriensis* cells cultured in the heterotrophic and phototrophic modes were disrupted, as explained above, except that no solvent was added, and the cells were suspended only in water. Also, *Chlorella vulgaris* cells from both metabolic regimes were analyzed.

After disruption, viability was assessed using fluorescein diacetate (FDA) and propidium iodide (PI), which exhibit complementary properties that facilitate the characterization of microalgae population viability. Specifically, the FDA distinguishes living cells (with valid esterase activity) from non-viable cells and debris. At the same time, PI enters dead cells and binds to their DNA, allowing the differentiation between non-viable cells (with compromised membrane permeability and DNA presence) and debris. The protocol described by Pozzobon *et al.* [220] was used to lead this assay. In brief, 200 μL of 120 μM FDA solution and 10 μL of 1 g L⁻¹ propidium iodide (Sigma Chemicals) were added to 790 μL of cell suspension at an optical density (750 nm) of 1.0 \pm 0.1. Afterward, cells were washed by centrifugation (15000 rpm, 4 °C, 5 min), and the supernatant was discarded. The pellet was resuspended in water before immediate analysis.

The dye signal was recovered using the yellow-green laser (561 nm, 610/20 nm detection) for propidium iodide and blue laser (588 nm, 530/50 nm detection) for FDA. Heat-treated (90 °C, 10 min) cells were used as positive control (*i.e.*, dead cells), and pristine cells were used as negative control (*i.e.*, alive cells). Each of the tested conditions was duplicated.

Flow cytometry analyses were carried out using a BD Fortessa x20 (with BD FACS Diva software). Four parameters were recorded: forward scatter (or FSC, blue laser at 488 nm) as a proxy of cell size, side scatter (or SSC, blue laser at 488 nm, 488/10 nm detection) as a proxy of cell complexity, chlorophyll fluorescence (red laser at 620 nm, 780/60 nm detection), and propidium iodide fluorescence (yellow-green laser at 561 nm,

610/20 nm detection). At least 100 000 events (FSC above 5000) were acquired for each run.

3.2.6 Pigment quantification

Pigments were quantified on an Ultima 3000 HPLC (Thermo Fisher Scientific) coupled with a U.V. detector. Separation was achieved on an Acclaim Polar Advantage II C18 column (4.6 x 150 mm, 3 μm , 120 \AA) from Thermo Fisher Scientific. The column temperature was maintained at 30 $^{\circ}\text{C}$. Pure methanol was the mobile phase. The flow rate was 0.5 mL min^{-1} , and the elution was set in isocratic mode. The injection volume was 5 μL , and the total run analysis was 40 min. Compounds were identified by comparing their retention time and UV-Vis spectra with standard solutions. UV-Vis spectra were recorded from 200 nm to 700 nm. Absorbance was recorded at 400, 450, 500, and 650 nm. Pigment quantifications were led using the area of the peaks in external calibration for the most sensible of the recorded wavelength. External calibration concentrations ranged from 0.25 to 5 mg L^{-1} . Pigment standards and methanol were purchased from Sigma-Aldrich. Standards had a purity greater than 97%. The five pigments of interest (chlorophyll a, b, lutein, violaxanthin, and zeaxanthin) were reported systematically for each sample. N.A. was used whenever one could not be detected or quantified.

3.2.7 Statistical analysis

Statistical significance was assessed using the ANOVA test. When the null hypothesis was rejected ($p < 0.05$), data were further analyzed using Tukey's Honestly Significant Difference (HSD) test. The following results are presented as the average of the replicate ($n = 5$ unless stated otherwise), while the error bars account for the standard deviation.

3.3 Results and discussion

3.3.1 Effect of biomass condition and storage

Lutein is a compound susceptible to oxidative degradation, accelerated by exposure to light and high temperatures. Under this scenario, most laboratory-level extractions seek to maintain the microalgal biomass in optimal conditions for lutein preservation while extraction processes are carried out. Traditionally, microalgal biomass is freeze-dried and stored at -20°C to avoid changes in its composition while it is being processed for content analysis. However, this drying process constitutes the primary energy consumption of the operation and is notably time-intensive [189]. On the other hand, industrial-level operations can only afford to store biomass for a short period. This circumstance invites the exclusion of the drying step, enabling a direct transition from harvesting to extraction in a shorter period by simply cooling the wet biomass. In this context, it becomes crucial to evaluate the impact of extraction techniques on wet biomass, whether frozen at -20°C or solely refrigerated at 4°C , which implies an energy reduction as well.

After removing the supernatant post-centrifugation, the samples utilized for wet biomass extraction exhibited a moisture content of $86.69 \pm 3.67\%$. Fig. 3.1 shows the concentration of photosynthetic pigments of *S. almeriensis* from samples stored at different temperatures and humidity percentages. After harvesting and centrifuging the samples, the wet ones were immediately stored at 4°C and -20°C , while those destined for drying were promptly

placed in the freeze-dryer. Three days post-harvest, extractions were performed for all conditions.

Samples Extractions from wet samples stored at $-20\text{ }^{\circ}\text{C}$ resulted in the highest yields of violaxanthin ($0.17\pm 0.01\text{ mg gDW}^{-1}$), lutein ($0.34\pm 0.03\text{ mg gDW}^{-1}$) and chlorophyll a ($1.13\pm 0.08\text{ mg gDW}^{-1}$), while chlorophyll b was recovered in higher amount from the wet samples preserved at $4\text{ }^{\circ}\text{C}$ ($1.40\pm 0.11\text{ mg gDW}^{-1}$). It can be observed that freeze-drying the biomass has a negative effect on the recovery of chlorophyll a and b compared to samples preserved with humidity, either stored at $4\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$, while storing the samples at $-20\text{ }^{\circ}\text{C}$ does not show a significant advantage compared to storage at $4\text{ }^{\circ}\text{C}$ for chlorophyll recovery. Regarding lutein and violaxanthin, storage of the wet biomass at $-20\text{ }^{\circ}\text{C}$ resulted in 1.18 and 1.27 times higher extraction than at $4\text{ }^{\circ}\text{C}$, respectively. On the other hand, no differences were found between the amount of lutein and violaxanthin recovered from freeze-dried samples and wet biomass preserved at $4\text{ }^{\circ}\text{C}$. These results are similar to those reported by Low *et al.* [178], where they obtained 1.39 times more lutein from wet biomass of *Scenedesmus sp.* compared to lyophilized biomass. Similar findings are reflected in the results presented by Gong and Bassi [221]. Hence, wet extraction is recommended due to its higher yield and potential cost reduction by eliminating a drying step. The following tests were performed with wet biomass stored at $4\text{ }^{\circ}\text{C}$.

While these results only show the effectiveness of the extraction process on the different conditioned samples, further studies are needed to determine the full impact of freezing and drying the samples. Additionally, more studies are necessary to validate the stability of lutein on wet biomass for relatively long time storage at $4\text{ }^{\circ}\text{C}$. Low *et al.* [178] demonstrated that the wet biomass of *Scenedesmus sp.* ANI-KL 8D can be preserved for at least 21 days without loss of pigments, but this might be species-dependent and should be studied on a case-by-case basis.

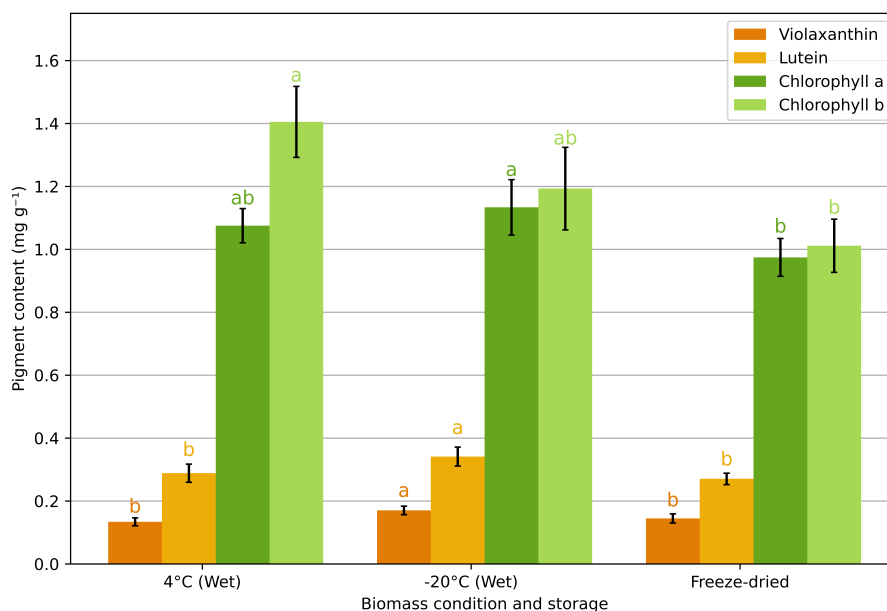


FIGURE 3.1: Pigment recovery from wet *S. almeriensis* biomass stored at $4\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$ compared to freeze-dried biomass. Letters above bars indicate differences between pigment recovery, $p < 0.05$. $n = 5$

3.3.2 Saponification effect

Naturally produced lutein can be found in two forms in plants and microalgae: esterified, with fatty acids at the ends of its 40-carbon chain, or in free form. In plants, it is commonly found esterified, and currently, the commercial process to obtain free lutein involves a process of saponification of marigold flower extracts [13]. Although there is no consensus on the form in which lutein is generally found in microalgae, some extraction protocols include a saponification process, either as a mechanism for cell disruption, to remove chlorophyll from the extract, or to release lutein from fatty acids if it is esterified [15]. However, the need to include this step is still under debate. As in many cases, this depends on the species and the conditions in which it was cultured, so it is necessary to define it for each process. Given that the predominant form of lutein in *S. almeriensis* is in free form [196], saponification in this instance aids primarily in disrupting the lipid membrane, thereby facilitating solvent penetration into the chloroplast.

Fig. 3.2 shows the amount of lutein, violaxanthin and zeaxanthin recovered from wet biomass of *S. almeriensis* grown in heterotrophy and treated with aqueous KOH solution after cell disruption. It can be observed that the higher the concentration of KOH, the higher the pigments collected. Lutein recovered was 1.19 times higher using 60% KOH than without KOH, while it was 1.17 and 1.04 times higher for violaxanthin and zeaxanthin, respectively. It can also be observed that the recovered value of lutein is much higher in this extraction than that obtained in the previous methanol extraction and without saponification. This could be attributed more to the nature of the solvent rather than to the saponification process itself, as even the extraction without KOH resulted in higher lutein content. The higher pigment results obtained by saponification are in agreement with results reported by Chan *et al.* [212], who achieved a 2-fold increase in the content of lutein from *S. obliquus* when employing a 2.5% KOH solution in contrast to omitting KOH. However, this same study reports that an increase in KOH concentration above 2.5% does not necessarily increase lutein recovery, which contrasts with the results reported in this work. In the same line, Gong *et al.* [216] exposes the need to use an alkaline solution to extract a higher content of lutein from wet biomass of *C. vulgaris*, however, in this case the authors emphasize that it is in order to obtain lutein in free form. Ceron *et al.* [196] also compared the concentration of KOH in the extraction of lutein from *S. almeriensis* and found that concentrations greater than 4% reduced lutein recovery. However, they did not compare it with lower concentrations.

Since lutein is not soluble in water, it has been suggested that using aqueous KOH presents the risk of precipitating part of the lutein in the saponification process [213]. For this reason, a comparison of carotenoid extraction using an aqueous KOH solution versus an ethanolic KOH solution was performed. An extraction using ethanol as solvent was also performed to complete the comparison and rule out any interaction between acetone and ethanol. Fig. 3.3 shows that the ethanolic KOH solution positively affects the recovery of carotenoids. Observed with the naked eye, using an aqueous solution results in the aqueous phase being divided from the organic phase by a thin opaque layer, which is not possible to characterize but may contain saponified lipids and, possibly, part of the pigments.

On the other hand, using the ethanolic solution avoids the formation of phases. Moreover, lutein recovered using acetone plus ethanolic KOH was 32.80% higher than using aqueous KOH. Similar increases were observed for violaxanthin and zeaxanthin (43.83% and 24.94%, respectively). These results are in agreement with D'Este *et al.* [213], who obtained three times more lutein with a solution of ethanolic KOH than aqueous

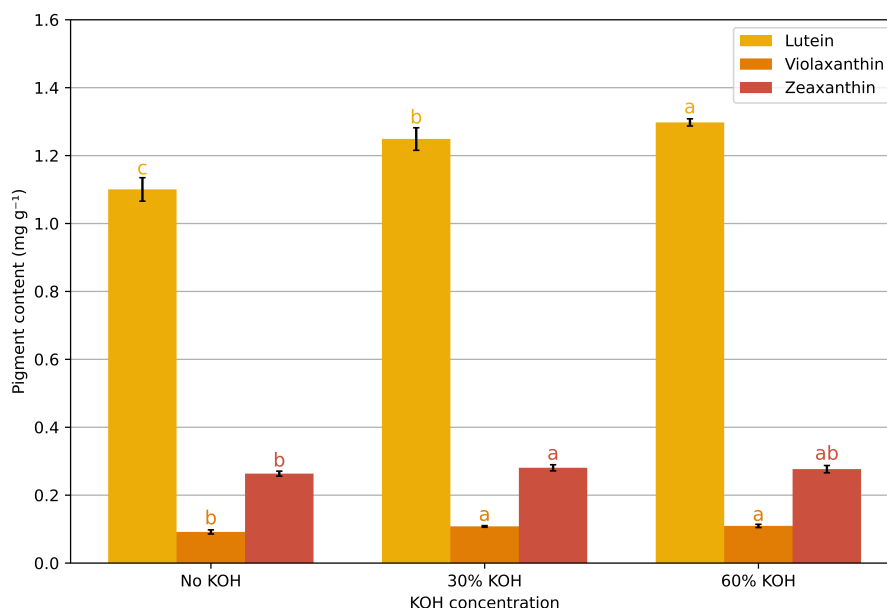


FIGURE 3.2: Carotenoids extracted with a saponification step after cell disruption of wet biomass using aqueous KOH and acetone as solvent. Letters above bars indicate differences between pigment recovery, $p < 0.05$. $n = 5$

KOH from *C. vulgaris* biomass. This is also explained by Low *et al.* [178], who argues that the mass transfer rate of lutein is limited by the insolubility of lutein in the aqueous KOH solution. Furthermore, no significant differences were observed when switching from acetone to ethanol as a solvent. Since ethanol is a solvent considered less toxic and is generally more accepted in the food industry, these results are promising for transitioning to a more environmentally sustainable process.

3.3.3 Solvent effect

Since the first results favored the use of wet biomass over dry biomass and the use of ethanolic KOH as the saponifying solution, it was necessary to go back in the solvent selection to rule out that any of these changes favored the extraction of carotenoids with solvents other than ethanol or acetone. Numerous studies have reported successful extraction processes using hexane, dichloromethane and other solvents that, due to their toxic nature, were discarded in this study, which aims to provide a more environmentally friendly and less toxic process for integration in food formulations.

Methanol, a solvent commonly used to extract polar pigments, such as xanthophylls, is considered by the U.S. Food and Drug Administration (FDA) to be Class 2, indicating that its use should be limited in pharmaceutical products due to its inherent toxicity. Ethanol, acetone and isopropanol are solvents also commonly used for pigment extraction and, according to the FDA, are solvents that pose no risk to human health at levels commonly accepted in pharmaceuticals (Class 3) [222]. According to this FDA report, no data assesses the Permitted Daily Exposure (PDE) of 2-MeTHF. However, according to Sicaire *et al.* [223], it is a solvent obtained from renewable sources by hydrogenation of hemicellulose from different feedstocks. In addition, it is reported to be biodegradable and easily recyclable.

As can be seen in Fig. 3.4, extraction with 2-MeTHF yielded the smallest amount

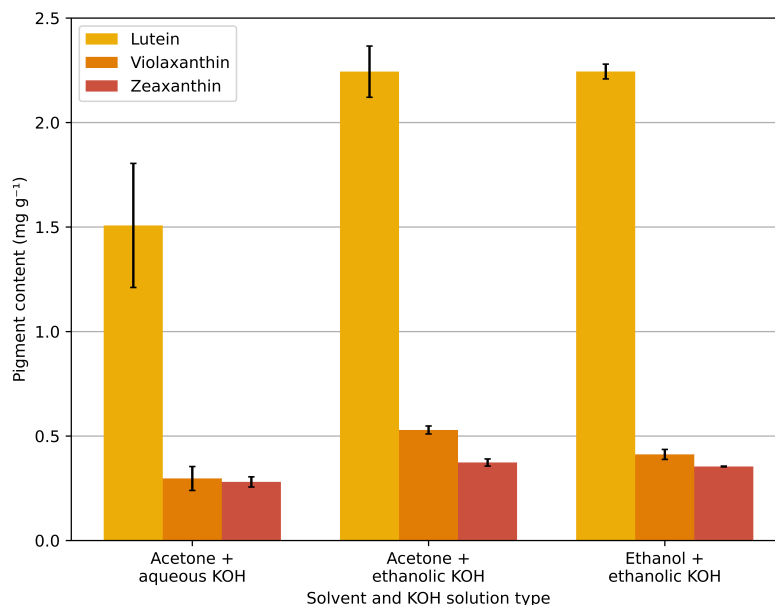


FIGURE 3.3: Carotenoids extracted using aqueous KOH during the saponification process compared to ethanolic KOH, either using acetone or ethanol as solvent. Data presented as the average of the two replicates, error bar covering the spread.

of lutein (0.38 ± 0.08 mg gDW⁻¹), while violaxanthin and zeaxanthin do not even appear in the HPLC chromatogram. Similar results were reported by Damergi *et al.* [224] on extracted lutein from *C. vulgaris*. Likewise, the values of lutein, violaxanthin and zeaxanthin extracted by methanol (0.61 ± 0.01 , 0.11 ± 0.005 , 0.20 ± 0.005 mg gDW⁻¹) did not show advantageous results compared to ethanol (0.85 ± 0.01 , 0.14 ± 0.006 , 0.24 ± 0.01 mg gDW⁻¹), acetone (0.91 ± 0.11 , 0.14 ± 0.02 , 0.26 ± 0.02 mg gDW⁻¹) and isopropanol (0.87 ± 0.01 , 0.14 ± 0.004 , 0.25 ± 0.009 mg gDW⁻¹). Although the extraction with ethanol was slightly lower than with acetone or isopropanol, this difference is not significant. In addition, the results showed less variation than with acetone.

Comparable results were achieved in a study conducted by Chen *et al.* [167], in which optimization of extraction conditions revealed that ethanol was better or at least as effective as other solvents studied in recovering lutein from *Chlorella* sp. Likewise, Ahmad *et al.* [179] obtained similar results in the extraction of xanthophylls using 90% acetone versus 90% ethanol from *C. luteoviridis* biomass. In contrast, Lee *et al.* [225] obtained 1.8 times more lutein from *Tetraselmis suecica* using methanol as solvent compared to ethanol or acetone. This difference in the solubility of lutein in various solvents may be caused by the difference in polarity of the solvents and the form in which the lutein is found. Gong *et al.* [216] explains that polar solvents extract lutein better from wet matrices, as in this study. In addition, they suggest using binary solvents, mixing one polar and one non-polar solvent to improve the solubility of lutein in its different forms (free and esterified). Moreover, Damergi *et al.* [224] suggests that the presence of water also influences the polarity of the extraction mixture, so it is necessary to differentiate between the use of wet and dry biomass in addition to the species in question. This shows that the choice of solvent is dependent on the species, and even more, on the conditions in which the microalgae were cultured. Furthermore, Low *et al.* [178] highlights the limitation of using ethanol as a solvent for lutein extraction because it lacks selectiveness, with the

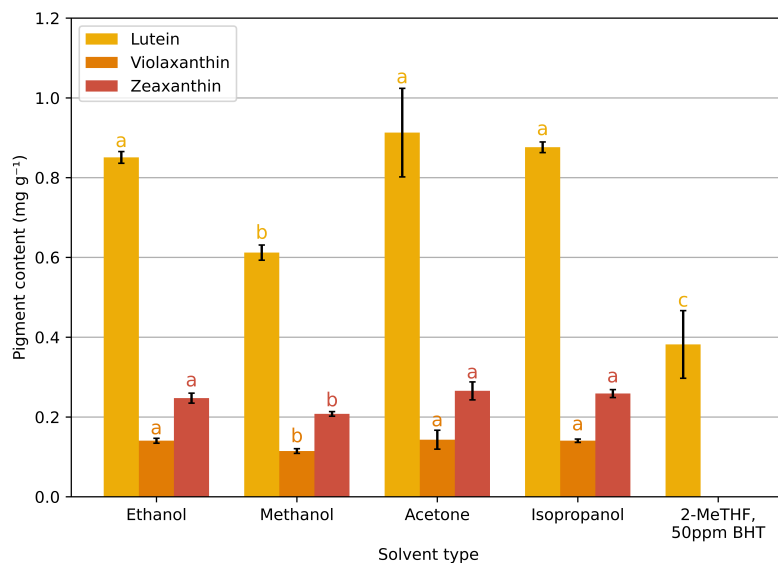


FIGURE 3.4: Carotenoids extracted from wet biomass using different solvents and 60% ethanolic KOH as saponification solution. Letters above bars indicate differences between pigment recovery, $p < 0.05$. $n = 5$

chlorophyll in the final product. However, this is also true for acetone. Thus, further procedures are necessary to improve the purity of lutein extracts when ethanol is used as a solvent.

Once it was defined that ethanol is as good as acetone and isopropanol for carotenoid extraction, a new step back was necessary to evaluate saponification's effect at different concentrations of ethanolic KOH. As shown in Fig. 3.5, low concentrations of KOH (between 2 and 10%) did not favor lutein recovery compared to the lack of an alkaline solution. Despite the increase in recovered lutein being up to 9.91% higher when using 10% ethanolic KOH compared to not using saponification, the difference is not statistically significant.

3.3.4 Cell wall integrity comparison

Several studies have shown *S. almeriensis* as a strain with potential for industrial lutein production. Many of these studies have used different processes to extract lutein and illustrate this potential [57, 124, 191, 195–197]. However, the culture conditions in all these cases had been in phototrophic mode, which implies a possible change in cell structure when the cells are cultured in heterotrophy. On the other hand, despite the lack of studies comparing the rigidity of the cell wall, there is a generalized idea (without literature support) that the cells of the genus *Scenedesmus* could be more recalcitrant than other genera of commercial interest, such as *Chlorella*. In this regard, Rashidi and Tindale [194] suggested that the amount of rhamnose in the cell wall is directly related to the rigidity of the cell wall. Additionally, Spain and Funk [193] reported that the concentration of rhamnose in the cell wall of *Scenedesmus* sp. was half that of *C. vulgaris* and Takeda [226] reports no rhamnose at all in eleven *Scenedesmus* species. This suggests that the cell wall of *S. almeriensis* may be less resistant to disruption than *C. vulgaris*.

For purposes of determining the cell wall resistance to the bead beating method described previously and without going further into the specific composition of each cell

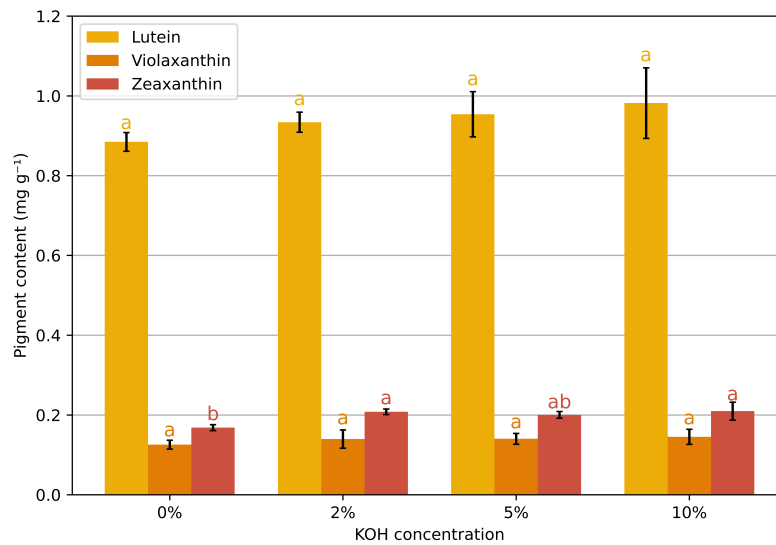


FIGURE 3.5: Carotenoids extracted from wet biomass using ethanol as solvent and different concentration of ethanolic KOH for saponification. Letters above bars indicate differences between pigment recovery, $p < 0.05$. $n = 3$

wall, comparisons were made between heterotrophic and phototrophic cells of *S. almeriensis* and *C. vulgaris* during the exponential growth phase. Disruption was carried out with the cells suspended only in water and not in a solvent to allow flow cytometer analysis and rule out the disruptive effect of the solvent. Essentially, the forward scatter (FSC), side scatter (SSC), FDA and PI signals were observed for pristine, broken but not stained, and stained broken cells.

Fig. 3.6 shows the percentage of viable cells after disruption treatment. It can be seen that, for both *S. almeriensis* and *C. vulgaris*, heterotrophic culture has a negative effect on cell disruption resistance. In the case of *S. almeriensis*, cultures grown in phototrophy retained 59.05% viability, whereas only 46.38% of cells remained alive under heterotrophic conditions. For *C. vulgaris*, the disparity is even more pronounced, with 88.45% of phototrophic cells remaining viable compared to only 49.29% of heterotrophic cells.

The comparison between the two species supports the argument above, confirming that *S. almeriensis* is less resistant than *C. vulgaris* when exposed to the same bead-beating treatment for cell disruption. To our knowledge, this is the first report that exposes the fragility of different species in two different culture modes to the same cell disruption treatment.

Although the results of this test determine the fragility of the cell wall of these species, resistance is not necessarily linked to composition, as it is known that other factors such as cell size and shape can determine the degree of disruption [227].

Most studies that analyze the relationship between cell wall composition and the effectiveness of different cell disruption methods perform this analysis on a single species and in a single culture condition [227, 228]. On the other hand, studies that characterize the cell wall rarely correlate it with analyses of resistance to disruption [229, 230]. Moreover, although it is recognized that changes in the structure and composition of the cell wall of microalgae occur at different times of culture (*i.e.*, exponential or stationary phase)

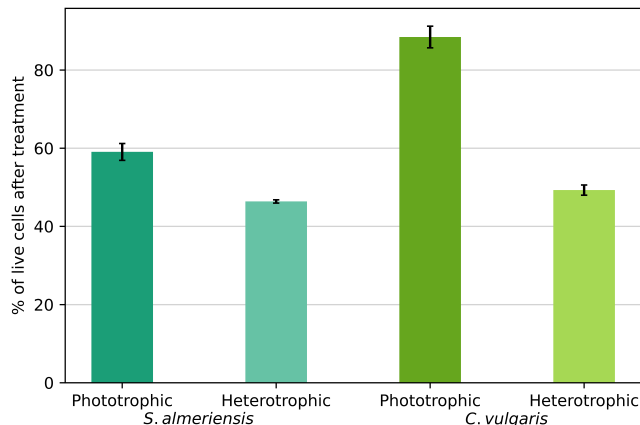


FIGURE 3.6: Percentage of viable phototrophic and heterotrophic cells of *S. almeriensis* and *C. vulgaris* after bead beating disruption. Data presented as the average of the two replicates, error bar covering the spread.

[230], there are no studies that make this comparison between cultures grown in different metabolic regimes or its impact on disruption resilience.

Further research is needed to elucidate the structural and biochemical alterations contributing to cell wall toughness under different metabolic regimes. Nevertheless, these findings provide valuable information for microalgal biomass downstream processing.

3.4 Conclusions

Microalgae culture is a promising and attractive source of lutein at the industrial level; however, the high costs of the process and the complexity of scaling it up to the industrial level present a barrier that needs to be addressed to make it commercially available. In addition, it is becoming increasingly necessary to address the challenge of making the process less toxic for product formulation and the environment.

The results of this study highlight that freeze-drying the *S. almeriensis* biomass cultured heterotrophically does not lead to increased lutein recovery, suggesting that alternative storage methods, such as refrigeration at 4 °C, can be viable options while maintaining extraction efficiency. Moreover, ethanolic KOH solution demonstrates superior efficacy for lutein extraction compared to aqueous solution, with saponification offering marginal benefits. Among the solvents examined, ethanol emerges as a promising alternative, outperforming methanol or 2-methyltetrahydrofuran and performing comparably to acetone or isopropanol. These findings not only contribute to reducing the environmental impact of lutein extraction from biomass but also enhance the feasibility of industrial-scale extraction. Additionally, observations regarding the lower resistance of *S. almeriensis* cells cultivated under heterotrophic conditions to disruption compared to their autotrophic counterparts suggest potential implications for downstream processing methods. Further studies should include the stability of the biomass and its components when stored wet at 4 °C in the medium and long term.

Culture scaling up and production intensification

The production of lutein from microalgae on a commercial level is waiting for a trigger to catapult it into the natural pigments and nutritional supplements market. The last 25 years have been decisive in finding species with lutein production potential, ideal culture conditions to obtain attractive yields and efficient and sustainable extraction methods. However, most of these discoveries have been on a laboratory scale and it is necessary to advance to the next critical step in order to expand production capacity. This chapter presents details of the first steps needed to reach industrial scale in the heterotrophic production of *S. almeriensis*. The increased volume and configuration of the culture system from flask to bioreactor present challenges that cannot be underestimated. In addition, culture intensification requires a careful balance between scale-up and modes of operation different from the flask batch, which raises the level of difficulty. The results presented here attempt to give a first overview of what needs to be considered to achieve an industrial level heterotrophic culture of *S. almeriensis* for lutein production in the future.

4.1 Introduction

Previous chapters have highlighted microalgae as a promising source of lutein and other products, such as bioactive compounds, renewable energy and sustainable solutions for various industrial processes. However, the transition from laboratory-scale cultivation to industrial-scale production presents significant challenges and opportunities. In addition, the intensification of biomass production and the compounds obtained by continuous or fed-batch cultivation allows us to envision more favorable scenarios for the industrialization of the processes.

While the studies carried out at a laboratory scale allow demonstrating the feasibility of a culture or the production of a metabolite, process scale-up is essential to materialize commercial projects from any species of microorganism, including lutein from microalgae. This transition, which necessarily goes through intermediate stages of scale-up, must consider several factors, such as the culture system, nutrient management, contamination

control, optimization of culture parameters and subsequent steps to recover the biomass or its compounds of interest.

As photosynthetic organisms, microalgae have been produced on a large scale phototrophically with some success for some applications, such as biomass for food and feed, astaxanthin and β -carotene production or nutrient removal from water [231]. While additional applications are awaiting full exploitation, the phototrophic regime encounters limitations that hamper its commercialization to the fullest extent.

The main obstacles are the low cell concentration and growth rate. This implies longer culturing times and more complex harvesting procedures, which results in higher production costs. Additionally, microalgae culture in open ponds requires significant amounts of water and large land areas, as they are usually grown at shallow depths to maximize light exposure for optimal growth. Borowitzka and Vonshak [231] states that for successful commercial production of microalgal biomass or by-products, the industrial capacity must be greater than 1 ton per year. Under these scenarios, the calculation of the volume of water and cultivation surface needed for production in any photobioreactor systems (bags, flat panels, tubular, open ponds) is incredibly high (up to $1 \cdot 10^6$ m³ in extensive ponds).

Scaling up algal cultures typically involves increasing the volume by a factor of 10 at each stage, progressing from 10 ml to 100 ml, then to 1 liter, and subsequently to 10 liters, and so forth. Under a phototrophic regime, microalgae cultures typically require approximately 5-7 days to reach the necessary growth level for subculturing to the subsequent stage. Therefore, it requires a minimum of one month to advance from a 10 mL stock culture to accumulate adequate inoculum for a 10,000 L culture [231]. This extended time and the need to perform multiple steps increases the cost of the process and the risk of contamination.

Alternatively, the heterotrophic capacity of some microalgae species can be used to cultivate them in fermenters, as is done with bacteria and yeasts. This method offers numerous advantages. Firstly, it allows cultures to reach significantly higher cell densities, exceeding 200 g L⁻¹, in contrast to the 0.5-2 g L⁻¹ typically achieved in phototrophic cultures within outdoor ponds. Secondly, the higher cell concentration simplifies biomass recovery during downstream processing. Lastly, it promotes higher growth rates compared to outdoor phototrophic cultures. Similarly, growth can be conducted 24 hours a day versus 8 hours a day for an outdoor photosynthetic production process. In addition, there is abundant knowledge and equipment development derived from traditional fermentation practices that can be used, since the culture of heterotrophic microalgae does not require specialized fermenters different from contemporary systems to grow bacteria and yeast.

Although the heterotrophic growth mode reduces the ability of microalgae to synthesize some compounds, such as lutein, it has been shown that higher biomass production yields better productivity of these compounds, as discussed in the previous chapters. Furthermore, microalgae cultured heterotrophically have been employed to enhance biomass and yields of non-photosynthetic biochemicals [149, 232]. They serve as the initial culture in two-stage systems to enhance the accumulation of specific molecules [58] or as a high-density inoculum for scaling to phototrophic-phase cultures [233]. Particularly in this last case, the authors highlight the suitability of using heterotrophic inocula, as the resulting cells did not undergo an acclimation phase when transferred to light conditions. Moreover, the higher growth rate and cell density obtained in the generation of the heterotrophic inoculum resulted in higher biomass productivity compared to autotrophic-grown inocula.

References cited in previous Chapters provide a first insight into the feasibility of

carrying out heterotrophic processes with microalgae at the laboratory scale. Moreover, scaling microalgal cultures in heterotrophy is not new. Since the 1980s, companies in the United States and Japan have achieved successful commercial production of microalgae (*Chlorella* sp., *Cryptocodinium* sp., *Schizochytrium* sp.) under heterotrophic growth for the production of high protein biomass and Poly-Unsaturated Fatty Acids (PUFAs) [234, 235]. However, developing efficient, economically feasible large-scale bioprocesses remains an obstacle to the commercialization of other promising microalgae species and byproducts.

On the other hand, the intensification of microalgal cultivation, i.e., the increase in biomass obtained per culture cycle, is essential to increase productivity and efficiency in producing biomass and bioactive compounds [236]. By achieving intensification, the use of resources such as water, nutrients and space can be optimized, making the scaling up of the process more viable [237, 238].

Two commonly employed methods for intensifying microalgae culture are fed-batch and continuous cultures. Fed-batch culture involves periodically supplementing the culture medium with nutrients. This method maintains optimal nutrient concentrations throughout the cultivation period, promoting sustained growth [239]. Moreover, it helps mitigate substrate inhibition effects and the buildup of inhibitory metabolites, thereby prolonging the exponential growth phase [240]. Since phototrophic cultures are quickly photo-limited by the amount of light reaching the cells, fed-batch models to obtain high-density cultures are more commonly reported in heterotrophic regimes [236].

Continuous culture involves continuously adding fresh medium to the culture system while simultaneously removing an equivalent volume of spent culture to maintain a constant culture volume. Continuous cultures enable steady-state conditions with constant cell density, nutrient concentrations, and growth rates, leading to stable and predictable biomass production [239].

This chapter presents the results of scaling up a *Scenedesmus almeriensis* culture in heterotrophy using a 5 L stirred reactor (4 L working volume). Subsequently, we describe the tests of the fed-batch culture with exponential feeding using a concentrated culture medium and the challenges to reaching high cell concentrations.

4.2 Materials and Methods

4.2.1 Culture medium

The modified B3N medium was concentrated four times and supplemented with 40 g L⁻¹ glucose for batch culture and the initial fed-batch cultures. The concentration of the feed medium for the fed-batch was defined based on our biomass production target of 100 g L⁻¹. The results in Chapter 2 indicate that the conversion rate from glucose to biomass is 0.5, so 800 g of glucose is necessary to reach the biomass target in 4 L. The concentrations of sodium nitrate and the rest of the salts were calculated accordingly. However, the initial culture starts with 40 g L⁻¹ of glucose, so the 3 L of feed medium contains 760 g of glucose, 57 g of sodium nitrate for a C:N ratio of 32, and the remaining salts of the B3N medium six times more concentrated to avoid limitation. The stock solution containing the glucose and sodium nitrate was prepared separately from the concentrated B3N to avoid the precipitation of salts in the feeding medium.

4.2.2 Microorganism and growth of seed culture

The microalgae *Scenedesmus almeriensis* was kindly donated by Prof. Francisco Gabriel Acien from Universidad de Almería. The strain is maintained in our laboratory in heterotrophic culture and subcultured weekly. The strain is kept in agitation at 150 rpm in 250 mL flasks with 50 mL of B3N medium (described previously) four times concentrated and added with 40 g L⁻¹ of glucose. It is kept in incubation at 30 °C without light.

Routine contamination controls are performed to rule out the presence of bacteria or yeast in the culture by microscopic observations and culture on microbial growth medium plates. Isolation processes are carried out if necessary to ensure that axenic cultures are always available.

Prior to the scale-up experiments, an active algal inoculum was prepared by growing the cells for four days (up to the late exponential phase) in eight flasks with 50 mL of medium (for a total of 400 mL. This was performed to avoid the culture's dissolved oxygen (DO) limitation.

4.2.3 Experimental conditions for scaling up the culture

Scale-up of the culture to 4 L was carried out using the same culture medium as the inoculum using a GPC[®] bioreactor with a total capacity of 5 L. The culture was inoculated with 400 mL of a 20 g L⁻¹ seed culture so that the initial biomass concentration was 2 g L⁻¹. The experiments were carried out at 30 °C, and the DO was maintained at 50% by automatically adjusting agitation rates ranging from 150 to 500 rpm in the first order of response, followed by modifications in aeration rates between 0.5 and 2 L per minute when the maximum agitation speed was not enough to maintain the DO setup. The pH was monitored and controlled at a value of 7 by injecting a sterile 4M HCl solution.

As contamination by fast-growing bacteria became hard to eradicate through normal autoclave procedures, the effect of antibiotic treatment on the growth rate of *S. almeriensis* was tested. The treatment consisted of applying a combination of 10 mg L⁻¹ penicillin, 0.4 mg L⁻¹ chloramphenicol, 3.2 mg L⁻¹ streptomycin and 100 µg L⁻¹ amphotericin at the beginning of the culture.

4.2.4 Experimental conditions for culture intensification

After demonstrating the feasibility of *S. almeriensis* to be cultured in a larger volume and more complex system (bioreactor vs. flask), investigations aiming at process intensification were carried out.

Biomass concentrations of *Scenedesmus acuminatus* of up to 286 g L⁻¹ in heterotrophy have been achieved using fed-batch cultures [151]. However, this work is the first report of *Scenedesmus almeriensis* in heterotrophy, and there are, therefore, no reports at high cell densities for this strain, so it was decided to reach a more moderate target of 100 g L⁻¹ in this first intensification step. This target was set based on two considerations. First, 200 g L⁻¹ is equivalent to the density of a pellet obtained by centrifugation. Hence, culture handling, gas supply/removal, and feed preparation might necessitate unique means. Second, from interactions of the team with industrial partners, 100 g L⁻¹ of cells is an achievable goal from an industrial biotechnology point of view.

To achieve this objective, it was decided to resort to a fed-batch culture strategy. Fed-batch operations can be used for many purposes, e.g., diluting a culture whose products are inhibitory, triggering metabolic shifts by supplying specific material to the cells after

a biomass amplification phase, or constantly feeding the cells to reach a high biomass density. In our case, the fed-batch culture aims to achieve high cell productivity, which boils down to achieving a high culture density while minimizing the time required to reach the targeted concentration. Therefore, we took advantage of the capacity of a specific fed-batch strategy (exponential feeding) to sustain cell exponential growth throughout the operation. To do so, high-concentration feed is supplied continuously to match the exponential decrease in substrate concentration, which results from the exponential growth of the culture. Still, to maintain a stable substrate concentration conducive to continuous exponential growth, it is essential to devise a tailored feeding strategy that prevents both substrate exhaustion and inhibitory concentrations.

Designing such a process is not a trivial thing; therefore, a modeling approach was used. The following equations summarize the envisioned operation of the system presented in Figure 4.1.

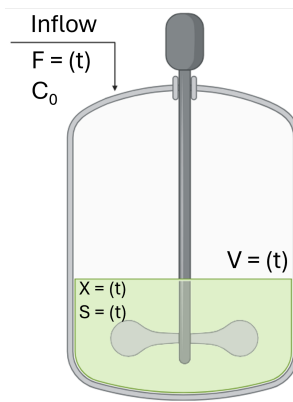


FIGURE 4.1: Simplified scheme of a fed-batch system.

These equations are based on a classical mass balance approach over the fed-batch system between two consecutive time points (t and $t+dt$), considering how the accumulation changes over time due to the combined effects of inflow, outflow, production, and removal (sink) processes (Eq. 4.1):

$$Accumulation = Inflow - Outflow + Production - Sink \quad (4.1)$$

From this general balance, the cell mass balance equation (Eq. 4.2) is presented to show the mass variation during the exponential growth.

$$\underbrace{m(t+dt) - m(t)}_{\text{Accumulation}} = \underbrace{0}_{\text{Inflow}} - \underbrace{0}_{\text{Outflow}} + \underbrace{\frac{dX}{dt} \cdot V(t)dt}_{\text{Production}} - \underbrace{0}_{\text{Sink}} \quad (4.2)$$

where the Inflow and Outflow are zero as the feed does not contain cells and the medium is not withdrawn from the reactor. The production represents the division of cells at a given rate (Eq. 4.3) described by the Monod law.

$$\frac{dX}{dt} = \mu(S, N)X \simeq \mu_{\max}X \quad (4.3)$$

where S is the glucose concentration and N the sodium nitrate concentration. In our case, $\mu(S, N)X \simeq \mu_{\max}$ simplification can be used as from Chapter 2 we obtain that glucose concentrations between 10 and 40 g L⁻¹ results in a stable growth rate of around

1.3 d⁻¹. A similar approach was calculated to define the nitrogen concentration for the feed based on the results reported in Chapter 2. Consequently, the variation in cell mass can be expressed as (Eq. 4.4):

$$dm = \mu_{\max} \cdot m(t) \cdot dt \quad (4.4)$$

where dm is the cell mass variation during culture and μ_{\max} is the maximum growth rate. Then, substrate balance was performed to obtain the variation of glucose during the exponential cell growth (Eq. 4.5):

$$\underbrace{s(t+dt) - s(t)}_{\text{Accumulation}} = \underbrace{F(t) \cdot C_0 \cdot dt}_{\text{Inflow}} - \underbrace{0}_{\text{Outflow}} + \underbrace{0}_{\text{Production}} - \underbrace{\frac{1}{Y_{x/s}} dm}_{\text{Sink}} \quad (4.5)$$

where in this case the Outflow and the Production are zero. The Inflow is the Feed at a given concentration over time and the Sink represents the substrate conversion rate to biomass. This can be simplified to (Eq. 4.6):

$$ds = F(t) \cdot C_0 - \frac{\mu_{\max}}{Y_{x/s}} m \quad (4.6)$$

where ds is the variation of substrate, $F(t)$ and C_0 define the feeding flow rate and feeding concentration of glucose and $Y_{x/s}$ is the conversion yield of glucose to biomass (mass conversion rate from glucose to biomass is 2 to 1, as shown in Chapter 2). Given that the objective in the fed-batch is to maintain a constant substrate concentration to promote a constant exponential biomass growth state, the flow rate to feed the culture can express it as follows (Eq. 4.7) to counter substrate exponential depletion:

$$F(t) = F_0 \cdot \exp(ft) \quad (4.7)$$

Leading the following expression for the glucose mass variation (Eq. 4.8), which we aim to keep close to zero to ensure constant substrate availability for the culture:

$$\frac{ds}{dt} = 0 = C_0 \cdot F_0 \cdot \exp(ft) - \frac{\mu_{\max} m_0}{Y_{x/s}} \cdot \exp(\mu_{\max} t) \quad (4.8)$$

where f is the feeding rate has to equal μ_{\max} , and $C_0 F_0$ has to equal $\frac{\mu_{\max} m_0}{Y_{x/s}}$ to ensure a constant value. One should keep in mind that Equation 4.8 govern mass within the tank. Still, microalgae are sensible to concentration, which is not constant. Yet, taking the assumption that at the beginning of the feeding phase, the glucose concentration is between 10 and 40 g L⁻¹, accounting for a 4-fold dilution (3 L added to 1 L), then the concentration would dwindle down to 2.5 to 10 g L⁻¹. Hence, adequate for optimal growth of *Scenedesmus almeriensis*. With this consideration, it is possible to derive the last unknown of our system (Eq. 4.9):

$$F_0 = \frac{\mu_{\max} m_0}{Y \cdot C_0} \quad (4.9)$$

which represents the initial feeding rate calculation based on the maximum specific growth rate, the initial biomass concentration, and the initial substrate concentration. This equation ensures that the feeding rate is adjusted according to the initial conditions of the culture.

From a practical perspective, we had no means to implement an exponential feed with

the bioreactor controller. Therefore, we chose to approximate the $F(t)$ exponential trend using a piecewise linear curve. Fig. 4.2 is the graphical representation of the exponential substrate feeding with the actual approximation given by the limitations of the feeding device. Furthermore, the F_0 was recomputed for every run based on actual initial biomass concentration measurement.

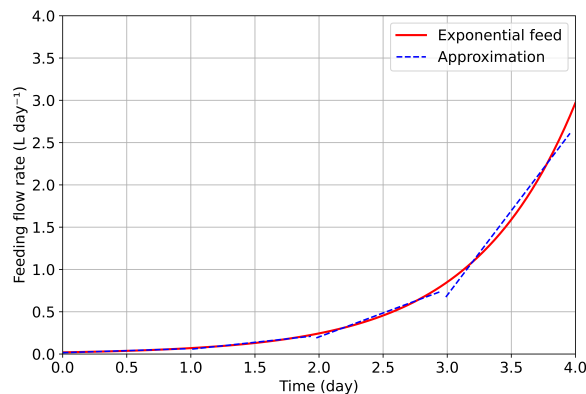


FIGURE 4.2: Graphical representation of the exponential feeding strategy along with actual approximation of the medium additions.

The experiment started with a 1 L culture medium inoculated with 100 mL of seed culture so that the initial biomass concentration was 2 g L^{-1} . The same conditions as the batch culture were maintained for the fed-batch in terms of agitation and bubbling to control DO, temperature regulations and pH control.

4.2.5 Growth measurements and lutein quantification

The culture was sampled twice daily by withdrawing 10 mL of culture. Of these, 1 mL was filtered to obtain the dry weight (DW) biomass and the remainder was immediately centrifuged. The supernatant was recovered and the pellet was washed with distilled water to remove traces of salts and glucose before centrifuging again. The final pellet and the supernatant were stored at $-20 \text{ }^\circ\text{C}$ until analysis. Pigment extraction and quantification were performed according to the results presented in Chapter 3.

4.3 Results and Discussion

4.3.1 Cultivation Scale-Up to a 5 L Bioreactor

In the pursuit of scaling up the culture of *S. almeriensis* to a 5 L bioreactor with a 4 L working volume, it became evident that transitioning from laboratory-scale (50 mL) cultures to larger volumes posed significant challenges.

Two main factors contributed to the complexity of this task. First, the intricate fluid flow dynamics in larger volumes required meticulous optimization to maintain optimal growth conditions. In particular, the amount of dissolved oxygen, as microalgae grow in heterotrophic conditions consumes it rapidly and it becomes a limiting component in the early stages of the culture. Secondly, susceptibility to contamination by bacteria and yeasts increased significantly with increasing volume and complexity of the system, posing challenges in maintaining the purity of the cultures.

The first bioreactor run (Run #1)(Fig. 4.3) presents a growth rate similar to that previously reported in flask cultures (1.2 d^{-1}). However, before all the glucose was consumed, the growth rate decreased and coincided with the detection of bacterial contamination.

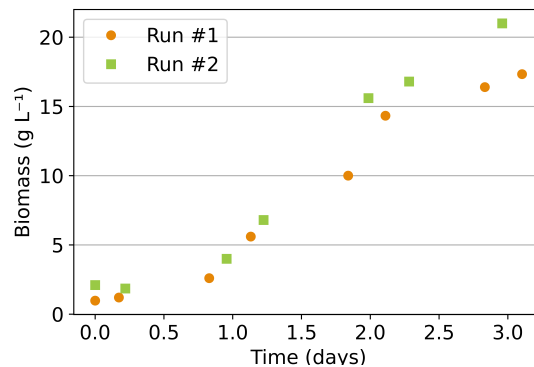


FIGURE 4.3: Scale-up of a batch culture of *Scenedesmus almeriensis* in a 3 L bioreactor. Run #1 showed signs of bacterial contamination before the end of the exponential phase (confirmed by microscope observations), which prompted an antibiotic treatment for run #2.

Despite exhaustive efforts and the implementation of various sterilization methods, contamination persisted until the introduction of an antibiotic treatment. Once it was confirmed that the antimicrobial treatment was effective in controlling the contamination, a second run (Run #2) was conducted to evaluate the effect of antibiotics on the growth of *S. almeriensis*. The results (Fig. 4.3) show that a similar growth rate (1.12 d^{-1}) was maintained, and the culture achieved complete substrate consumption to reach a cell density of 21 g L^{-1} . This achievement underscores the feasibility of achieving equivalent productivity at larger scales and highlights the importance of meticulous control in scaling up microalgal cultivation processes.

While the scaling-up of microalgal cultures has been more extensively studied in phototrophic cultivation mode [44], there are few reports on scaling-up in heterotrophic regime [14]. Focusing on phototrophic cultures first, the ease of implementing cultivation systems in open ponds to harness solar energy for photosynthetic metabolism has facilitated the exploration of scaling up phototrophic cultures. Fuentes *et al.* [241] successfully scaled up the acidophilic microalgae *Coccomyxa onubensis* in phototrophy to 800 L, achieving biomass and lutein productivity values similar to those found at laboratory scale. Similarly, Morillas-España *et al.* [192] scaled up the cultivation of *S. almeriensis* in a thin-layer bioreactor up to 126 m^{-2} with results comparable to those found with the same species cultivated in the laboratory. However, both studies agree that limiting or poorly controlled factors, including temperature, light intensity, and CO_2 availability for the cells, require special attention when scaling up. Nevertheless, none of these factors pose obstacles to cultivation in heterotrophy, making it challenging to apply what has been achieved in scaling up phototrophic cultures to scaling up in heterotrophy.

Jacob *et al.* [242] scaled up the heterotrophic production of *Chlorella pyrenoidosa* from laboratory flasks as seed inoculum, to 300 L tanks, through 100 and 200 L intermediate tanks. Although the results between the three pilot tanks show similar behaviors regarding biomass production, oxygen demand and pH changes, the authors do not compare the results with those obtained at laboratory scale or the challenges they faced in transferring from flask to fermenter. In the same line, Xu *et al.* [243] reported a biomass concentration of up to 3.92 g L^{-1} of *Chlorella protothecoides* in 500 mL flask under heterotrophic regime,

while in a 5 L bioreactor they obtained 15.5 g L^{-1} in only 40 more hours of culture. The results are also not comparable since the bioreactor culture was supplied with a concentrated glucose solution, whereas the flask culture was conducted as a batch process. Although it is not possible to compare the efficiencies of flasks versus fermenter culture, it is clear that heterotrophic culture of microalgae has been successful at pilot (750 L) to commercial (8000 L) scales [244]. However, publications are scarce due to the fact that the information is proprietary [231, 232].

On the other hand, the results shown here highlight the robustness of *S. almeriensis* against the antibiotic concentrations typically utilized for contamination control in microalgae, without compromising its growth performance. This finding resonates with prior studies from Mustapa *et al.* [245], which conducted comprehensive screenings to determine the optimal antibiotic and concentration for achieving axenic microalgal cultures. Such investigations are vital, as contamination issues pose significant challenges to heterotrophic culture scale-up, particularly considering the rapid growth of bacteria and yeasts, which often outcompete microalgae for nutrients.

However, on the lutein aspect, Figure (4.4) shows that there are marked differences between the accumulated lutein between the two runs. In Run #1, 1.57 mg gDW^{-1} of lutein was obtained, a concentration similar to that obtained in flask (1.43 mg gDW^{-1}) and reported in Chapter 2. On the other hand, the maximum amount of lutein obtained in Run #2 is 0.8 mg gDW^{-1} . This may be due to the antibiotic treatment used in Run #2. Inhibitory compounds for key enzymes in carotenoid metabolism have been documented, directing synthesis towards various branches of the pathway [175]. However, the impact of antibiotics on carotenoid synthesis pathways is poorly understood, and no specific reports were found regarding lutein. Therefore, further investigation into the effects of antibiotics on carotenoid synthesis is necessary to create new knowledge in this area. Ultimately, the best approach in scaling up involves achieving axenic cultures and material sterility without the need for antibiotics, a goal that can be attained through industrial processes.

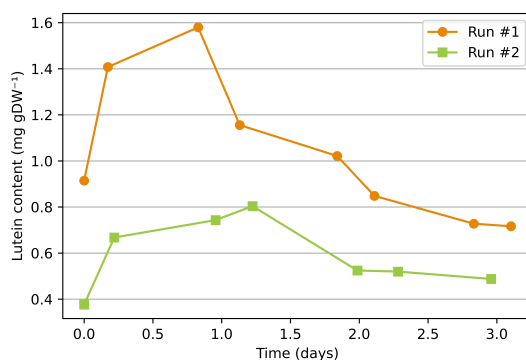


FIGURE 4.4: Lutein content in *S. almeriensis* during bioreactor batch cultures.

Another remarkable finding is that the maximum lutein content in Run #1 occurs in only 19.8 hours of culture, while it took three days in the flask to reach the maximum content. This may be due to the higher inoculum concentration (2 g L^{-1}) than in the flask (0.5 g L^{-1}) or the rigorous control of the dissolved oxygen and pH, which were kept constant in the bioreactor culture and not in the flask.

In terms of productivity, this high lutein content in a short period of time results in a productivity of $3.09 \text{ mg L}^{-1} \text{ d}^{-1}$, well below the $9.79 \text{ mg L}^{-1} \text{ d}^{-1}$ obtained in flask after three days of culture. This is because the high lutein content in the bioreactor occurred

when the biomass density was still very low. As the cell concentration increased, so did the productivity, reaching a maximum of $5.36 \text{ mg L}^{-1} \text{ d}^{-1}$ on day 2.1, even though the lutein content had decreased to 0.84 mg g^{-1} . For the run #2, the low lutein productivity ($3.56 \text{ mg L}^{-1} \text{ d}^{-1}$) is due to low lutein content, probably derived from the interaction with antibiotics.

4.3.2 Fed-batch process for culture intensification

The batch results carried out in the bioreactor validated the same growth rate of *S. almeriensis* and an equivalent glucose-to-biomass conversion rate as those obtained in flasks. This enabled the design of an exponential feeding strategy of concentrated culture medium, corresponding to the exponential growth of the cells. Accordingly, after the initial 40 g of glucose, the remaining 760 g needed to reach 100 g L^{-1} of biomass were added following the model derived from the equations presented in this chapter's materials and methods section.

To optimize experimental times, the batch Run #2 was used to start the first fed-batch run. For this, three of the four liters of the culture were discarded and one liter was kept in the bioreactor. The feeding strategy began immediately to supply the cells with glucose and other nutrients already consumed during the batch stage, since the cells had reached the stationary phase during the initial batch culture, producing 20 g L^{-1} of biomass while consuming the 40 g L^{-1} of glucose present in the medium (Fig. 4.3).

Figure 4.5 shows the exponential increase in glucose due to the feeding of the concentrated medium. However, it can be observed that the biomass concentration decreases. This may be due to the fact that the growth rate had already declined when the feedings started and does not counterbalances the induced dilution. Subsequently, although the glucose concentration increased in the medium, the cells entered a new lag phase, which was not considered in the feeding strategy. As the glucose concentration rose more rapidly than the biomass growth, it eventually reached inhibitory levels, resulting in the collapse of the culture.

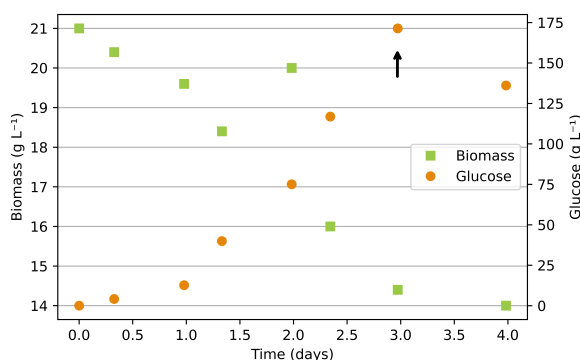


FIGURE 4.5: Biomass and glucose concentrations during the first fed-batch test using one liter from the previous batch run as inoculum. The arrow indicates the time at which the feeding of the culture medium was stopped, since the glucose concentration increased too much to allow for cell growth.

As a result of this trial, it was decided to initiate a second fed batch, starting from one liter in batch culture mode to initiate substrate feeding during the exponential growth phase. The culture was grown for 2.7 days until it reached 15.6 g L^{-1} of biomass and a decrease in glucose from 40 to 8 g L^{-1} . The feeding strategy started at this time.

TABLE 4.1: Glucose feeding per day. The corresponding volume was distributed in intermittent pulses during the day to prevent each day's addition from causing inhibitory glucose concentrations. Day 1 indicates the start of the feeding, considering that the culture had already been in batch for 2.6 days.

	Feeding volume (mL)	Glucose per day (g)
<i>Day 1</i>	272	71
<i>Day 2</i>	695	180
<i>Day 3</i>	1,982	514

The model presented in the previous section allowed the prediction of daily substrate feedings with concentrated glucose exponentially to maintain a stable concentration of approximately 40 g L^{-1} , with the growth rate being set as equivalent to the glucose consumption rate. Thus, the daily feedings were distributed throughout the day to avoid increasing the glucose concentration above the inhibitory limit (Table 4.1).

Figure (4.6) shows that feeding increased the glucose concentration exponentially, as expected. However, the biomass did not respond positively to the medium's glucose increase. Although growth is observed, it is not sufficiently accelerated to avoid over-accumulation of glucose.

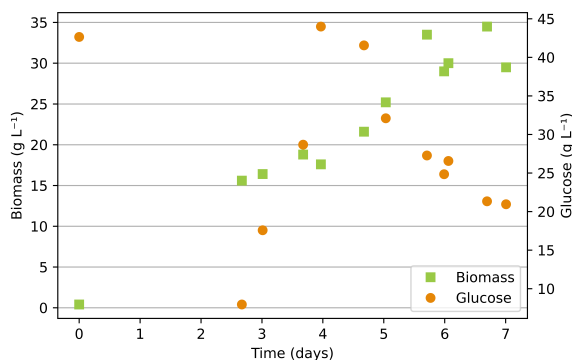


FIGURE 4.6: Biomass and glucose concentrations during the second fed-batch test. The feeding strategy was interrupted at day 4 after a decrease in growth rate induced an over-accumulation of glucose.

Under these circumstances, the automatic addition of medium was stopped on day 4 to give the biomass time to increase its growth rate and consume the substrate before adding more glucose. This allowed for biomass growth and a decrease in glucose during the following two days (from days 4 and 6). However, growth rate decreased and biomass concentration stabilized at around 35 g L^{-1} from day 6. The culture was stopped due to electrical problems on day 7, but interesting findings were drawn.

Although the addition of glucose started when the culture was still in its exponential phase, it is possible that the cells had already sensed the low concentration of glucose remaining (8 g L^{-1}) and started to change their metabolism. This resulted in a lower growth rate than calculated for the feeding strategy and led to a faster-than-expected increase in glucose concentration. Different results were reported by Nagy *et al.* [239], who cultured *C. vulgaris* under a heterotrophic regime with exponential glucose feeding. The authors initiated substrate additions (including glucose, nitrate and phosphate) once the initial batch had consumed all the glucose and reported a sustained increase in biomass. However, biomass accumulation matched the increasing phosphate trend in the medium, suggesting an important role in achieving high biomass densities of this species. Although

the substrate feeding in our protocol includes phosphate, it will be important to identify its role in the growth and biomass accumulation of *S. almeriensis*.

In another study, Shi *et al.* [246] also obtained constant biomass increase after starting the feeding of a *C. protothecoides* culture in the late exponential phase. They fed glucose and urea, maintaining a glucose concentration between 10 and 30 g L⁻¹ without observing an acclimation phase, which does not agree with our results. However, even though they started feeding in the late exponential phase, about 20 g L⁻¹ of glucose remained in the medium. The fact that our culture was found with a residual concentration of 8 g L⁻¹ at the beginning of feeding could have caused the lag phase shown in our results.

Another remarkable finding is the change in concentration of lutein, chlorophyll a and chlorophyll b throughout the second fed-batch culture and according to glucose availability in the medium. In Figure (4.7), it can be seen how the concentration of the three main pigments analyzed behaves similarly to the glucose concentration. Although no changes are perceived during the first 2.6 days of the initial batch culture, an increase of pigments with the same trend as glucose is observed from the first addition of the culture medium. On day 4, when feeding was stopped to allow the biomass to consume the glucose, pigments also decreased and then increased with the last addition of medium.

It is necessary to point out that, although pigment dynamics are only compared to that of glucose, other elements may also play a role in this variation. However, we only have data for glucose, and further studies will be necessary to verify whether this phenomenon is linked to glucose concentration alone or to other nutrients as well.

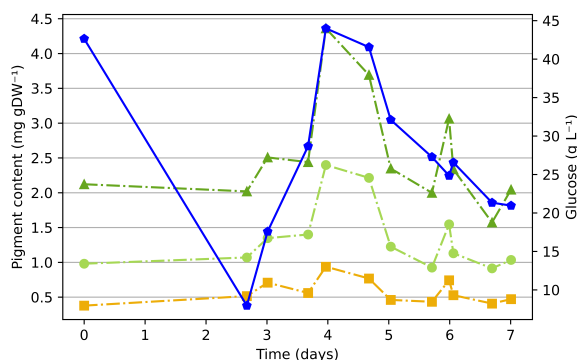


FIGURE 4.7: Comparison of glucose concentration with the amount of lutein, chlorophyll a and b during the second fed-batch test.

These dynamics are different from the results reported by other authors. Wang *et al.* [165] carried out a fed-batch culture of *C. protothecoides* fed with waste monascus fermentation broth medium to increase biomass and lutein productivity. The authors compared three different feeding time intervals following the depletion of organic carbon, which then ranged from 4 to 12 g L⁻¹ during the culture and following feeding. However, it was observed that both biomass and lutein content maintained an increasing trend to reach 23.9 g L⁻¹ of biomass and 9.16 g L⁻¹ of lutein.

Similar results were reported by Xie *et al.* [170] with a mixotrophic culture of *C. sorokiniana*. The authors report higher lutein content when the feeding interval is longer, suggesting that a carbon source in the medium during mixotrophic culture interferes with photosynthetic pigment synthesis; therefore, once the substrate is consumed, the lutein concentration increases. However, our protocol is purely heterotrophic, and the reduction

of glucose concentration in the medium negatively impacts lutein content. Further studies will be necessary to determine the effect of mixotrophic culture on *S. almeriensis*.

Most of the studies on heterotrophic production of microalgae using fed-batch as the operation mode have been carried out with species of the genus *Chlorella*, as they have proved to be fast-growing species with high lutein contents [146, 150, 159, 165, 170, 246]. It is evident that the strategies that are useful for the production of *Chlorella* species under these conditions do not apply to *Scenedesmus almeriensis*, as the response to glucose feeding is very different and further studies are needed to understand the metabolism of this species in heterotrophy.

On the other hand, the lutein content throughout the fed-batch culture does not exceed 0.7 mg g^{-1} . This is half of what is obtained in the fed-batch culture without antibiotics. Therefore, it will be necessary to confirm experimentally the effect that antibiotics have on lutein synthesis in *S. almeriensis*, followed by a process optimization to achieve axenic cultures without the need for antibiotics to evaluate the maximum productivity that can be obtained with *S. almeriensis* in fed-batch mode.

4.4 Conclusions

The results presented in this chapter allow us to identify advances in using *Scenedesmus almeriensis* for lutein production. The possibility of maintaining the same growth rate in the scale-up from 50 mL culture in flask to 4 L in a bioreactor is a necessary step to achieve higher production and to evaluate the feasibility of scaling up to even larger volumes. In addition, the ability to synthesize lutein has been maintained, although further studies are needed to test how more stable pH and dissolved oxygen conditions in the bioreactor influence the ability to produce lutein from this strain.

On the other hand, while antibiotics did not impact biomass production, they may have a negative effect on lutein synthesis. Therefore, fine adjustments in the type and concentration of antibiotics used, or improvements in the process, will be necessary to mitigate this effect.

Finally, although the fed-batch mode of operation offers the best way to achieve high biomass densities in heterotrophically grown microalgae, the factors that have prevented the intensification of the culture of *Scenedesmus almeriensis* need to be further analyzed. Although it was possible to obtain a biomass concentration 1.5 times higher than in batch mode (33.5 g L^{-1}), the model developed to obtain a concentration of 100 g L^{-1} needs to be improved to take into account the unexpected response of the strain to glucose additions. In this regard, it is proposed that the effect of glucose on the cell cycle be analyzed in a closer way. If the conditions that lead the cell to increase size, accumulate reserves, synthesize photosynthetic pigments and divide can be identified, it will be possible to determine the best time to add nutrients to the medium and to intensify the culture.

These conclusions call for immediate perspectives to be addressed before the thesis defense. Rapid tests will be conducted to determine the effect of antibiotics on lutein synthesis by *S. almeriensis* on a small scale. In addition, experiments will be carried out to gain a close insight into how *S. almeriensis* cells respond to the addition of different feed substrates, i.e. glucose, nitrates and minor elements.

Conclusion

Conclusion

The primary aim of this thesis was to investigate the potential of the microalgae *Scenedesmus almeriensis* for industrial-scale lutein production, addressing three primary research questions: Is the quest for higher lutein content in microalgae coming to an end and should the focus now be on increasing overall productivity, does *S. almeriensis* hold the capacity to produce high lutein yields with downstream process benefits, and is it feasible to scale up this strain to achieve high-density cultures in fed-batch mode?

While phototrophic cultures of microalgae have traditionally been emphasized for inducing high lutein content, the low biomass density achieved when depending on light calls for a paradigm shift. Optimal phototrophic culture parameters, such as light quality and quantity, nitrogen levels, and temperature, have little influence on lutein accumulation. Moreover, pushing these parameters to their extremes often induces cell stress, resulting in diminished biomass. Therefore, the focus should be placed on overall productivity rather than just lutein content. Heterotrophic and mixotrophic cultures have shown superior lutein productivities, especially those incorporating photoinduction in two-stage processes and varied culture medium feeding strategies. Since the species that have demonstrated this capacity (mainly from the genus *Chlorella*) present complex harvesting and extraction processes, searching for alternative species will facilitate the industrialization of processes to produce lutein from microalgae. However, future research should focus on validating the techno-economic feasibility and scalability of these alternative methods to ensure commercial viability.

The species *Scenedesmus almeriensis* has demonstrated potential for lutein production in phototrophic cultures; however, its slow growth relative to other species has relegated it to a less prominent role in the lutein production race. In this work, the results demonstrated that heterotrophic conditions can lead *S. almeriensis* to achieve high biomass concentrations and increased lutein productivity, despite the higher lutein content in phototrophic cultures. The higher overall productivity in heterotrophic conditions stems from the rapid biomass increase, comparable to other reports using *Chlorella* species. A photoinduction step on the dense culture further enhanced lutein productivity. These findings also highlighted the need to optimize nutrient supplementation and light intensity during photoinduction to maximize lutein yields and analyze its benefits.

Moreover, exploring solvent alternatives for lutein extraction revealed ethanol as a promising and environmentally friendly option, outperforming other solvents and enhancing industrial feasibility. Future studies should continue optimizing these parameters and

exploring the long-term stability of biomass stored at 4 °C. Furthermore, *S. almeriensis* cultured on heterotrophy showed benefits regarding the downstream process to recover lutein from its biomass. The larger cell size and the greater fragility of its cell wall, compared to species of the genus *Chlorella*, make *S. almeriensis* a promising candidate for scaling up the culture to obtain lutein.

Successfully maintaining the growth rate from small-scale flask cultures to larger bioreactors is a critical step toward industrial-scale production. During the scaling up of the heterotrophic cultures of *S. almeriensis*, from 50 mL to 4 L, the growth rate remained the same, proving the potential to increase the operating volume further. Additionally, the ability to synthesize lutein under this regime was retained, although further research is required to understand the influence of stable pH and dissolved oxygen conditions in bioreactors. The fed-batch mode of operation demonstrated the potential to achieve higher biomass densities than in batch mode. However, further analysis is needed to address factors that hinder the complete intensification of *S. almeriensis* cultures. The model developed to achieve 100 g L⁻¹ biomass concentration requires refinement to account for the strain's unexpected response to glucose additions. Investigating the effect of glucose on the cell cycle could lead to optimized nutrient addition timing, enhancing culture productivity.

In conclusion, this thesis comprehensively explores *S. almeriensis* as a viable microalgae for lutein production. The findings underscore the need for a balanced approach between lutein content and biomass production to attain higher lutein productivity, considering both heterotrophic and two-stage cultivation methods with a photoinduction step. Furthermore, addressing the challenges of scaling up and intensification through continued research and development will be crucial in unlocking the full potential of *S. almeriensis* for industrial-scale lutein production, paving the way for its commercial success.

French extended abstract

Introduction

Avec l'avènement de l'industrialisation de l'alimentation, la science a cherché à conserver les couleurs vives des aliments transformés pour qu'elles correspondent à celles des aliments frais. Cependant, les pigments naturels se sont révélés instables pour ces processus, ce qui a conduit à l'utilisation de colorants industriels, dont beaucoup étaient toxiques. Les organismes de réglementation ont fini par interdire ces colorants nocifs, ce qui a conduit à l'utilisation de dérivés du goudron de houille, moins toxiques mais qui ont tout de même suscité des inquiétudes sur le plan de la santé au fil du temps. Aujourd'hui, les colorants synthétiques sont réglementés sur la base de leur dose journalière admissible, ce qui garantit la sécurité à des niveaux de consommation spécifiques. Toutefois, l'apport cumulé des aliments transformés peut dépasser les limites de sécurité, en particulier chez les enfants, ce qui peut entraîner des problèmes de santé tels que des troubles gastro-intestinaux, une anémie, des réactions allergiques, voire des tumeurs.

En réponse à ces risques pour la santé, les pigments naturels tels que les caroténoïdes suscitent un regain d'intérêt. Les caroténoïdes, que l'on trouve dans les plantes, les algues et certaines bactéries, sont responsables des couleurs vives de nombreux fruits et légumes et offrent des avantages pour la santé grâce à leurs propriétés antioxydantes. Les principaux caroténoïdes sont le β -carotène, le lycopène, la lutéine et la zéaxanthine. Les régimes riches en ces composés sont associés à une réduction des risques de maladies chroniques telles que les maladies cardiaques et certains cancers.

La lutéine, en particulier, est appréciée pour son rôle dans la promotion de la santé oculaire en protégeant la rétine de la lumière bleue nocive. Elle est également associée à une réduction des risques de maladies chroniques et possède des propriétés anti-inflammatoires. La lutéine est produite commercialement à partir de fleurs de souci, mais cette méthode soulève des problèmes environnementaux et sociaux. La culture de microalgues est envisagée donc comme une alternative durable. Les microalgues poussent plus vite que les plantes, nécessitent moins de terre et d'eau et peuvent être intégrées au traitement des eaux usées, ce qui réduit l'impact sur l'environnement.

Malgré des recherches approfondies, la production industrielle de lutéine à partir de microalgues se heurte à des difficultés liées aux limites métaboliques des cellules. Les efforts actuels se concentrent sur l'optimisation des conditions de croissance afin de maximiser la productivité en lutéine. Des stratégies récentes, telles que la culture mixotrophe et hétérotrophe, se sont révélées prometteuses, permettant d'obtenir des concentrations cellulaires élevées et une productivité comparable à celle d'autres caroténoïdes produits commercialement.

Cette étude vise à déterminer si la phototrophie reste une méthode viable pour produire des quantités significatives de lutéine microalgale ou si d'autres méthodes doivent être explorées. Une analyse de l'état de l'art présentée dans le premier chapitre met en évidence la nécessité de réorienter les recherches vers la productivité globale de la lutéine plutôt que vers sa seule accumulation dans la biomasse. Ce changement est crucial pour le potentiel industriel des microalgues en tant que producteurs de lutéine.

Les deuxième et troisième chapitres étudient le potentiel de l'espèce de microalgue *Scenedesmus almeriensis* pour la production hétérotrophe de lutéine. *S. almeriensis*, connue pour sa forte teneur en lutéine dans des conditions phototrophiques, n'avait pas été étudiée pour sa croissance hétérotrophe. Les résultats montrent que *S. almeriensis* peut atteindre une productivité en lutéine comparable à celle des espèces *Chlorella*, avec des avantages tels qu'une taille de cellule plus importante et une paroi cellulaire plus faible, ce qui facilite la récolte de la biomasse et l'extraction de la lutéine.

Le chapitre quatre montre les limitations de la concentration en biomasse dans les cultures en mode batch de *S. almeriensis* en raison de l'inhibition du glucose. Une stratégie fed-batch a été conçue pour maintenir des niveaux optimaux de glucose, améliorant la productivité en biomasse et la production de lutéine. Ce chapitre présente les performances de cette stratégie et discute des nouvelles pistes de recherche qui émergent de ces expériences.

Dans l'ensemble, cette étude contribue à la compréhension et à l'amélioration de la production de lutéine microalgale, en abordant les limitations métaboliques et en explorant des stratégies de culture pour améliorer la productivité. Ces connaissances sont essentielles pour développer des procédés industriels durables et efficaces pour la production de lutéine, avec des implications significatives pour la sécurité alimentaire et la santé.

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Titre: Production de lutéine et amélioration de l'extraction à partir d'une culture hétérotrophe de *Scenedesmus almeriensis*

Mots clés: Biotechnologie, *Scenedesmus almeriensis*, Lutéine, Culture hétérotrophe, Extraction

Résumé: Les microalgues sont considérées comme une solution biotechnologique aux problèmes majeurs de ce siècle, offrant des réponses et des alternatives dans des domaines tels que la malnutrition, les pénuries alimentaires, le changement climatique et la pollution. En tant qu'organismes photosynthétiques, les microalgues synthétisent de nombreux composés afin d'exploiter la lumière du soleil pour leurs fonctions métaboliques, notamment la lutéine, un caroténoïde jaune essentiel pour l'alimentation humaine. Bien que la lutéine soit un composé associé à l'activité photosynthétique des microalgues, certaines espèces conservent la capacité de synthétiser cet important pigment même dans des conditions de culture hétérotrophe, ce qui permet d'augmenter la productivité de la biomasse et de la lutéine. *Scenedesmus*

almeriensis a été rapportée comme une bonne productrice de lutéine dans des conditions phototrophiques, mais son faible taux de croissance par rapport à d'autres espèces de microalgues a entravé son potentiel. Cette thèse présente les résultats de la productivité en lutéine obtenue à partir d'une culture hétérotrophe de *Scenedesmus almeriensis*, à la fois à l'échelle du laboratoire et à l'échelle pré-pilote. En plus d'atteindre des valeurs élevées de productivité en lutéine, sa taille cellulaire plus grande et sa fragilité simplifient la récolte de la biomasse et l'extraction des pigments. Une méthode simplifiée d'extraction de la lutéine adaptée à cette espèce est également présentée. Ce procédé utilise l'éthanol comme solvant d'extraction et nécessite moins de temps et d'énergie, ce qui représente des avantages pour l'environnement et l'acceptation dans l'industrie.

Title: Lutein production and extraction improvements from a heterotrophic culture of *Scenedesmus almeriensis*

Keywords: Biotechnology, *Scenedesmus almeriensis*, Lutein, Heterotrophic culture, Extraction

Abstract: Microalgae are positioned as a biotechnological solution to major problems of this century, offering answers and alternatives in areas like malnutrition, food shortages, climate change, and pollution. As photosynthetic organisms, microalgae synthesize numerous compounds to harness sunlight for their metabolic functions, including lutein, a yellow carotenoid crucial for the human diet. Although lutein is a compound associated with the photosynthetic activity of microalgae, some species maintain the capacity to synthesize this important pigment even under heterotrophic conditions, allowing increased productivity of both biomass and lutein in cultures supplemented with an organic carbon source. *Scenedesmus almeriensis*

has been reported as a good lutein producer under phototrophic conditions, but its low growth rate compared to other microalgae species has hindered its potential. This thesis presents results of the lutein productivity obtained from a heterotrophic culture of *Scenedesmus almeriensis*, both at laboratory and pre-pilot scale. In addition to achieving high lutein productivity values, its larger cell size and fragility simplify biomass harvesting and pigment extraction. A simplified method for lutein extraction adapted to this species is also presented. This process uses ethanol as an extraction solvent and requires less time and energy, which represents environmental and product acceptance advantages.