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Research review paper

Evolving perspectives on lutein production from microalgae - A focus on productivity and heterotrophic culture

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ABSTRACT

Increased consumer awareness for healthier and more sustainable products has driven the search for naturally sourced compounds as substitutes for chemically synthesized counterparts. Research on pigments of natural origin, such as carotenoids, particularly lutein, has been increasing for over three decades. Lutein is recognized for its antioxidant and photoprotective activity. Its ability to cross the blood-brain barrier allows it to act at the eye and brain level and has been linked to benefits for vision, cognitive function and other conditions. While marigold flower is positioned as the only crop from which lutein is extracted from and commercialized, microalgae are proposed as an alternative with several advantages over this terrestrial crop. The main barrier to scaling up lutein production from microalgae to the commercial level is the low productivity compared to the high costs. This review explores strategies to enhance lutein production in microalgae by emphasizing the overall productivity over lutein content alone. Evaluation of how culture parameters, such as light quality, nitrogen sufficiency, temperature and even stress factors, affect lutein content and biomass development in batch phototrophic cultures was performed. Overall, the total lutein production remains low under this metabolic regime due to the low biomass productivity of photosynthetic batch cultures. For this reason, we describe findings on microalgal cultures grown under different metabolic regimes and culture protocols (fed-batch, pulse-feed, semi-batch, semi-continuous, continuous). After a careful literature examination, two-step heterotrophic or mixotrophic cultivation strategies are suggested to surpass the lutein productivity achieved in single-step photosynthetic cultures. Furthermore, this review highlights the urgent need to develop technical feasibility studies at a pilot scale for these cultivation strategies, which will strengthen the necessary techno-economic analyses to drive their commercial production.

1. Introduction

Lutein is a yellow-coloured carotenoid pigment produced naturally by some plants and microoragnisms. Due to its antioxidant activity, there have been longstanding claims about its health benefits (Granado-Lorencio et al., 2009; Fernández-Sevilla et al., 2010). Their recent demonstrations for eye vision (Christaras et al., 2019; Demmig-Adams et al., 2020), brain health, and cognitive functions (Stringham et al., 2019; Gazzolo et al., 2021), 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 (MMR, 2023)). 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 (Stringham et al., 2019). 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 (Stringham et al., 2019). 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 (Junghans et al., 2001; Gazzolo et al., 2021).

Humans, and animals, do not synthesize lutein and need to acquire it

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through their diets. In the case of a human diet, lutein can 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 (Ochoa Becerra et al., 2020). 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 (Hajizadeh-Sharafabad et al., 2019). 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 (Lin et al., 2015; Ochoa Becerra et al., 2020). 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 (Lin et al., 2015; Ochoa Becerra et al., 2020). 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 (Bosma et al., 2003). 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 (Willoughby et al., 2000) and processing (Britton et al., 2001) flowers, 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 m^3 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 (Vechpanich and Shotipruk, 2010; Lin et al., 2015). 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 bares 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%) (Mayer and Rüttimann, 1980; Khachik and Chang, 2009). 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 (Takemura et al., 2021). Microbial fermentation exhibits fast growth rates, making its combination with genetic engineering a promising substitution pathway for the production of value-added compounds (Bian et al., 2021, 2023). A lutein titer of 11 mg L^{-1} in its free form was obtained from a genetically engineered *Escherichia coli* (Takemura et al., 2021). 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} (Bian et al., 2021). 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 (Fernández-Sevilla et al., 2010; Lin et al., 2015). 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 (Alam et al., 2020; Levasseur et al., 2020). 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 (Safi et al., 2014).

The content of carotenoids in microalgae, including lutein, has been studied since the 1960s (Iwata et al., 1961). 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 (Borowitzka et al., 1984; Vonshak, 1985). 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 (Pick et al., 2019). These species can accumulate such a large amount of pigments due to cellular mechanisms that respond to stress conditions (Esteban et al., 2015).

However, lutein content among studied microalgal species varies considerably between 0.19 and 0.72% DW (Ho et al., 2014) and only some strains stand out as lutein producers under certain conditions, such as *C. vulgaris* CS-41 (0.94% DW) (McClure et al., 2019), *D. salina* (0.88% DW) (Fu et al., 2014) or *Parachlorella* sp. JD-076 (1.18% DW) (Heo et al., 2018).

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 (Xie et al., 2021).

In recent years, numerous studies have focused on studying lutein content in microalgae, mainly from the genus *Chlorella* (*C. vulgaris* (McClure et al., 2019), *C. pyrenoidosa* (Sampathkumar and Gothandam, 2019), *C. protothecoides* (Wei et al., 2008; Ribeiro et al., 2017; Xiao et al., 2018; Shi et al., 2000), *C. sorokiniana* (Cordero et al., 2011; Chen et al., 2017a), *C. zofingiensis* (Liu et al., 2014)) and *Scenedesmus* (*S. obliquus* (Wiltshire et al., 2000; Ho et al., 2014), *S. almeriensis* (Sánchez et al., 2008), *S. incrassatulus* (Flórez-Miranda et al., 2017)), but also on *Chlamydomonas reinhartii* (Ma et al., 2020b), *Muriellopsis* sp. (Del Campo et al., 2000) *Coccomyxa onubensis* (Vaquero Calañas, 2013; Bermejo et al., 2018; Soru et al., 2019), *Dunalliela salina* (Fu et al., 2014). 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. (2015) suggest that potential microalgal strains must have a lutein content of at least 1% DW to be economically feasible. Furthermore, Xie et al. (2021) 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 (Cordero et al., 2011; Chen et al., 2022). 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% (Patel et al., 2022). For example, Rathod et al. (2020) reported a lutein percentage increase of 83% on *Chlamydomondas 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 (Hu et al., 2020). 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 (Xie et al., 2021). Although, in recent years, some critical genes for these processes have been identified in plants and microalgae, 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 focused on assessing lutein content within microalgae, and only a few have highlighted the ability of heterotrophic and mixotrophic growth to increase productivity. Most of them highlight relevant findings and suggest strategies such as inducing oxidative stress, genetic engineering and nutrient concentration variations to increase the cells' lutein content.

For example, Hu et al. (2018) clearly explains the metabolism associated with pigment synthesis when microalgae are grown in heterotrophy and enumerate methods by which enhancements in lutein content have been attained, yet they do not delve into how these approaches affect biomass production and, consequently, lutein productivity. Similarly, reviews from Saha et al. (2020) and Patel et al. (2022) explain in detail the metabolic pathways associated with lutein synthesis, both in the presence and absence of light, as well as the genetic engineering tools reported to increase lutein content, but pay little attention to productivity values. 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 (Fig. 1), raising a pivotal question: should we continue focusing primarily on content, or is it time to shift our collective attention towards enhancing lutein productivity? The subtle distinction between content and productivity holds immense significance.

Recently, reviews from Zheng et al. (2022a), Fu et al. (2023), and Leong and Chang (2023) have highlighted the importance of heterotrophic and mixotrophic growth modes along with the potential of two-stage cultures to enhance lutein productivity. Furthermore, these reviews offer detailed explanations of three highly relevant topics: different bioreactor systems for microalgae cultivation and lutein production, in-situ accumulation of lutein in fermenters using metabolic engineering, and lutein production at pilot scale. However, these reviews primarily document the high productivity values from the references without deeply analyzing the interplay between lutein content, biomass production, and lutein productivity, which is the focus of the current review.

Hence, 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 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

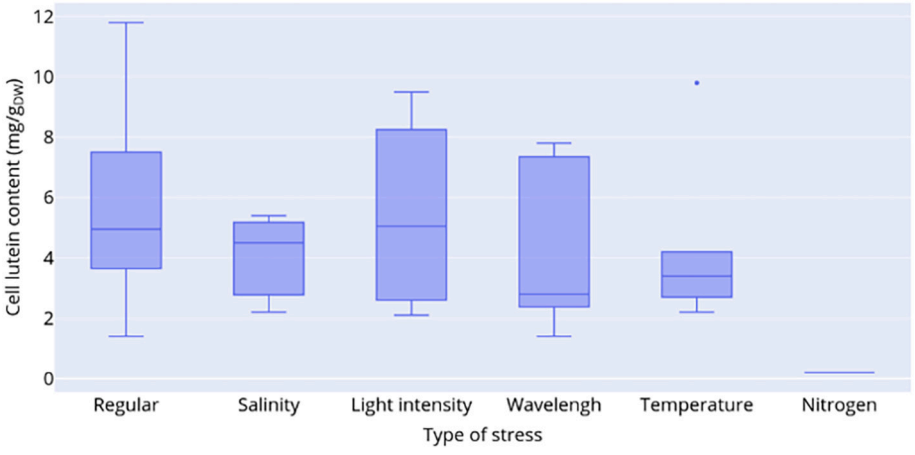

Fig. 1. The boxplot illustrates the variation in lutein content in microalgae resulting from different stress factors, including light intensity, light wavelength, nitrogen quantity, temperature and salinity. Each box represents the interquartile range (IQR) of lutein content for a specific stress condition, 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 of the impact of various stress conditions on lutein content in microalgae cultures.

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.

2. Lutein synthesis and function in microalgae

Lutein is associated with photosystem proteins and participates in the light-harvesting complex, where it fulfills three main roles: (i) it plays an important role in the correct folding of photosystem II proteins (Formaggio et al., 2001; Luciski and Jackowski, 2006); (ii) it absorbs blue-green light (maximal absorption at 446 and 476 nm in acetone (Esteban et al., 2015)) to optimize photosynthetic capacity under low light conditions, transmitting the energy it receives to the chlorophyll (Fu et al., 2014; Sun et al., 2018); (iii) it acts as an antioxidant. Its structure and position in the light-collecting complex allow it to quench harmful oxidative species and excited chlorophyll (Sun et al., 2018; Larkum et al., 2020). 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)(Larkum et al., 2020). 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 (Sun and Li, 2015). Moreover, lutein can scavenge ROS that may be formed otherwise (Jahns and Holzwarth, 2012).

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 (Esteban et al., 2015). 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 (Hirschberg, 2001; Sun and Li, 2015).

In microalgae, carotenoids in the photosynthetic apparatus that participate in light harvesting and photoprotection are termed primary carotenoids. Likewise, the secondary are carotenoids synthesized and accumulated under stress conditions, such as high light stress, nutrient deprivation or salinity stress (Shi et al., 2020). Secondary carotenoids accumulate in lipid bodies within the chloroplast or in the cytoplasm (Sun and Li, 2015; Pick et al., 2019). A good example of this is the accumulation of the carotenoids astaxantin and β-carotene in *Haematococcus pluvialis* and *Dunaliella salina*, respectively (Pick et al., 2019; Tamaki et al., 2021). 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 (Lemoine and Schoefs, 2010; Liu et al., 2014; Pick et al., 2019). 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 (Ota et al., 2018). In addition, these large amounts of stored carotenoids offer great protection against oxidative stress during harsh conditions (Lamers et al., 2010; Lemoine and Schoefs, 2010). To date, no report has shown that lutein can accumulate in lipid bodies.

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 same strength. Care was therefore taken in specifying the context of the subsequently reported findings.

3.1. Eye and vision performance

In the eye, lutein is found in the macula 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 (Christaras et al., 2019; Demmig-Adams et al., 2020).

These two eye carotenoids are referred to as the Macular Pigments (MP) and are measured in-vivo through psychophysical methods (Christaras et al., 2019). High MP values are associated with better visual performance, like tolerance to extreme light intensity, a faster speed of visual processing and better image contrast sensitivity (Stringham et al., 2019; Demmig-Adams et al., 2020).

Due to its antioxidant potential, it has been shown that lutein supplementation in the diet of mice minimizes age-related macular degeneration (AMD) Izumi-Nagai et al. (2007), the most frequent source of human blindness in developed countries (Bressler, 2004; Izumi-Nagai et al., 2007). Additionally, Feng et al. (2019) reported that an intake of 10–20 mg day^{-1} for more than six months significantly increases MP measurements and improves vision in patients with AMD. Moreover, Gazzolo et al. (2021) states that the blue light filtering capacity of lutein is of vital importance in the development of ocular tissue in children.

3.2. Brain and cognitive function

In recent years, a multitude of studies conducted in both animals (do Prado Silva et al., 2017; Gunal et al., 2021; Nazari, 2022) and humans (Johnson et al., 2008; John et al., 2015; Alonso-Garrido et al., 2020) have identified lutein as a significant contributor to brain health 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 (Stringham et al., 2019; Gazzolo et al., 2021). 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 (Dhas and Mehta, 2020) results in the highest concentration of these carotenoids being detected at various developmental stages and persisting into later life (Stringham et al., 2017).

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 (Demmig-Adams et al., 2020; Gazzolo et al., 2021). Moreover, lower Alzheimer's mortality has been reported in individuals with higher serum levels of lycopen and lutein+zeaxanthin (Min and Min https://www, 2013).

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 (John et al., 2015).

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 (Demmig-Adams et al., 2020). 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 (Buscemi et al., 2018).

These assumptions have led to numerous studies to assess the effects of lutein against various diseases. The results of these studies position lutein as an important compound mainly for the prevention of cancer (Park et al., 1998; Kim et al., 2019; Sumantran et al., 2000; Kavalappa et al., 2021; Omar et al., 2021) and cardiovascular diseases (Buscemi et al., 2018; Hajizadeh-Sharafabad et al., 2019).

3.4. 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 (Gazzolo et al., 2021). The results of these studies have been considered by authorities to determine the maximum recommended daily doses. The Food and Agricultural Organization (FAO) (World- Health-Organization, 2004) 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) (European-Food-Safety-Authority, 2008) 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 (Administration, 2014).

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 (Pick et al., 2019). 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 (Liu et al., 2014; Shah et al., 2016). 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) (Wang et al., 2013).

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 (Fu et al., 2014).

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 (Cirulis et al., 2013; Tamaki et al., 2021). 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 (Tamaki et al., 2021). To deal with the oxidative damage that this represents for macromolecules, microalgae employ a variety of antioxidant compounds, like carotenoids (Shi et al., 2020). 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.

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 (Gatamaneni Loganathan et al., 2020). 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 (Gatamaneni Loganathan et al., 2020).

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 (Atta et al., 2013).

4.1.1. Light intensity

Because outdoor microalgae production scale-up is generally carried out at intensities given by the sun (up to 2000 μmolPhoton m^{-2} 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 (Esteban et al., 2015). In a general context, it has been argued that the lutein content in plants tends to increase under conditions of intense illumination (Hirschberg, 2001).

In microalgae, it has been found that low and moderate light intensities (50–400 μmolPhoton m^{-2} s^{-1}) generally promote relatively high lutein content (Vaquero et al., 2014). 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 (Vaquero et al., 2014; Schüler et al., 2020). Yet, the photoprotective role of lutein would also indicate an increase in cell content when microalgae are subjected to high light intensities (Jahns and Holzwarth, 2012).

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 (Esteban et al., 2015). *Scenedesmus almeriensis* is a microalgae isolated from southern Spain that was reported to have high tolerance to high light intensity (Sánchez et al., 2007). When cultured under controlled conditions, this strain showed a maximum lutein content of 0.43% under 1700 μmolPhoton m^{-2} s^{-1}. Additionally, biomass productivity was also higher under this light intensity, giving a total lutein productivity of 3.8 mg L^{-1} d^{-1}.

Total carotenoid content in the marine microalgae *Tetraselmis* sp. CTP4 was 1.5-fold higher under a light intensity of 33 μmolPhoton m^{-2} s^{-1} compared to 170 μmolPhoton m^{-2} 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 μmolPhoton m^{-2} s^{-1} compared to 33 μmolPhoton m^{-2} s^{-1}, possibly due to the photoprotecting role of this pigment for this species collected in the south of Portugal (Schüler et al., 2020). In this study, biomass concentration was also higher at 170 μmolPhoton m^{-2} s^{-1}, resulting in lutein productivity of 1.83 mg L^{-1} d^{-1}, compared to 0.35 mg L^{-1} d^{-1} obtained at 33 μmolPhoton m^{-2} s^{-1}.

Parachlorella sp. JD-076 has been reported as a species tolerant to high light intensities (Heo et al. Liu et al., 2018). When cultured in a tubular photobioreactor at 1000 μmolPhoton m^{-2} s^{-1} achieved a biomass concentration of 8.45 g L^{-1} and a lutein content of 11.8 mg g^{-1} DW, which led to a productivity of 25 mg L^{-1} 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 μmolPhoton m^{-2} s^{-1} compared to 100 μmolPhoton m^{-2} s^{-1} (Couso et al., 2012). 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 μmolPhoton m^{-2} s^{-1}, the carotenoid that increased was β-carotene, while the lutein content decreased (Lamers et al., 2010).

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. (2021) reported almost 4-fold higher lutein content in *Scenedesmus* sp. SVMIICT1 under 50 μmolPhoton m^{-2} s^{-1} compared to 250 μmolPhoton m^{-2} s^{-1}.

On the same line, Gong and Bassi (2017) cultured *Chlorella vulgaris* UTEX265 in a coiled tubular photobioreactor and found that at low light intensity of 25 μmolPhoton m^{-2} s^{-1} the lutein content was 22.9% higher than at 85 μmolPhoton m^{-2} s^{-1}. However, as the growth rate is higher at 85 μmolPhoton m^{-2} s^{-1}, the specific lutein production is also higher (11.98 mg g^{-1} d^{-1}).

Ho et al. (2014) 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 μmolPhoton m^{-2} s^{-1} led to an increase in biomass productivity and growth rate (+858% and + 266%, respectively), lutein content was higher between 30 and 75 μmolPhoton m^{-2} s^{-1} (0.54–0.55% of DW). Accordingly, the highest lutein productivity was found at 300 μmolPhoton m^{-2} s^{-1}, with 4.08 mg L^{-1} d^{-1}.

McClure et al. (2019) reported that the increase in light intensity (from 160 to 440 μmolPhoton m^{-2} 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 μmolPhoton m^{-2} s^{-1} (0.58 mg L^{-1} d^{-1}).

Dineshkumar et al. (2016) 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.

4.1.2. Light wavelengh

It has been proposed that different wavelengths can produce diverse impacts on the metabolism of microalgae (Zhao et al., 2019). 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 (Gatamaneni Loganathan et al., 2020). 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 (Kandilian et al., 2013).

Additionally, carotenoids exhibit photoprotective functions against wavelengths that can be particularly damaging to the photosynthetic system (Zarekarizi et al., 2023). 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 (Zarekarizi et al., 2023). However, there is evidence that this is not always the case. Atta et al. (2013) 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 μmolPhoton m^{-2} s^{-1}. Additionally, cultivation time was reduced by two days. In contrast, Fu et al. (2013) 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 μmolPhoton m^{-2} s^{-1} compared to red light alone at 128 μmolPhoton m^{-2} 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. (2019) found that lutein content in *Chlorella* sp. AE10 under high-intensity blue light (850 μmolPhoton m^{-2} s^{-1}, peak at 457 nm) was 1.63 times higher to that of white light. However, lutein productivity (4.44 mg L^{-1} 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. (2020) reported a 25% decrease in biomass yields using low-intensity blue light (40 μmolPhoton m^{-2} 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 (Xiao et al. Liu et al., 2018).

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 Zhao et al. (2019), 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 mg L^{-1} 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 (Zarekarizi et al., 2023). 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. (2018) 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 μmolPhoton m^{-2} s^{-1}). In a similar way, Salguero et al. (2005) reported that *Dunaliella bardawil* cultured under white light (100 μmolPhoton m^{-2} s^{-1}) plus UV-A (70 μmolPhoton m^{-2} 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 should focus on determining whether overall lutein productivity can be increased.

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 (Ramanna et al., 2017). 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 (Levasseur et al., 2022; Shareefdeen et al., 2023). Microalgae rely on photosynthesis to convert light energy into chemical energy, and the presence or absence of light profoundly influences this process (Zarekarizi et al., 2023). 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 (Levasseur et al., 2022). 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 (2017) and Zheng et al. (2022b) reported an increase in lutein concentration in *Chlorella vulgaris* and *Chlorella sorokiniana*, respectively, when reducing the hours of light in a 24-h photoperiod, however, the results are not comparable. (Zheng et al., 2022b) used a culture medium with corn starch, suggesting that their cultures were under mixotrophic conditions, while Gong and Bassi (2017) 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. (2021) cultured *Chlorella salina* under light/dark periods of 24 h:0 h, 16 h:8 h and 12 h:12 h. Although they report a 1.5-fold increase in productivity at the 16 h:8 h 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. (2021) 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, Schüler et al. (2022) 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 (2022) reports an increase of 39% on lutein content in a *Chlorella vulgaris* culture under flashing lights at 7000 μmolPhoton m^{-2} s^{-1} and a similar growth rate than the continuous light at 200 μmolPhoton m^{-2} s^{-1}. The author suggests that, among the three functions of lutein, including ensuring the folding of antenna proteins, transferring energy to chlorophyll *a*nd 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.

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 (Liu et al., 2022). 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 (Liu et al., 2022). 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 (Schüler et al., 2020). On the other hand, a sufficient supply of nitrogen causes high growth rates and biomass accumulation, thus obtaining higher carotenoid contents (McClure et al., 2019).

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 mg L^{-1} d^{-1} and 8.87 mg g^{-1} DW) were obtained at values established as optimal also for biomass growth (Fu et al., 2014). *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 (Ho et al., 2014). This was confirmed by Pozzobon et al. (2020), who cultured *Desmodesmus pleiomorphus* under moderate light (150 μmolPhoton m^{-2} 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 (Ho et al., 2014).

On the other hand, culture medium with high nitrogen ($NaNO_3$) content or medium renewal strategies increased lutein content and productivity in *Chlorella vulgaris* (2.44 and 4.21 fold, respectively) (McClure et al., 2019). 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 (Schüler et al., 2020). Similarly, Xie et al. (2017) 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 (Shi et al., 2020).

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 (Cirulis et al., 2013; Shi et al., 2020).

It has been observed in plants that lutein synthesis generally decreases under low temperatures (Esteban et al., 2015). 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 (Sánchez et al., 2008).

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 (Schüler et al., 2020), suggesting that lutein synthesis is linked to optimal culture conditions for biomass accumulation on this strain. In a similar way, Del Campo et al. (2000) 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. (2020a) in cultures with *C. sorokiniana*.

In contrast, Gong and Bassi (2017) 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. (2019) 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. (2020b) 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. (2008), 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. (2018) 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. (2021) 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. (2007) report only a 15% increase in lutein content of *Scenedesmus almeriensis* when increasing NaCl from 0 to 5 g L^{-1}. McClure et al. (2019) 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 (Del Campo et al., 2000; Fu et al., 2014). 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 (2019) 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 30*th* day of cultivation, significantly reducing productivity. Similarly, Blanco et al. (2007) 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. (2008), 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.

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)(Perez-Garcia and Bashan, 2015). 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 (Perez-Garcia and Bashan, 2015). 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 (Perez-Garcia and Bashan, 2015). According to a review article by Perez-Garcia and Bashan (2015), 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* (Jin et al., 2021) and *Scenedesmus acuminatus* (Jin et al., 2020) 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 (Perez-Garcia and Bashan, 2015; Hu et al. Liu et al., 2018; Do et al., 2022). 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 (Perez-Garcia and Bashan, 2015; Yun et al., 2021).

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 (Wu et al., 2009; Hu et al., 2018). 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 (Liu et al., 2018). 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 (Kamalanathan et al., 2017; Liu et al., 2018). 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 (Hu et al., 2018).

Since each regime (photo- and heterotrophic) 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 (Perez- Garcia and Bashan, 2015; Hu et al., 2018). 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 (Matsukawa et al., 2000). 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 (Cordero et al., 2011; Chen et al., 2017b, 2018; Ma et al., 2020a; Yun et al., 2021; Do et al., 2022; Van and Dinh, 2022; Vadrale et al., 2023). In phototrophic culture, Van and Dinh (2022) reported a maximum lutein productivity of 4.57 mg L^{-1} d^{-1}. Still, Chen et al. (2018) obtained 7.14 mg L^{-1} d^{-1} in heterotrophic culture while Ma et al. (2020a) under mixotrophic conditions obtained 4.79 mg L^{-1} d^{-1}. Later, Do et al. (2022) 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% CO_2 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 CO_2 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 (Shi et al., 1999) and nitrogen (Shi et al., 2000) 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. (2018) 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. (2022) 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. (2023) compared biomass and lutein production in the microalgae *Chlorococcum amblystomatis* 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. (2022) 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. (2021) 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 CO_2.

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. (2019) obtained a lutein content and lutein productivity of 7.27 mg g^{-1} and 7.34 mg L^{-1} 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. (2022b) 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}.

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 (Liyanaarachchi et al., 2021).

Using a feeding strategy, Wang et al. (2019) increased lutein productivity by 45% in a heterotrophic culture of *C. protothecoides* compared to a batch culture. Similarly, Chen et al. (2016) reported a lutein productivity of 5.67 mg L^{-1} 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. (2013) 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. (2019) reported a lutein productivity of 4.96 mg L^{-1} 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. (2019) 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. (2018) 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 mg L^{-1} d^{-1} was achieved under light and a nitrogen (glycine) enriched medium.

Chen et al. (2018) 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 mg L^{-1} d^{-1}) than the batch culture and 56% higher than the semi- and fed-batch strategies separately.

Similarly, Ma et al. (2020a) obtained better lutein productivity results (8.25 mg L^{-1} 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. (2019) 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 mg L^{-1} d^{-1}, one of the highest values reported.

On the same line, Flórez-Miranda et al. (2017) 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 mg L^{-1} 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. (2022) 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. (2012) 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 μmolPhoton m^{-2} 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. (2024) obtained a productivity of 11.68 mg L^{-1} d^{-1} in a two-stage culture of *Scenecesmus 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 (2004) 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 h 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 (1965) 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.

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 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. (2021) 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 (Xiao et al., 2018). 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 (Wu et al., 2009), 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 (Patel et al., 2022). Yildirim et al. (2017) 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.

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

Table 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 mg g^{-1} d^{-1} **Strain obtained by mutation of the wild type.

Species	Culture mode	Metabolic regime	# of stages	Lutein content (% DW)	Lutein productivity (mg L^{-1} d^{-1})	Ref.
Chamydomonas sp. JSC4	Batch	Phototrophic	One-stage	0.23	3.27	Ma et al. (2020b)
Chlamydomonas sp.	Batch	Phototrophic	Two-stage	0.42	3.25	Zhao et al. (2019)
Chlorella minutissima	Semi-continuous	Phototrophic	One-stage	0.8	5.35	Dineshkumar et al. (2016)
Chlorella sorokiniana TH01	Batch	Phototrophic	One-stage	0.98	4.57	Van and Dinh (2022)
Chlorella sp. AE10	Batch	Phototrophic	One-stage	0.95	4.44	Li et al. (2019)
Chlorella vulgaris UTEX266	Batch	Phototrophic	One-stage	0.79	11.98*	Gong and Bassi (2017)
Desmodesmus sp. F51	Batch	Phototrophic	One-stage	0.55	5.22	Xie et al. (2017)
Dunaliella salina	Batch	Phototrophic	One-stage	0.7	3.68	Fu et al. (2014)
Parachlorella sp. JD-076	Batch	Phototrophic	One-stage	1.18	25.03	Heo et al. (2018)
Scenedesmus almeriensis CCAP276/24	Continuos	Phototrophic	One-stage	0.54	4.77	Sánchez et al. (2008)
Scenedesmus obliquus FSP-3	Batch	Phototrophic	One-stage	0.48	4.08	Ho et al. (2014)
Scenedesmus sp. FSP3	Batch	Phototrophic	Two-stage	0.64	2.3	Li et al. (2022)
Teraselmis sp. CTP6	Batch	Phototrophic	One-stage	0.31	1.83	Schüler et al. (2020)
Chlorella protothecoides	Fed-batch	Heterotrophic	One-stage	0.91	10.57	Wang et al. (2019)
Chlorella sorokiniana MB-1	Semi-batch	Heterotrophic	Two-stage		5.67	Chen et al. (2016)
Chlorella sorokiniana MB-1-M12	Fed-batch	Heterotrophic	Two-stage	0.49	7.14	Chen et al. (2018)
Chromochloris zofingiensis	Fed-batch	Heterotrophic	Two-stage	0.13	19.68**	Chen et al. (2022)
Auxenochlorella protothecoides	Batch	Heterotrophic/ photoinduction	Two-stage	0.49	12.36	Xiao et al. (2018)
Scenedesmus incrassatulus	Batch	Heterotrophic/ photoinduction	Two-stage	0.14	3.1	Flórez-Miranda et al. (2017)
Scenedesmus obliquus	Batch	Heterotrophic/ photoinduction	Two-stage	0.15	6.5	Koh et al. (2022)
Scenedesmus almeriensis	Batch	Heterotrophic/ photoinduction	Two-stage	0.16	11.68	Camarena-Bernard et al. (2024)
Chlorella sorokiniana	Batch	Mixotrophic	One-stage	0.38	3.97	Chen et al. (2017a)
Chlorella sorokiniana	Batch	Mixotrophic	One-stage	0.58	2.39	Chen et al. (2017b)
Chlorella sorokiniana Kh12	Batch	Mixotrophic	One-stage	1.73	0.45	Vadrale et al. (2023)
Scenedesmus obliquus CWL-1	Fed-batch	Mixotrophic	One-stage	0.1	4.96	Chen et al. (2019)
Chlorella sorokiniana FZU60	Fed-batch	Mixotrophic/ photoinduction	Two-stage	0.95	11.57	Xie et al. (2019)
Chlorella sorokiniana FZU63	Fed-batch	Mixotrophic/ photoinduction	Two-stage	1.12	8.25	Ma et al. (2020a)
Chlorella sorokiniana C16	Batch	Mixotrophic	One-stage	1.74	9.04	Patel et al. (2023)

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, CO_2 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 (Leong and Chang, 2023). 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 (Kamalanathan et al., 2017, 2018; Yun et al., 2021), 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 (Saini and Keum, 2018), 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 (Gong and Bassi, 2017; Low et al., 2022). Similarly, exploring the utilization of less hazardous solvents with reduced ecological footprints presents a viable alternative for mitigating adverse environmental effects during the process (Ahmad et al., 2021). 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 (do Prado Silva et al., 2017; Dhas and Mehta, 2020); however, it is necessary to focus efforts on finding alternatives that can be industrially scalable.

Furthermore, integrating a biorefinery framework presents an opportunity in this context (Safi et al., 2014). 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 (Janssen et al., 2022), lipids and carbohydrates for biofuel generation (Hussain et al., 2021) and the formulation of biostimulants adapted to agricultural applications (Alvarez et al., 2021).

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 (Perez-Garcia and Bashan, 2015). 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 (Jacob-Lopes et al., 2020; Iwamoto et al., 2024). 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. (2013) coupled the production of lipids, carotenoid pigments and hydrogen from the processing of *Nannochloropsis* sp. biomass. Using supercritical CO_2 extraction and 20% ethanol the authors were able to extract 4.5 g_{lipids} g^{-1}DW 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. (2012) 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. (2021) 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 (Davis et al., 2011; Vázquez-Romero et al., 2022); 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.

6. Conclusions

The pursuit of optimal lutein production from microalgae necessitates a shift in focus towards 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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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