applied sciences

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Review

Marine Microalgae for Potential Lutein Production

Sushanta Kumar Saha *, Hande Ermis and Patrick Murray

Shannon Applied Biotechnology Centre, Limerick Institute of Technology, Moylish Park, V94 E8YF Limerick, Ireland; Hande.Ermis@lit.ie (H.E.); Patrick.Murray@lit.ie (P.M.)

* Correspondence: Sushanta.Saha@lit.ie; Tel.: +353-61-293-536

Received: 8 September 2020; Accepted: 13 September 2020; Published: 16 September 2020

Abstract: Lutein is particularly known to help maintain normal visual function by absorbing and attenuating the blue light that strikes the retina in our eyes. The effect of overexposure to blue light on our eyes due to the excessive use of electronic devices is becoming an issue of modern society due to insufficient dietary lutein consumption through our normal diet. There has, therefore, been an increasing demand for lutein-containing dietary supplements and also in the food industry for lutein supplementation in bakery products, infant formulas, dairy products, carbonated drinks, energy drinks, and juice concentrates. Although synthetic carotenoid dominates the market, there is a need for environmentally sustainable carotenoids including lutein production pathways to match increasing consumer demand for natural alternatives. Currently, marigold flowers are the predominant natural source of lutein. Microalgae can be a competitive sustainable alternative, which have higher growth rates and do not require arable land and/or a growth season. Currently, there is no commercial production of lutein from microalgae, even though astaxanthin and β-carotene are commercially produced from specific microalgal strains. This review discusses the potential microalgae strains for commercial lutein production, appropriate cultivation strategies, and the challenges associated with realising a commercial market share.

Keywords: commercial microalgae cultivation; dietary supplements; lutein production; marine microalgae

1. Introduction

Microalgae are photosynthetic microscopic organisms that possess several accessory light-harvesting carotenoid pigment molecules such as astaxanthin, canthaxanthin, lutein, zeaxanthin, and β-carotene, which have commercial value. Lutein is a xanthophyll and one of 1178 known naturally occurring carotenoids [1]. It is an oxygenated carotenoid found primarily in plants such as spinach, kale, and marigold as well as certain microalgal species such as *Scenedesmus almeriensis*, *Chlorella zofingiensis*, and *Muriellopsis* sp. [2]. Lutein is a lipid-soluble primary carotenoid that humans obtain from their diet and has several known health benefits including aiding in the prevention of macular degenerative disease, reducing the risk of stroke and heart attack, and mitigation against other debilitating metabolic syndromes. In photosynthetic species, xanthophylls act to modulate light energy and free radical quenching agents which are produced during photosynthesis under high light intensity. Lutein is found to accumulate in the macula of the eye, acting as a light filter protecting cells against free radical damage, and has also been implicated in ameliorating the damaging effects of macular degenerative disease in ageing adults [3]. These health-promoting effects of lutein as well as its potential as a natural food colourant have led to increased investigations on the potential of lutein as a high-value nutraceutical functional food ingredient. The growth of the global nutraceutical market is driven by an increase in demand for healthy and organic food products and a surge in awareness of dietary health promoting supplements. Furthermore, the rise in disposable income allows consumers

to purchase healthy alternatives to regular food products. The global lutein market is expected to reach at EUR 409 million by 2027 at a Compound Annual Growth Rate (CAGR) of 6.10% over the predicted period 2020–2027 [4].

Primarily, commercial natural lutein production has been reliant on extraction from marigold flower oleoresin. However, marigold flower harvesting and extraction is seasonal and labour intensive and recent data have suggested that microalgal species under controlled cultivation conditions can have much higher lutein productivity rates when compared to marigold cultivars [5]. There is, therefore, the strong potential for these organisms to be an alternative production route for natural lutein. Microalgae are attractive lutein producers; concomitantly, they function as a carbon dioxide capturing system reducing greenhouse gases, they can be cultivated all year round depending on the selected reactor cultivation conditions chosen, and environmentally friendly solvent-free extraction strategies can be tailored to enriched-lutein extraction e.g., supercritical fluid extraction methodologies [6].

The use of microalgae as human food is not unusual as it can be traced back many years in indigenous populations from China, Japan, and the Republic of Korea [7]. The traditional knowledge of microalgae use by these indigenous people has now disseminated throughout the world population through migration. Further, growth in microalgal consumption has been due to the significant amount of research on the health and nutritional benefits of microalgae and these health benefits are especially relevant for our modern-day lifestyle [8]. As a result, presently, several microalgae are commercially cultivated for various nutraceutical products that are available in the market. *Arthrospira* (*Spirulina*) *maxima*, *Arthrospira* (*Spirulina*) *platensis*, and *Aphanizomenon flos-aquae* are used as "whole cells biomass" in supplement products as well as for extraction of blue food colourant phycocyanin, *Chlorella vulgaris* biomass for health supplement products, and *Dunaliella salina* and *Haematococcus pluvialis* for commercial production of natural carotenoids such as β-carotene and astaxanthin [9].

2. Health Benefits of Lutein

Lutein, also known as the "eye vitamin", functions as a light protector by sheltering the eye tissues from harmful sunlight damage. The natural lens in the eye, which must remain clear, mainly collects and focuses light on the retina, where oxidation of this lens (clouding of the lens) is a major cause of cataracts. Antioxidants, such as lutein, neutralise free radicals that are connected with oxidative stress and retinal damage [10]. Because of its antioxidant potential, lutein is gaining popularity as a functional food ingredient to prevent age-related macular degeneration (AMD) [11]. Moreover, lutein has beneficial health effects other than eye health including anti-inflammatory, anti-atherogenic, antihypertensive, antidiabetic, antiulcer, and anticancer effects [12]. Lutein is one of the two main carotenoids found in human milk [13] and is very important for infant visual and cognitive development [14]. Lutein is one of the predominant carotenoids found in the new-born brain—about 66–77% of the total carotenoids [15]. These health-promoting effects of lutein (Figure 1) as well as its potential as a natural food colourant have led to increased investigations on the potential of lutein as a high-value nutraceutical functional food ingredient [16]. However, the health claim related to the cause and effect relationship between the consumption of lutein and maintenance of normal vision has not been approved by the European Food Safety Authority (EFSA) at the European level. From the scientific evidence, EFSA, however, agree that lutein consumption can increase macular pigment density in most of the healthy subjects (https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2012.2716); therefore, the challenge to prove the health benefits of lutein for the approval of health claims remains open.

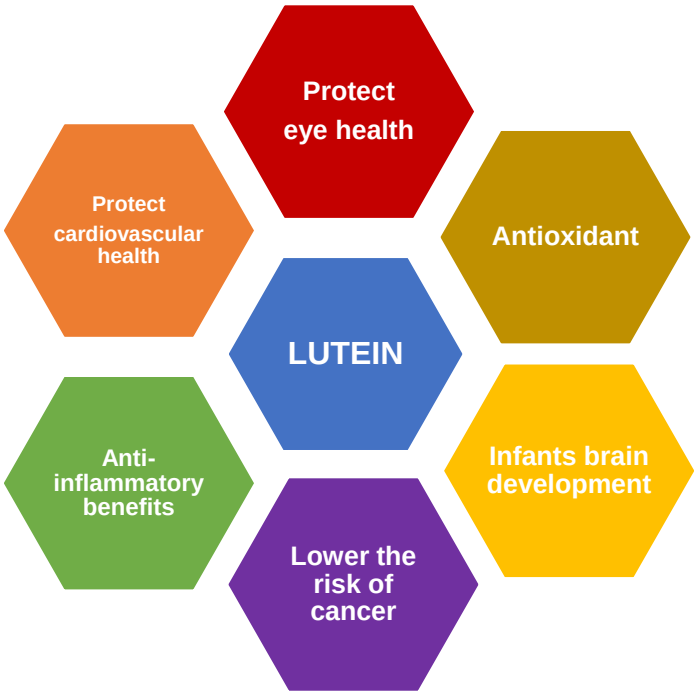

Figure 1. Schematic showing multiple potential health benefits of lutein.

3. Putative Biosynthetic Pathway of Lutein in Microalgae

Lutein is found within the members of Chlorophyta, Chlorarachniophyta, Cryptophyta, Euglenophyta, and Rhodophyta algal species [17]. Knowledge of the biosynthetic pathways for lutein biosynthesis in microalgae is limited. The current understanding is based on identified chemical structures of carotenoids found in microalgae. It is now believed that all types of carotenoids, including lutein, are obtained from common five-carbon (C_5) starting molecules isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These common metabolic precursors (IPP and DMAPP) might be derived from either of the two independent pathways: (i) the cytosolic mevalonate (MVA) pathway starting from Acetyl-CoA, or (ii) the plastidic (chloroplast in microalgae) methylerythritol 4-phosphate (MEP) pathway starting from pyruvate [18]. However, there is evidence to suggest that the precursors for microalgal carotenoids including lutein biosynthesis proceed from the MEP pathway in *Dunaliella salina*, *Chlorella vulgaris*, *Scenedesmus* sp. [18], and *Haematococcus pluvialis* [19] species.

Lutein biosynthesis in microalgae begins in the MEP pathway (Figure 2) through the 5-carbon building block molecule isopentenyl pyrophosphate (IPP), which isomerises to dimethylallyl pyrophosphate (DMAPP) by the action of the enzyme IPP isomerase. Elongation of the carbon chain then takes place through continuous head-to-tail condensation of IPP to DMAPP followed by growing of the polyprenyl pyrophosphate chain by the action of the enzyme prenyltransferase [20–22]. As a result, geranylgeranyl PP (GGPP, C_{20}), the first immediate precursor of lutein, is synthesised following a condensation reaction of GPP (C_{10}) by the action of GGPP synthase. Next, the colourless C_{40} carotenoid phytoene is formed through condensation of two GGPP (C_{20}) molecules by the action of phytoene synthase (PSY). Next, lycopene (a coloured carotenoid with 13 double bonds and a chromophore of 11 conjugated double bonds) is formed by the conversion of phytoene (nine double bonds molecule) through stepwise desaturation reactions (or dehydrogenation reactions) catalysed by phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) enzymes. Lycopene is the precursor to the formation of both α-carotene and β-carotene after two cyclisation reactions. The enzyme lycopene β-cyclase (*lcy-b*) catalyses the cyclisation of both ends of lycopene to make β-carotene with two β-rings. Meanwhile, the action of lycopene β-cyclase (*lcy-b*) and lycopene ε-cyclase (*lcy-e*) enzymes catalyse the cyclisation of both ends of lycopene to make α-carotene with a β-ring and an ε-ring. This is an important branching point of the carotenoid biosynthesis pathway in microalgae, where one branch

leads to the biosynthesis of α-carotene and the other branch leads to the biosynthesis of β-carotene. The former is then converted to lutein in two hydroxylation steps, while the latter is converted to zeaxanthin and subsequently, to other carotenoids. The hydroxylation of α-carotene at the C-3 and C-3′ positions results in the formation of lutein and the enzymes involved in these processes are β-carotene hydroxylase and ε-carotene hydroxylase, respectively [23].

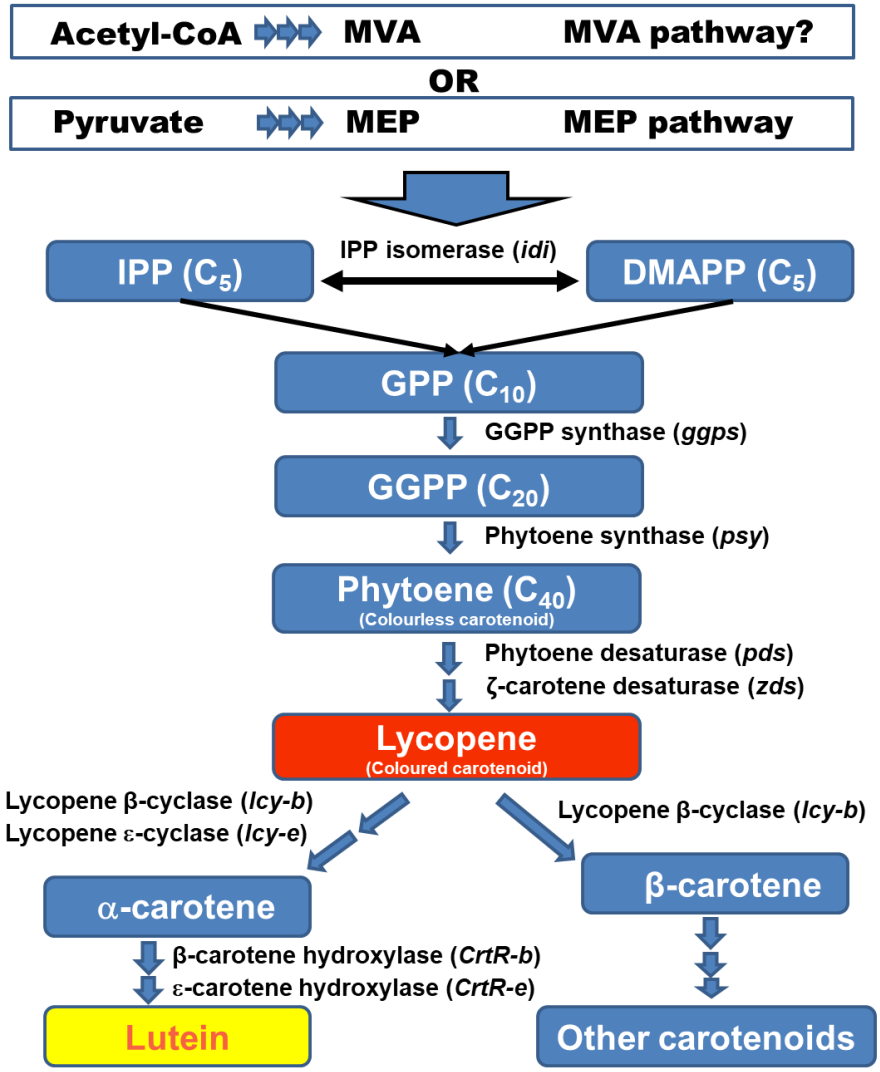

Figure 2. Schematic overview of lutein biosynthesis in microalgae. Enzymes involved in each biochemical conversion step are listed and their corresponding genes are indicated in parenthesis.

From the biotechnological perspective, a number of chemical inhibitors have been tested to regulate the carotenoid biosynthetic pathway in microalgae. For examples, Yildirim et al. [24] studied the effect of the addition of 2-methylimidazole in *Dunaliella salina* and found an increase of 1.7-fold lutein content and a related decline in β-carotene content. This study suggested that 2-methylimidazole preferentially alters lycopene β-cyclase (*lcy-b*) activity and thus, shifts the carotenogenic pathway from β-carotene to the α-carotene branch. Liang et al. [25] tested triethylamine, which triggered lycopene production in *Dunaliella bardawil* as a lycopene cyclase inhibitor that inhibited the expression levels of *lcy-b* and *lcy-e*, and upregulated the upstream carotenogenic genes. Likewise, nicotine was also tested as a possible lycopene cyclase inhibitor. A low concentration of nicotine resulted in a significant decrease in β-carotene, while triggering the accumulation of lycopene in *Chlorella regularis* Y-21 and *Dunaliella salina* CCAP 19/18 [26,27].

4. Engineering of Biosynthetic Pathways in Microalgae for Lutein Production

Microalgae as biomolecule production platforms have long been explored through engineering of their biosynthetic pathways by chemical mutagenesis as well as targeted genetic engineering of specific genes. Chemical mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and Ethyl Methane Sulfonate (EMS) have been successfully used for generating microalgal mutants with high contents of carotenoids including lutein [21,28–30]. In a recent study, a lutein-deficient *Chlorella vulgaris* (CvLD) strain was generated by chemical random mutagenesis and the strain was identified as an enhanced producer of violaxanthin [21]. Sequence analysis of the lycopene ε-cyclase gene (*lcy-e*) of the mutant strain CvLD revealed a single mutation (A336V), which might have resulted in a conformational change of the conserved region of CvLCYE (lycopene ε-cyclase) with reduced biochemical activity.

For genetic engineering of microalgae, robust genetic transformation protocols for the nuclear, chloroplast, or mitochondrial genomes are available [31]. The key methods for delivering DNA to microalgae are electroporation, shaking of cells with glass beads, and the biolistic (particle gun bombardment) method [32]. Most of the enzymes of secondary metabolism are encoded within the nuclear DNA; however, some of these enzymes are actually targeted to function in the chloroplast [33]. Since most of the enzymes of the carotenogenic pathway are found in the microalgal chloroplast, it was suggested that nuclear and/or chloroplast transformation can be used for the metabolic engineering of the carotenoid biosynthetic pathway [34]. Genetic engineering of microalgae through chloroplast transformation was mostly achieved by the biolistic method [35]. Although the low expression level of the target gene is the main drawback of nuclear transformation, it is the method of choice when appropriate post translational modifications of the target protein are essential [31].

The phytoene synthase gene (*psy*) from *Dunaliella salina* [28] and *Chlorella zofingiensis* [36] was nuclear transformed to the model microalga *Chlamydomonas reinhardtii*, and the derivative strains, respectively, produced 2.6- and 2.2-fold higher amounts of lutein compared to the wild type strain. Another study with the microalga *Chlamydomonas reinhardtii* involved a point mutation of the native gene for phytoene desaturase (*pds*) which was then nuclear transformed. The derivative strain, consisting of the mutant enzyme with increased desaturase activity biosynthesised, increased the amount of lutein, β-carotene, zeaxanthin, and violaxanthin [37]. In a very similar approach, Liu et al. [38] nuclear-transformed *Chlorella zofingiensis* with a mutant version of the native *pds* gene. The derivative strain, consisting of the mutant phytoene desaturase enzyme with increased desaturase activity, accumulated a higher amount of total carotenoids (32%) and astaxanthin (54%) compared to the parent strain. In a recent metabolic engineering study with *Chlamydomonas reinhardtii*, the gene for phytoene-β-carotene synthase (*pbs*) from the red yeast *Xanthophyllomyces dendrorhous* was cloned into pMS188 vector and nuclear transformed [39]. This derivative strain possesses the bifunctional enzyme with both phytoene synthase (*psy*) and lycopene cyclisation (*lcy-b*) activities. This is the first heterologous expression system for carotenoids biosynthesis, which resulted in a simultaneous increase in lutein (60%) and β-carotene (38%) under low light conditions.

In the lutein biosynthetic pathway, the lycopene ε-cyclase gene (*lcy-e*) acts as a key regulator of the α-branch, while lycopene β-cyclase (*lcy-b*) acts in both the α-branch and β-branch. The findings from chemical mutagenesis and genetic engineering studies suggest that an altered lycopene β-cyclase with enhanced activity in α-branch and reduced or no activity for β-branch, as well as an altered lycopene ε-cyclase with enhanced activity, might be a target for creating a genetically engineered strain with enhanced lutein productivity. Additionally, the cytosolic MVA pathway of microalgae can be genetically engineered to express a whole heterologous metabolic pathway for lutein biosynthesis. This approach was recently successfully demonstrated in tobacco (*Nicotiana tabacum* L.) plant, where a viral vector was used to express three enzymes (GGPP synthase (*crtE*), phytoene synthase (*crtB/psy*), and phytoene desaturase (*crtI*)) from the soil bacteria *Pantoea ananatis* to biosynthesise lycopene [40].

5. Synthetic Production of Lutein

Since the development of the chemical synthesis method for carotenoids production in 1950, β-carotene has long been chemically synthesised industrially for meeting global demand [41]. The chemically synthesised carotenoids list has been growing and includes astaxanthin, canthaxanthin, lycopene, zeaxanthin, and β-carotene [42,43]. The commercial production of lutein through the chemical synthesis method has not been viable due to poor overall yield. However, the technology news of the University of Maryland, USA reported that their researchers have published a chemical synthesis method for lutein with overall yields >20% from readily available precursor molecules [43,44]. At present, most of the commercially produced carotenoids are produced chemically and only a small portion are obtained from the biotechnological process, including extraction from microalgae, but not lutein by either method. Considering the fact that there is high demand and consumer preference for natural carotenoids, there is a huge opportunity for natural lutein production from microalgae.

6. Microalgae Cultivation for Commercial Lutein Production and Challenges

There are several microalgae species both from marine and freshwater habitats identified as potential lutein producers. Some of these species could produce up to 5 g of free lutein per kg biomass [16]. Microalgae are considered as important industrial candidates for lutein bioproduction. They have higher biomass productivity, high lutein content, are suitable for cultivation in freshwater, wastewater, brackish, or seawater, and/or are dependent neither on arable lands nor on local weather. However, the main challenge in carotenoid production from microalgae in general is low biomass growth rate due to the stress conditions necessary for carotenogenesis. Therefore, large-scale microalgal cultivation mostly operates in two steps, which are the cultivation of microalgae in optimum conditions for fast growth in complete growth medium followed by stress conditions to improve the desired carotenoid in microalgae [45]. There are many strategies to improve microalgal lutein content such as different light intensities, light colour, growth conditions, and nutrient limitations. In the study by Ho et al. [46], *Scenedesmus obliquus* FSP-3 was subjected to light-related strategies to increase cell growth and lutein production, where the best lutein productivity (4.08 mg L^{-1} d^{-1}) was observed at a light intensity of 300 µmol photons m^{-2} s^{-1} with white light. In another study by Ho et al. [47], the effects of nitrogen sources on the cell growth and lutein content of *Scenedesmus obliquus* FSP-3 were examined, where the highest lutein content (4.61 mg g^{-1}) and lutein productivity (4.35 mg L^{-1} d^{-1}) were obtained when using 8.0 mM calcium nitrate as the nitrogen source. After determining the nitrogen source condition, Ho et al. [47] applied two bioreactor strategies which were semi-continuous and two-stage; and observed that semi-continuous operation with a 10% medium replacement ratio achieved the highest biomass productivity (1304.8 mg L^{-1} d^{-1}) and lutein productivity (6.01 mg L^{-1} d^{-1}).

Zhao et al. [48] examined the marine microalga *Chlamydomonas* sp. JSC4 under different environmental conditions for lutein production and concluded that the optimal lutein content was obtained under blue light and a lower temperature of 20–25 °C. Schüler et al. [49] also examined different environmental factors on marine microalga *Tetraselmis* sp. CTP4 for carotenoid biosynthesis and examined that lutein amount increased 1.5-fold under higher light intensities (170 and 280 µmol photons m^{-2} s^{-1}), but in contrast to Zhao et al. [48], found a temperature increase from 20 to 35 °C, after only two days at a light intensity of 170 µmol photons m^{-2} s^{-1}, yielded the highest lutein productivity (3.17 ± 0.18 mg g^{-1} dry weight biomass). Ma et al. [50] also examined light stress on the marine microalga *Chlamydomonas* sp. JSC4 as a potential lutein production. High lutein productivity (5.08 mg $L^{-1}d^{-1}$) was attained under high light irradiation of 625 µmol photons m^{-2} s^{-1}, where lutein amount started to decrease at 750 µmol photons m^{-2} s^{-1} due to downregulation of *lut1* and *zep* genes, which respectively encode the enzymes ε-carotene hydroxylase and zeaxanthin epoxidase that are responsible for lutein biosynthesis.

Chen et al. [51] also examined light-related carotenogenesis strategies along with different nitrogen concentrations and growth conditions on *Scenedesmus obliquus* CWL-1 under mixotrophic cultivation. In contrast to Ho et al. [46], maximum lutein yield (1.43 mg L^{-1} d^{-1}) was achieved in 12 h/12 h light/dark

conditions under blue/red light. In addition to the light strategy, the addition of 4.5 g L^{-1} of calcium nitrate increased lutein productivity (3.06 mg L^{-1} d^{-1}), while the addition of 1.5 g L^{-1} of calcium nitrate increased the lutein content to 2.45 mg g^{-1}. Finally, compared to a batch cultivation system, a fed-batch cultivation strategy had 11-fold higher lutein productivity (4.96 mg L^{-1} d^{-1}), which was the highest productivity that was achieved compared to all strategies.

In the study by Florez-Miranda et al. [52], a two-stage cultivation strategy was applied, where heterotrophy with different temperatures was followed by photoinduction to improve biomass and lutein production in *Scenedesmus incrassatulus*. In the initial stage, where different nitrogen sources and temperatures were applied, the highest lutein content was observed using urea plus vitamins at 30 °C; during the second stage, after 24 h of photoinduction, lutein content increased seven times. According to the comparison between autotrophic and heterotrophic growth, Florez-Miranda et al. [52] concluded that lutein productivity was 1.6 times higher compared to autotrophic cultivation. Hence, heterotrophic and/or mixotrophic growth for lutein production can be suggested as a better cultivation method compared to autotrophic growth. Xie et al. [53] observed different types of media and concentrations of sodium acetate and nitrate on *Chlorella sorokiniana* FZU60 to improve mixotrophic growth and lutein production, where highest lutein content (9.57 mg g^{-1}) and productivity (11.57 mg L^{-1} d^{-1}) were obtained in BG-11 medium supplemented with 1 g L^{-1} acetate and 0.75 g L^{-1} nitrate. Moreover, pulse feeding with 1 g L^{-1} acetate every 48 h led to the alternation between mixotrophy and photoinduction, which resulted in a lutein production of 33.6 mg L^{-1}.

In the study by Shi et al. [54], *Chlorella protothecoides* was cultivated heterotrophically (40 g L^{-1} glucose and 3.6 g L^{-1} urea) with nitrogen limitation in a fed-batch culture, which was followed by an increased lutein production without drastically reducing the biomass amount. Afterwards, this N-limited fed-batch culture was successfully scaled up from 3.7 to 30 L, and temperature stress was applied. Higher temperature (32 °C) for 84 h resulted in a 19.9% increase in lutein content but a 13.6% decrease in biomass amount, as compared to the fed-batch culture (30 L) without any treatment.

In the study by Shinde et al. [55], the lutein content of *Auxenochlorella protothecoides* SAG 211-7a was observed under heterotrophic conditions, where the Taguchi Orthogonal Array method, a statistical technique for screening and optimisation of medium components at small-scale, was applied to select the six independent variables (yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, EDTA, and pH) that affect lutein production. Sucrose, yeast extract, $MgSO_4.7H_2O$, and EDTA were selected as the significant factors affecting lutein production. According to the experimental results, 1303 ± 25.32 μg L^{-1} lutein was produced when the medium was supplemented with 14 g L^{-1} sucrose, 3 g L^{-1} yeast extract, 0.8 g L^{-1} $MgSO_4.7H_2O$, and 0.76 g L^{-1} EDTA.

In conclusion, compared to all strategies mentioned above, the best approach for a microalgae cultivation system for maximum lutein productivity is a fed-batch cultivation strategy with different light and temperature stress depending on the selected freshwater culture and salinity for marine culture, since the maximum level of lutein in the cell is dependent on the salinity [56].

There is no commercial production system for lutein from microalgae yet; however, some outdoor productions of *Muriellopsis* sp. and *Scenedesmus* sp. at a pilot scale have been created, where *Muriellopsis* sp. was cultivated in a 55 L tubular photobioreactor and the highest lutein productivity was 40 g m^{-2} d^{-1}. Moreover, *Scenedesmus almeriensis* was also cultivated in a 4000 L tubular photobioreactor for lutein production and 290 mg lutein m^{-2} d^{-1} was observed [57]. Besides lutein, the main commercial carotenoid productions are β-carotene from *Dunaliella* sp. and astaxanthin from *Haematococcus* sp. Commercially, β-carotene from *Dunaliella* sp was extracted by Betatene (Australia) and Western Biotechnology (Australia) in 400 and 240 ha extensive unmixed ponds, where 13–14 and 4–5 tonnes of β-carotene per year have been extracted, respectively. For commercial astaxanthin extraction, Cyanotech (Hawaii) uses a closed tubular photobioreactor followed by open ponds, whereas Algatechnologies (Israel) applies a closed tubular photobioreactor for *Haematococcus* sp. cultivation [57]. In conclusion, a tubular photobioreactor can be suggested for commercial lutein production as was experimentally

tested for *Muriellopsis* and *Scenedesmus* species, and also due to the fact that tubular photobioreactors have been successfully used for other carotenoid-producing microalgae.

7. Extraction of Lutein from Microalgae and Challenges

The extraction of intracellular microalgae products is most commonly conducted by conventional means. This involves using dry biomass coupled with maceration and thermal extraction employing either organic or aqueous solvents (Table 1), depending on the polarity of the desired compound to be extracted. Compounds, such as carotenoids, display varying polarities and chemical natures. Hence, an appropriate solvent must be chosen with regards to extracting target carotenoids based on the selectivity, efficiency, and purity required. The extraction of carotenoids is typically conducted using non-polar solvents such as n-hexane, dichloromethane, and dimethyl ether, with other solvents such as acetone and octane also used for extraction [58].

The use of these non-polar solvents is due to the high hydrophobicity of carotenoids. Recently, green solvents such as ethanol and biphasic water solvent mixtures have also been researched for the extraction of carotenoids from microalgae [59]. The conventional extraction process of microalgae products still bears limitations such as extraction efficiency, selectivity, and high solvent consumption. Lin et al. [5] discussed that the energy consumption for microalgal cell disruption ranged from 30 to 500 MJ kg^{-1}. This energy consumption was reliant on the disruption process, where the energy demand is determined by factors such as cell wall compositions, cell wall thickness, and cell size. This value is 1000 times higher than the crushing energy for marigold flower (800 kJ kg^{-1}). To overcome this problem, the development and application of a multistage extraction process, combined with varying physical and chemical methods, may prove useful in selectively targeting the carotenoids of interest [58]. Microalgal biomass consists of a rich cellular composition and much thicker cell wall than marigold flowers. Dry-milled marigold petals are usually processed using solvent extraction to produce oleoresin-containing carotenoids in their ester form. This is followed by a multistep purification process which frees the hydroxylated carotenoids from the accompanying fatty acids and finally, a recrystallisation process occurs which results in pure lutein/zeaxanthin. Lutein is sold in oily extracts ranging from 5% to 60% as its crystalline form poses management and stability problems, where microalgal biomass can be extracted into an oleoresin-like extract with 25% lutein in free form that could be used directly for the commercial products [60].

Araya et al. [61] applied two cell disruption methods, glass bead vortexing and ball mill grinding, on *Chlorella vulgaris*, *Chlorella zofingiensis*, and *Chlorella protothecoides* to improve the extraction yield of lutein, where the yield of *C. vulgaris* (0.51 mg L^{-1} d^{-1}) and *C. zofingiensis* (0.53 mg L^{-1} d^{-1}) was higher compared to *C. protothecoides* (0.37 mg L^{-1} d^{-1}). Chen et al. [62] also applied two different cell disruption methods (bead-beating and high pressure) on *Chlorella sorokiniana* MB-1 and the lutein was extracted by a reduced pressure extraction method. High-pressure pretreatment extracted with tetrahydrofuran (THF) as the solvent resulted in high lutein recovery efficiencies of 87.0% (at 20 min incubation) and 99.5% (at 40 min incubation) at 850 mbar pressure and at temperature 25 °C.

Solvents such as hexane and/or ethanol are the most commonly employed methods for lutein extraction due to the easy removal of the solvent from the extract while also retrieving a high content of lutein. In contrast, direct extraction with vegetable oil was described by Nonomura [63] and does not allow for solvent removal. This patent involves direct extraction on wet biomass using the addition of vegetable oil, followed by emulsification and a resting period. However, in regard to microalgae with thick cell walls, such as *Murielopsis* sp. or *Scenedesmus* sp., it is unlikely this methodology would be efficient.

Low et al. [64] described a microwave-assisted binary phase solvent extraction method (MABS) for the recovery of lutein from microalgae. The method was established and optimised to specifically attain the highest lutein recovery from microalgae *Scenedesmus* sp. biomass, with a total of 11.92 mg g^{-1} lutein recovered. The optimal binary phase solvent composition was a 60% potassium hydroxide solution with acetone in the ratio of 0.1 (mL/mL). The highest lutein content was at 55 °C treatment temperature,

36 min extraction time, 0.7 (mg mL^{-1}) biomass:solvent ratio, 250 Watt microwave power, and 250 rpm stirring speed. This optimised novel procedure can increase lutein recovery by approximately 130%, along with also shortening the overall extraction time by 3-fold.

According to an optimised extraction method of lutein from microalgal biomass conducted by Ceron et al. [65], it was concluded that cell disruption was necessary and the use of a bead mill with alumina in a 1:1 (*w*/*w*) proportion as a disintegrating agent for 5 min was the preferred method amongst other varying treatments tested.

Studies conducted by Gong et al. [66] investigated lutein extraction from wet *Chlorella vulgaris* UTEX 265, where various extraction parameters such as sample size, drying method, and cell disruption method were studied and lutein production was monitored throughout the microalgal growth phase. From the analysis of varying solvent performances on lutein extraction using Nile Red as a solvatochromic polarity probe, it was determined that 3:1 (*v*/*v*) ethanol/hexane was the optimal solvent for lutein extraction, which resulted in 13.03 mg g^{-1} lutein.

Studies carried out by Li et al. [67] involved the use of high-speed counter-current chromatography (HSCCC) for the isolation and purification of lutein from microalgae. Analytical HSCCC was used for the preliminary selection of a suitable solvent system composed of n-hexane:ethanol:water (4:3:1, *v*/*v*), where HSCCC was successfully performed, resulting in lutein yield at 98% purity from 200 mg of crude extract in a one-step separation.

The use of supercritical CO_2 fluid is gaining increasing interest in regard to the recovery of pharmaceutical or nutraceutical compounds due to clean extracts and lack of toxicity of CO_2 in comparison to organic solvents. However, because the polarity of free lutein is high and excess amounts of organic solvents are required for lutein extraction from microalgae due to binding of light-harvesting complexes, supercritical fluid extraction (SFE) should not prove efficient. However, according to Yen et al. [68], the addition of polar co-solvents such as methanol or ethanol may increase lutein extraction efficiency. When coupled with a co-solvent, SFE may seem a promising lutein extraction method compared to organic solvent extraction. Miguel et al. [69] recommended the use of supercritical CO_2 following a solvent extraction of the carotenoids. The solvent containing the carotenoids was then mixed with supercritical CO_2 and the conditions of pressure and temperature were adjusted to promote the precipitation of lutein.

Macías-Sánchez et al. [60] aimed to clarify the influence of temperature and pressure parameters on the supercritical fluid extraction of lutein and β-carotene from a freeze-dried powder of the marine microalga *Scenedesmus almeriensis*. The extracts were analysed by HPLC and empirical correlations were also established. The results determined an optimal pressure of 400 bar and optimal temperature of 60 °C resulted in a significant yield of pigment extraction. In comparison with the referenced extraction processes used, the results obtained from this study displayed that improved yields were obtained in the extraction of β-carotene, where it was possible to extract 50% of the total of this pigment contained in the microalga studied.

Di Sanzo et al. [70] also investigated the use of supercritical fluid extraction using CO_2 for the extraction of astaxanthin and lutein from disrupted red phase biomass of the *Haematococcus pluvialis*. A bench-scale reactor in a semi-batch configuration was employed. Parameters such as extraction time (20, 40, 60, 80, and 120 min), CO_2 flow rate (3.62 and 14.48 g min^{-1}) temperature (50, 65, and 80 °C), and pressure (100, 400, and 550 bar.) were investigated. The results indicate the maximum recovery of astaxanthin and lutein obtained was 98.6% and 52.3%, respectively, at 50 °C and 550 bars.

Mehariya et al. [71] employed supercritical fluid for the extraction of lutein from *Scenedesmus almeriensis*. The optimisation of the main parameters affecting the extraction, such as biomass pretreatment, temperature, pressure, and CO_2 flow rate, was conducted. Firstly, the effect of mechanical pretreatment with diatomaceous earth (DE) and biomass mixing in the range of 0.25–1 DE/biomass, grinding speed varying between 0–600 rpm, and pretreatment time changing from 2.5 to 10 min, was evaluated on lutein extraction efficiency. Next, the influence of SFE extraction parameters such as pressure (250–550 bar), temperature (50 and 65 °C), and CO_2 flow rate (7.24 and 14.48 g min^{-1}) on

lutein recovery and purity was examined. According to the results, increases in the temperature, pressure, and CO_2 flow rate improved the lutein recovery and purity. The maximum lutein recovery (~98%) with a purity of ~34% was accomplished running at 65 °C and 550 bar with CO_2 flow rate of 14.48 g min^{-1}.

Research conducted by Yen et al. [68] in regard to utilising SFE for the extraction of lutein from *Scenedesmus* sp. biomass found that an increase in both pressure and temperature resulted in an increase in lutein yield, but an increase in temperature resulted in increased impurity within the HPLC profile. This increase in yield was not as significant when compared to the yields from conventional extraction methods. Optimum parameters for lutein recovery yield were determined as 400 bar pressure and temperature at 70 °C while using ethanol as the co-solvent at a ratio of 30 mol%. These parameters resulted in lutein recovery of 76.7%, which was compared to conventional methanol extraction methods.

Research carried out by Ruen-ngam et al. [72] involved the use of a pretreatment process using alcohol aimed at removing chlorophyll *a*, *b*, and β-carotene from *Chlorella vulgaris*. This process was developed to enhance the yield and selectivity of lutein in the extract obtained by subsequent supercritical fluid extraction. SFE was carried out in the pressure range 200 to 400 bar and the temperature range of 40 to 80 °C, with methanol and ethanol trialled as the co-solvents, with ethanol the most suitable for lutein extraction. Lutein yield within the extract increased with pressure but decreased with temperature. The optimal parameters for lutein recovery yield were determined as 400 bar and 40 °C, using ethanol as the co-solvent. Under these conditions, the maximum recovery percentage and selectivity percentage of lutein resulted in approximately 52.9 ± 0.02% and 43.1 ± 0.02%, respectively.

Fan et al. [23] aimed to extract lutein from *Chlorella pyrenoidosa* using ultrasound-enhanced subcritical CO_2 extraction (USCCE). As part of this research, parameters such as pretreatment process, pressure, temperature, CO_2 flow rate, and ultrasonic power were examined, alongside orthogonal analysis, to study the effects of varying parameters on lutein extraction yields. The developed USCCE method was conducted as part of this study and it was compared to other common extraction methods. Optimal extraction conditions were determined as temperature at 27 °C, pressure at 210 bar, 1.5 mL g^{-1} ethanol, and ultrasound power at 1000 W. Under these conditions, a maximum extraction yield of 1.24 mg lutein g^{-1} crude material was obtained and when compared to other methods, it was found that USCCE could result in increasing lutein extraction yield significantly at much lower extraction pressure and temperature.

Wu et al. [73] also applied supercritical fluid extraction followed by HPLC and LCMS analysis to lutein extraction from *Chlorella pyrenoidosa.* Under optimum conditions, temperature at 50 °C, pressure at 250 bar, and 50% ethanol as the co-solvent, the extraction yield recovery of lutein was 87.0%. From the results, it was concluded that SFE yielded in high purity lutein recovery and the process developed during this study might be suitable for the commercial production of lutein.

In conclusion, in regard to the large-scale production of lutein, only solvent extraction has, to date, achieved high degrees of efficiency and purity. However, new advances in methods and techniques such as selective precipitation with supercritical CO_2 and new advantageous solvents, such as ethyl lactate, which have been proposed for the extraction of other plant matter [74], may also be applied to microalgae and prove beneficial.

Table 1. List of lutein-producing microalgae and their lutein yield associated with tested cultivation and stress conditions.

Microalgae	Biomass	Cultivation Conditions	Lutein Yield	Stress Conditions	Extraction Methodologies	References
Marine cultures						
Chlamydomonas sp. JSC4	1271 mg L^{-1} d^{-1}	1-L glass photobioreactor	3.27 mg L^{-1} d^{-1}	Temperature (35 °C)	Solvent extraction	[75]
Chlamydomonas sp.	1500 mg L^{-1} d^{-1}	1-L glass photobioreactor	5.08 mg L^{-1} d^{-1}	Light intensity (625 µmol photons m^{-2} s^{-1})	Solvent extraction	[50]
Chlamydomonas sp. JSC4	560 mg L^{-1} d^{-1}	1-L glass photobioreactor	3.42 mg g^{-1}	Salinity gradient	Solvent extraction	[76]
Chlamydomonas sp. JSC4	490 mg L^{-1} d^{-1}	1-L glass photobioreactor	2.95 mg g^{-1}	Light wavelengths (blue light)	Solvent extraction	[50]
Chlamydomonas acidophila	-	Batch growth	20 mg L^{-1}	UV-A radiation (10 µmol photons m^{-2} s^{-1}), or heated at 40 °C.	Solvent extraction	[77]
Chlorella salina	-	3-L glass flask	2.92 mg g^{-1}	-	Microextraction coupled with ultrasonication	[78]
Dunaliella salina	2.2 g m^{-2} d^{-1}	Tubular photobioreactor	15.4 mg m^{-2} d^{-1}	None	Solvent extraction	[79]
Muriellopsis sp.	40 g m^{-2} d^{-1}	Outdoor tubular photobioreactor	6 mg g^{-1}	None	Solvent extraction	[80]
Muriellopsis sp.	12.9 g m^{-2} d^{-1}	Open ponds	100 mg m^{-2} d^{-1}	None	Solvent extraction	[57]
Tetraselmis sp. CTP4	-	5-L reactors	3.17 mg g^{-1}	Light intensity (170 and 280 µmol photons m^{-2} s^{-1}) and temperature (35 °C)	Solvent extraction	[49]
Freshwater cultures						
Chlorella minutissima	0.117 g L^{-1} d^{-1}	2-L airlift photobioreactor	5.58 mg g^{-1}	None	Solvent extraction	[81]
C. vulgaris, *C. zofingiensis* and *C. protothecoides*	0.131, 0.122, 0.103 g L^{-1} d^{-1} (respectively)	In indoor vertical alveolar panel photobioreactor	3.86, 4.38 and 3.59 mg g^{-1} (respectively)	None	Glass bead vortexing and ball mill grinding	[61]
Chlorella protothecoides	31.2 g L^{-1}	Heterotrophic growth in a 3.7-L fermenter	1.90 mg g^{-1}	80 g L^{-1} glucose addition	Solvent extraction	[82]

Table 1. *Cont.*

Microalgae	Biomass	Cultivation Conditions	Lutein Yield	Stress Conditions	Extraction Methodologies	References
			Freshwater cultures			
Chlorella pyrenoidosa	-	-	1.24 mg g^{-1}	None	Ultrasound-enhanced subcritical CO_2 extraction	[23]
Chlorella sorokiniana	1.98 g L^{-1} d^{-1}	Two-stage mixotrophic cultivation	7.62 mg L^{-1} d^{-1}	None	Solvent extraction	[83]
Chlorella vulgaris	-	Batch	3.36 mg g^{-1}	None	Ultrasound extraction with enzymatic pretreatment	[84]
Chlorella sorokiniana	2.4 g L^{-1}	Semi-batch mixotrophic cultivation.	5.21 mg g^{-1}	None	Reduced pressure extraction method.	[85]
Chlorella protothecoides	28.4 g L^{-1}	Heterotrophic batch growth in a 3.7-L fermenter	0.27 mg g^{-1}	Nitrogen limitation and high temperature	Mechanical method	[54]
Chlorella zofingiensis	7 g L^{-1}	Batch growth	4 mg g^{-1}	None	Solvent extraction	[86]
Desmodesmus sp.	939 mg L^{-1} d^{-1}	1-L glass vessel	5.22 mg L^{-1} d^{-1}	Different C/N ratios (1:1 and 150 mg L^{-1})	Solvent extraction	[87]
Muriellopsis sp.	5.37 g L^{-1}	Batch growth	29.8 mg L^{-1}	None	Solvent extraction	[88]
Scenedesmus incrassatulus	17.98 g L^{-1}	Two-stage heterotrophy photoinduction culture	1.49 mg g^{-1}	Glucose concentration increase (30.3 g L^{-1})	Solvent extraction	[52]
Scenedesmus sp. CCNM 1028	0.47 g L^{-1}	Batch growth (1L)	2.12 mg g^{-1}	Two-stage nitrogen starvation	Solvent extraction	[89]
Scenedesmus obliquus CWL-1	9.88 g L^{-1}	Mixotrophic cultivation	1.78 mg g^{-1}	Light-related strategies (12/12 L/D, blue to red light)	Solvent extraction	[51]
Scenedesmus almeriensis	0.95 g L^{-1}	Vertical bubble column photo-bioreactor	8.54 mg g^{-1}	Different CO_2 Content (3.0% *v*/*v*)	Accelerated solvent extraction	[90]
Scenedesmus sp.	1.1 g L^{-1}	20 L photobioreactor	1.794 mg g^{-1}	Different pressure and temperature in the SFE operation (400 bar, 70 °C and ethanol as the co-solvent)	Supercritical CO_2 extraction	[68]
Scenedesmus almeriensis	0.63 g L^{-1}	Bubble column photobioreactors (2.0 L)	3.6 mg L^{-1}	Salinity (5 g L^{-1})	Solvent extraction	[91]
Scenedesmus obliquus	2.44 g L^{-1}	1-L glass vessel	3.63 mg g^{-1}	Light-related strategies	Solvent extraction	[46]

8. Current Market Demand, Value and Sources

Currently, the global lutein market is valued at EUR 255 million and is expected to reach EUR 409 million by 2027 [4]. Lutein appears as a slightly lower value carotenoid compared to the market price for astaxanthin. As per ICIS market research, the price for 100% pure lutein may range from EUR 1688.00 to EUR 2532.00 per kg, while the available products with dry forms of lutein may range from EUR 126.00 to EUR 253.00 per kg, and the products with the liquid forms of lutein may range from EUR 422.00 to EUR 590.00 per kg (https://www.icis.com/explore/resources/news/2003/05/16/195956/lutein-eyes-robust-growth-in-food-and-nutraceuticals/ accessed on 12 September 2020). The growth of the global lutein market is driven by an increase in demand for healthy and organic food products and a surge in awareness towards dietary supplements. Furthermore, the rise in disposable income allows consumers to purchase healthy alternatives to regular food products.

Asia Pacific is projected to account for 23.2% of the global market. Developing nations such as China and India are expected to observe high growth. High occurrence of eye disorders coupled with the growing demand for dietary supplements is expected to accelerate the need [92]. Dietary supplements held 29% of the overall market share in 2019 and are expected to keep their dominance over the forecast period. Europe held 36.2% of the industry in 2018 and is projected to grow significantly in the coming years [92].

9. Overall Discussion and Future Prospects

Although marigold meets the global demand for lutein to some extent, there is still a huge opportunity to contribute to the global demand for natural lutein. This is where microalgae can play a significant role because there are several microalgae that produce 0.5–1.2% lutein of their cell dry weight [5]. Moreover, microalgae have more free lutein than marigold, which is preferable since it is easily absorbed compared to the esterified forms found in marigold flowers. In addition, there are several microalgal commercial technologies for cultivation, and optimum extraction of carotenoids is available. Importantly, microalgae are considered as one of the most promising biofactories, with 5–10 times higher growth rate than land plants, high-potential CO_2 scavenging, and their commercial cultivation technologies can be based on all types of water sources such as freshwater, brackish water, and seawater, and do not require arable land. Microalgae as commercial lutein producers are still waiting for the involvement of enthusiast entrepreneur biotechnologists who would be willing to adopt existing microalgal cultivation technologies. It is good that there are already a number of marine as well as freshwater microalgal strains identified as lutein producers through various research studies (Table 1), which can serve as a starting point.

The commercially optimum method and the safety of chemically synthesised lutein for human consumption are still questionable; therefore, the natural lutein market is gaining interest. Even though there is no commercial production of lutein, microalgae are an attractive source for the mass production of lutein due to their high growth rates and high pigment content. However; there are some challenges to be focused on that are mostly related to the cost [93]. Particularly, these are due to the current market price for lutein as well as the lutein yield in known microalgae being lower compared to the market price and yield of astaxanthin, which is currently produced economically and reliably from *Haematococcus pluvialis*. Although heterotrophic cultivation improves cell growth and lutein content, the cost of glucose and other carbon sources prevent microalgal lutein production from being commercially profitable [94]. Not only the carbon addition cost, but also the downstream processes such as harvesting and drying increase the cost, where energy saving approaches are essential for both processes. Moreover, to improve the growth capacity of the selected culture, high efficiency photobioreactors should be designed, where optimal growth conditions such as temperature or light can be applied. Based on the current cultivation technologies for *Dunaliella* sp. and *Haematococcus* sp. that were also found suitable at experimental-scale for *Muriellopsis* sp. and *Scenedesmus* sp., it appears that tubular photobioreactors can also be the choice for lutein production from these microalgae.

In addition, advanced metabolic engineering approaches may be applied to improve the lutein synthesis pathways in microalgae and increase its cellular accumulation to be commercially competitive. In summary, microalgal lutein has a great potential to be commercially produced with some challenges mentioned above to be overcome. In the future, better engineering reactor designs should be created for higher culture growth, new innovations should be applied for low-cost harvesting/drying processes, and selected cultures should be metabolically engineered (by chemical mutagenesis or targeted genetic engineering) to produce higher lutein without decreasing the culture biomass yield.

Overall, considering the growing demand for natural lutein, available potential microalgal strains, and their cultivation technologies available, it is high time to initiate industrial involvement along with some research and development activities for microalgal lutein production, along with other value-added bioproducts, using a biorefinery approach. The approach would at least need to achieve, through the rapid cultivation of selected microalgal strains by tweaking their abiotic growth factors to enhance lutein content followed by simple harvesting of biomass, extraction of lutein at affordable costs and valorisation of lutein-extracted biomass for additional bioproducts development.

Author Contributions: Conceptualisation, S.K.S. and P.M.; methodology, S.K.S. and H.E.; formal analysis, S.K.S. and H.E.; investigation, S.K.S. and H.E.; writing—original draft preparation, S.K.S., H.E., and P.M.; writing—review and editing, S.K.S. and P.M. All authors have read and agreed to the published version of the manuscript.

Funding: Recent literature search and analysis were carried out in conjunction with one of the ongoing funded project (19/KGS/006) on the "Development of commercialisation pipeline of Microalgal bioFACTORIES starting from biodiscovery screening (M-FACTORIES)".

Acknowledgments: All authors acknowledge the APC waiver for contributing to this invited review article.

Conflicts of Interest: The authors declare no conflict of interest.

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