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Enhancement Techniques for Microalgal Carotenoids: A Review

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Abstract
Carotenoids are a diverse group of naturally occurring pigments having significant biological and industrial importance. However, the commercial supply of these pigments still remains dominated by synthetic forms that lack the bioefficacy of natural counterparts. Microalgae have emerged as promising and a sustainable source of natural carotenoids. Their rapid growth, photosynthetic efficiency, and adaptability to variable environments makes them more beneficial to culture and extract carotenoids. This review highlights the recent advances in the microalgal carotenoid production. It focuses on the cultivation systems, stress-induced biosynthesis, and genetic engineering strategies to enhance the yield and quality of the carotenoids. Open pond and photobioreactor (PBR) systems are the two principal cultivation methods, each with distinct advantages and limitations regarding cost, scalability, and contamination control. Hybrid systems integrating both approaches show superior productivity and improved stability. Carotenoid accumulation in microalgae is influenced by several abiotic and biotic factors, including light intensity, temperature, nutrient availability, metal ions, and oxidative stress. Furthermore, two-stage cultivation, mutagenesis, adaptive laboratory evolution (ALE), and metabolic and genetic engineering approaches have proved to be effective in augmenting carotenoid synthesis. Recent breakthroughs such as CRISPR-Cas9 genome editing, transcriptional engineering, and co-culture techniques offer novel routes to optimize metabolic pathways for high-value pigment production. Thus together, these integrated biotechnological and cultivation strategies can pave the way forward. The focus would be on producing natural carotenoids from microalgae in a sustainable, large-scale, and economical manner for use in bioindustrial, medicinal, and nutraceutical applications.

Keywords: Microalgae, Carotenoids, Photobioreactors, Adaptive Laboratory Evolution, CRISPR-Cas9, Random Mutagenesis, Transcriptional Engineering

Introduction
Carotenoids comprise a diverse group of taxonomically well-known, multifunctional, biotechnologically crucial pigments. Apart from various vegetative sources, more than 1100 structurally different carotenoids with similar biological functions have been derived from microbes. Carotenoids exhibit multiple health benefits and are one of the vital substances acquired through diet as they are not synthesized in the human body [Gupta, R. *et al*., 2024; Gammone *et al*., 2015] [38, 8]. Although carotenoids have a broad range of industrial applications, many of these carotenoids are being chemically synthesized, which is in high demand in many countries. Natural carotenoids obtained from micro-organisms, plants and animals are in small amounts in comparison to synthetic ones with the advantage of fast growth and reduced cost. These artificial carotenoids are less effective in promoting health, which makes them less popular as a commodity [Das *et al*., 2025] [36]. For instance, microalgal βcarotene, an all-9-cis and trans isomer naturally occurring compound, loses its health benefits when artificially synthesised as it turns to an all-trans isomer. Hence, the market value of synthetic carotenoids is much less, almost half as compared to the worth of a microalgal-derived [Koller *et al*., 2014] [44]. However, due to low cost and affordability, they have a broad market in the Asia-Pacific region. However, North America and Europe have a high demand for natural carotenoids [Markets and Markets Report 2024].
The advancement of biotechnological tools has led to enhanced natural carotenoid production, reducing processing costs. To fully exploit the potential of microalgae as commercial green hosts, the scientific community is now moving towards a better understanding of these microorganisms from a systems biology perspective.

Microalgae Cultivation System

Microalgae are the potential feedstock for the biofuel as well as carotenoid production for commercial market. Microalgae cultivation began early in the 1960s, as a source for single-cell protein and for biomass. *Chlorella* along with *Spirulina* was the first microalgae to be commercialized as "health food" in Japan, Taiwan and Mexico. This followed an establishment of culturing and harvesting facility of *Arthrospira* in the early 1970's. The 1980's saw increase in the mass production of *Chlorella*, *Dunaliella salina*, *Haematococcus pluvialis* and cyanobacteria (blue-green algae) across globe including USA, China, Thailand, Australia and Israel. *Dunaliella salina* was used extensively for the extraction of beta-carotene [Faraloni *et al*., 2025] [37]; astaxanthin extraction was done majorly from *Haematococcus pluvialis* and *Crypthecodinium cohnii* was used for extraction of docosahexaenoic acid [Ambati R.R *et al*., 2019] [1]. The late 1990s saw a continuous trend and setting up of Cyanotech in Hawaii for the extensive commercial production of *Haematococcus pluvialis*. Subsequently the heterotrophic production of *Crypthecodinium cohnii*, for extraction of eicosapentaenoic acid also commenced in the USA.

Microalgal carotenoid production is a multi-step process that involves various strategies. The first step is to screen the microalgal genera to decide on a species/strain that naturally produces elevated levels of carotenoids. An option to the first step is to select a suitable species to genetically engineer so to increase carotenoids production (where legislature allows). The genetic change can either be introduced directly via targeted engineering or indirectly through mutagenesis. The second step is to cultivate the selected species to enhance production of carotenoids, keeping the biotic and abiotic factors in control. Large-scale microalgal cultivation first involves a rapid cultivation of cells to accumulate large biomass volume and then apply a stress factor to drive the synthesis of carotenoids. Large-scale microalgae cultivation contributes to the development of a sustainable industry for generating cost-effective high-value products as well as for biomass production. Many species of microalgae as mentioned above show potential for large-scale cultivation. Various factors considered when selecting the method of cultivation include operational and capital cost, available are for cultivation, climate (temperature, light, rainfall etc.), contamination risk, water availability, level of automation and system efficiency, culture mixing strategy and the purpose of cultivation.

Microalgae are cultured by various methods and under different conditions. The fundamental design and infrastructure of the engineered facilities of the cultivation methods depends on the growth requirements of the microalgae of interest as well as the nature and commercial value of the final product. Light is needed as energy source for conversion of the absorbed water and CO2 into biomass through photosynthesis, along with nitrogen and phosphorus as major nutrients, macronutrients Ca, Na, Mg, and K; and micronutrients, such as Zn, Mo, B, Co, Mn and Fe. Wastewater is a good source of these required nutrients for microalgae cultivation.

Natural and artificial are the two principal production methods of microalgae. Natural method includes shallow ponds, raceway ponds and circular ponds. The artificial methods of culturing include photobioreactor of different shapes. These methods of mass production of microalgae whether done in open ponds or enclosed photobioreactors or a combination of both, have contrasting advantages and disadvantages. However another factor to be attended when selecting the most apposite cultivation system is the nature of the microalgae to be cultured, whether it is a wild type species that is naturally occurring or if it is a genetically modified organism (GMO) with specific enhanced properties such as for carotenoid production. Farming of GMOs requires enclosed PBRs and has stringent monitoring and regulation and containment plans are installed to prevent any escape of the GMOs.

Natural Method of Cultivation

Natural open methods for the commercial production of microalgal biomass are the method of choice owing to their potential benefits. This open system of cultivation includes natural, circular and raceway ponds along with inclined systems, which are either constructed in excavated pits or raised above the ground level, with incorporated air pumps, paddlewheels, or water jets to allow for better flow and circulation of water, nutrients and algae. The natural lakes and lagoons with spontaneous blooms of specific microalgae have also been exploited such as the Lake Kossorom, where the locals consume *Arthrospira* as food and annually about 40 t yr^{-1} is harvested [Abdulqader *et al*., 2000] [45].

Raceway ponds the most widespread commercial system, are either built in concrete or dug in the ground and covered with a plastic liner. It consists of a circuit of parallel channels, where the microalgae suspension is circulated by the paddle wheel. An advanced raceway pond with pumps rather than the traditional paddle wheel has been designed which reduces the water and energy consumption per square meter of pond in the cultivation phase, leading to a higher algae concentration and increased efficiency [Ren *et al*., 2025] [25]. Circular ponds having a centrally pivoted rotating agitator are being used for *Chlorella* production in Japan, Taiwan and Indonesia. While its complete culture is done in the outdoor ponds, at some places such as the Chlorella Industry Co., Japan, the culture is shifted to ponds after the inoculums are produced in fermenters. However the restricted size of the pond to about 10000 m^2 due to the mechanical issue of the rotating arm and non-homogeneous mixing [Borowitzka M.A. 2013] [46, 48] limits the widespread usage of circular ponds. The inclined (cascades) system of open ponds allows for the high biomass densities resulting in economical harvesting of microalgae. The system requires the flow of culture suspension from the top with falling in a retention tank and further pumped back resulting in high turbulence and effectively utilizing light [Schädler *et al*., 2020] [42].

Microalgal biomass production for industrial purposes for animal feed ingredients, health food market, and for wastewater treatment is dependent on these engineered open ponds. These open ponds are also used for unialagal cultures based on the competitive ability of the species. For instance, *Dunaliella* species predominates in saline culture, and *Spirulina* in highly alkaline water which makes the conditions unsuitable for the competing species. Chlorophytes such as *Scenedesmus* and *Ankistrodesmus* grow in wastewater treatment systems that depend on microalgae for O_2 production, as these can tolerate environments with high dissolved and particulate organic compounds concentration [Singh *et al*., 2024] [28, 40].

However large-scale commercial production in open ponds is limited to certain species of microalgae such as those of genera *Chlorella, Dunaliella and Arthrospira*, as they have the advantage of high growth rate and a selective medium which limits contamination. Hence majority of the species which do not require a selective or a specific growth medium cannot be cultivated in the open outdoor systems for prolonged periods as it increases the contamination rate [Ferreira *et al*, 2025] [39]. This method requires large arable land as a continuous operation is maintained near the ponds to ensure of nutrients and CO_2 circulation, while biomass harvesting. These ponds are easier to construct and operate, but due to more energy requirement for nutrient homogenization and failure of maintenance of selected strains in ponds makes this method disadvantageous as compared to artificial method. Unregulated temperature, poor utilization of cell light, predator contamination, CO2 diffusion into the atmosphere, evaporation loss and rainfalls diluting the available nutrients and biomass are the other hindrances which makes this method suitable only for production of certain large microalgal quantities (Table 1).

Table 1: Summary of the Open Ponds

Advantages	Disadvantages
Simple design, Low capital cost/ operational cost, low energy input	Lower productivity (10-25 g/m²·d) with highest net yields around 38 g/m²·d
Scalability- suitable for large-scale outdoor use	Poor environmental control
Can leverage extreme environmental conditions such as high salinity, alkalinity for contamination resistance	High contamination risk- Susceptible to bugs, evaporation, CO_2 losses, and weather variability
Utilizes natural sunlight	

Artificial Method of Cultivation

Photobioreactors

Photobioreactors (PBR) are the closed systems for algae cultivation and provide a safe controlled environment for microalgal cultures. These are lit fermentors where photons, the energy source for growth do not invade directly on the culture surface but pass through the reactors transparent walls, before reaching the cultivated cells. This limits the direct exchange of particles (microbes, insects, dust), liquids (e.g., rain) and gasses between the culture and the atmosphere [Tredici *et al*., 2010] [50]. PBRs have different categories of designs such as (i) tubular or flat; (ii) vertical, horizontal, inclined or spiral; (iii) floating; (iv) biofilm; (v) manifold or serpentine; and (vi) hybrid reactors. These are used for the production of high value products such as the reagent grade phycobilins and isotopically-labeled research compounds [Lee & Lee 2016] [51]. This helps to prevent any chemical or biological contamination such as predation, parasitism, and the competition from redundant microalgae ('weeds'). Further it also allows for a more precise control of the culture parameters (temperature, pH, pO2, CO2 etc.), ensuring the cultivation of specific microalgal strains with improved productivity via a complete control process. The photobioreactors designs, mode of operation and construction material are based on the type of the species to be cultivated, the location, and the end products to be achieved. Vertical reactors internally illuminated also called as annular columns, have a culture chamber made of two transparent concentric cylinders of variable diameter sealed at the base. Various microalgae and cyanobacteria including *Nannochloropsis* sp, *Nostoc* strains [Rodolfi *et al*., 2003] [47], *Isochrysis* sp. [Zittelli C.G. *et al*., 2013] [48], among many more have been cultured under natural, artificial or combined illumination. These annular columns have been found out to produce high-quality algae biomass at harvesting on a regular basis, as compared to the traditional systems used in hatcheries. Floating photobioreactors initially devised in the 1980s are deployed in the water bodies and anchored to piers to prevent them from drifting away with currents and the waves in the water body. These PBRs allow for the microalgae cultivation without competing with land and exploit water surfaces, hence proving to be very useful for the highly urbanized coastal areas with less land availability and abundant nutrients from waste water and flue gases to enhance microalagal productivity. *Chlorella vulgaris* and *N. oleoabundans*, have been cultured obtaining average productivities of about 50 g m−2 d−1 for both algae, with harvesting three times a week 10-30% of the volume.

Mainly used for research or small-scale applications, further advancements in PBRs have helped to achieve high photosynthetic efficiencies and push productivity beyond that is currently attainable. The limitations and challenges that affect the natural cultivation methods are eliminated in these photobioreactors. However, the major drawbacks are the soaring capital and operating costs, the negative energy balance, and a restricted possibility of being scaled up [Tredici *et al*., 2010] [50]. Although these limitations have hindered the scalability of PBRs for production of food, biofuels etc. from microalgae, various companies targeting biofuel production have developed innovative applications of PBR designs [Zittelli C.G. *et al*., 2013] [48]. An intensive research done on it highlighted the vertical systems that dilute photosaturation and photoinhibition due to light minimizing. This maximizes the photosynthetic efficiency and areal productivity [Tredici *et al*., 2010] [50]. (Table 2).

Table 2: Summary of the Photobioreactor

Advantages	Disadvantages
Better control of growth factors productivity up to \~24 g/m²·d (higher biomass and carotenoid productivity)	Very high capital costs (€0.5-0.8M/ha),
Reproducible yields	
Higher photosynthetic efficiency (up to \~5%),	energy intensive
valuable for high-purity products like carotenoids	high operational expenses
Controlled conditions, low contamination risks	cost of biomass up to \~\$10/kg

Hybrid System (Open Pond + PBR)

With advancements in technology, a hybrid system of coupling open ponds and photobioreactors, and even floating photobioreactors, have been put in use, which aims at exploiting water bodies rather than the land for algae cultivation [Chuka-ogwude, *et al*., 2022] [43]. The raceway ponds are more prone to algal and grazers contamination but more cost effective than the PBR. The PBRs though minimize contamination but the high installation and operation costs make them a secondary choice. Hence, a combination of both these systems is a promising strategy for the cost-effective cultivation of the desired microalgal strains. This combined system works efficiently for the two-stage cultivation processes, wherein for the first stage, PBR

is used for the inocula production and in the second stage the desired products such as biomass, oil etc are obtained from the open ponds. This integrated system results in a higher productivity than the individual pond and panel system operating separately, for example in microalgae *H. pluvialis* an average biomass productivity of 38 metric tons per annum and an oil production at rate of 10 metric tons was obtained [Huntley & Redalje 2007] [52]. In a study by Li W. *et al*., [Li W. *et al*., 2018] [31] *Scenedesmus dimorphus* was cultivated using an open pond-PBR setup. It resulted in increased efficiency and productivity of the microalgae. The biomass concentration was found to be 116% higher than a non-hybrid system indoors whereas the outdoor productivity was 46-74% higher as compared to the open ponds. In another study with *Tetraselmis* sp. the hybrid systems produced significantly more lipid-rich biomass per solar irradiance with reduced contamination risks. Further study by Huang G., *et al*. [2010] [53] suggested that long chain fatty acid of high value could be achieved through artificial cultivation method, from microalgae which was contaminated, due to the enhanced environmental control of photobioreactors. However this achievement would be marred by the increased operating cost and the technicalities involved. Though the photobioreactors have the capability to produce as well as control algal biomass with enhanced production rates than the open ponds, but they are currently restricted to pilot scale plants and research laboratories. A key to the commercial success of these photobioreactors is by understanding their energy consumption as its significant reduction will increase the cost effectiveness of the culture by saving time and money and increasing productivity [Xu *et al*. 2018] [54]. (Table 3).

Table 3: Summary of Hybrid Systems

Advantages	Limitations
Combine benefits of both systems Combines cost-effectiveness of ponds with productivity and control of PBRs	More complex operation, moderate costs, requires integration management
Higher biomass outputs (+46-74% vs open ponds).	Moderate contamination risks
Better quality, lipid-rich biomass comparable to PBRs	
Mitigation of contamination risk and downtime	
Higher initial capital and operational coordination—balanced by improved productivity and stability	

Comparison of Economics and Productivity

In the Open Raceway Ponds, the typical biomass productivity ranges between10-25 $g \cdot m^{-2} \cdot d^{-1}$. The major causes of low productivity of the biomass are inefficient carbon dioxide usage and poor light distribution. However in certain modified open ponds with ultra-thin layer, the boost in the productivity was achieved with the culture of *Chlorella*. The biomass productivity reached almost 38.2 $g \cdot m^{-2} \cdot d^{-1}$ [Liu, W. *et al*., 2018] [31]. Further, based on the visible light the photosynthesis efficiency of 7.05% was also achieved.

Table 4: Comparison of Microalgae Cultivation Systems

Cultivation System	Yield/ Productivity ($g/m^2 \cdot d^2$)	Operational Cost (\$/kg biomass)	Photosynthetic Efficiency	Water Use	Scalability
Open Ponds	Low-moderate yield 10-25 (up to 38) (in ultrathin inclined pond design)	\$9-25/kg	~1.5%	High (~58,061 $m^3/ha \cdot yr$)	Very high scalability with low cost
PBR	High yield - 19-24	High ~\$10/kg	~3-5%	Lower (~16,872 $m^3/ha \cdot yr$)	Moderate scalability with high costs involved, energy-intensive
Hybrid Systems	Very high yield +46-74% as compared to open ponds	Intermediate	Comparable to PBRs	Moderate	High scalability with balanced yield

Source: Lin-Lan Zhuang *et al*., (2023) [34]

Strategies used to augment Carotenoid accumulation in Microalgae

Overview of carotenoid accumulation in microalgae: The advent of modern biotechnology has led to the microorganisms from diverse lineages being used for production of bio-based feedstocks and many bioactive compounds. However the commercial success of these compounds in their respective industry warrants further investigations into improving their production through strain development. The photosynthetic microalgae have the potential to be called as "cell factories" [Fu W. *et al*., 2019] [7], due to their ability to synthesize commercial compounds such as carotenoids. However, microalgal strain improvement necessitates appropriate engineering methods which would enable the complete biotechnological potential of microalgae come to realization.

Factors influencing carotenoid production: Along with the culture types and conditions there are various other culture-stimulating factors as well as environmental factors which are required for the effective stimulation of the biosynthesis of carotenoids for increased microalgal yield per unit volume and accumulation of microalgal carotenoids. These significant factors are temperature and light regulation, addition of salts, metal ions, nanoparticles and other chemical substances in the culture medium. These factors directly affect the level and the activity of the biosynthetic enzymes in variable intensities.

Further arrays of stress methods have been developed to increase the carotenoid synthesis in microorganisms. A two-stage cultivation process is a potent strategy which balances both cell growth and metabolite accumulation has been studied with promising results whereby in the first stage optimum growth conditions are provided to obtain maximum biomass production and in the second stage accumulation of carotenoids and lipids under various stress conditions are obtained [Li Q. *et al*., 2021] [16]. Using a two-stage heterotrophy/ photoinduction strategy for culture of

Scenedesmus incrassatulus, the lutein productivity was found to be increased by 60% as compared to the autotrophic fed-batch culture [Sun X. *et al*., 2018] [25]. Temperature affects growth and development of the organisms along with the enzymes concentration thereby controlling the carotenoids level in microalgae [Gammone M. A. *et al*., 2015] [8]. For example the ß-carotene content of *Rhodotorula* sp. varies with change in temperature. In R. glutinis the ß-carotene content increases at low temperatures. The intensity of light too has a stimulating effect on carotenoids synthesis as well as accumulation in fungi and algae, though varying with the microorganisms. The growth of microorganisms improves with the increase in carotenoids also simultaneously causing intense activity of the enzymes involved in carotenoid biosynthesis. For example, *Dunaliella* sp. known for β-carotene synthesis accumulates large amount of carotenoids, under various abiotic stress conditions, including high salinity, high light and nitrogen consumption. An increased accumulation of β-carotene by 50.6% due to nitrogen depletion and increased lutein synthesis of 744.6 µg/mL because of high light were obtained as compared to the control group [Wu Z.*et al*., 2009] [28]. Most of the microalgae have a positive correlation between the carbon/nitrogen ratio and carotenoid accumulation. In microalgae *Nephroselmismis* sp. carotenoid lycopene showed 3 times increased productivity with 2.4 times increased antioxidant activity due to presence of sufficient nitrogen [Cordero, B. F *et al*., 2012] [6]. Also the presence of metal ions such as potassium, magnesium and sodium which are the active group of few enzymes, along with calcium, zinc and iron are essential for microbial growth and increased carotenoid production as observed in *H. pluvialis which had increased* astaxanthin production when cultured in a iron rich medium [Li, J *et al*., 2011] [15]. Oxidative stress created due to the chemicals or salts or by photooxidation leads to the microalgae accumulating carotenoids [Hu Z. *et al*. 2008] [10]. *Chlorella zofingiensis* accumulataed canthaxanthin when exposed to ferrous salt-based oxidative stress while ß-carotene accumulation was observed in *Dunaliella salina* [Rammuni M.N.*et al*., 2019] [21]. Photooxidation generated by high light irradiance (UV), produces free active oxygen molecules which generates oxidative stress, thereby eliciting the carotenogenesis process and leading to astaxanthin synthesis and accumulation [Hu Z.. *et al*. 2008] [10].

Another approach to enhance carotenogenesis is by heterotrophic and mixotrophic type of microalgal cultivation which also increase microalgal biomass. In heterotrophic cultivation which is done in absence of light, only carbon source such as acetate, glycerol, glucose and other sources of carbon are used which leads to enhanced carotenoid production and biomass, by utilizing organic carbon in media. In mixotrophic cultivation system microalgae perform photosynthesis to fix carbon as external source of both carbon and light are provided, to accumulate carotenoids [Zhan *et al*., 2017] [29]. *H. pluvialis* is able to accumulate astaxanthin in both heterotrophic conditions and mixotrophic conditions, whereas *D. salina* growth is able to accumulate β-carotene only in mixotrophic cultivation system [Goswami *et al*., 2021] [9].

Further methods for carotenoid accumulation are the mutual cultivation or cohesive cultivation or the coculture cultivation techniques, where in one or more different organisms shown to have mutual benefits are grown simultaneously in the same reactor or same culture [Sun X. *et al*., 2018] [25]. For example bacteria and microalgae when grown together results in carotenoid accumulation in microalgae. However this process requires careful selection of organisms due to production of toxic metabolites. Furthermore, another approach to enhance carotenoids accumulation in microalgae is using metabolic and genetic engineering techniques to obtain the desired end products. Various mutagenic techniques such as the random mutagenesis where UV rays or different radiation is used as a mutagen to enhance carotenoid production as in *H. pluvialis* which on exposure to radiations accumulates lutein and astaxanthin [Cordero B.F. *et al*., 2012] [6]. Genetic engineering of genes to manipulate metabolic pathways involves both up and down regulation of the factors involved in transcription and translation which leads to desired pigment enhancement [Saini D.K.*et al*., 2020] [22]. Genetic modification of upregulation of the enzyme phytoene desaturase in *C. zofingiensis* lead to an enhancement of total carotenoids by 32.1%, whereas a 26% enhancement of astaxanthin was observed in *Hematococcus pluvialis* [Saini D.K.*et al*. 2020] [22]. Adaptive laboratory evolution (ALE) is also being extensively used for developing novel phenotypic and biological functions along with strain improvement in microalgae leading to increased carotenoid and lipid accumulation [Fu W. *et al*., 2019] [7]. The ALE experiments done under controlled laboratory conditions followed by genome re-sequencing allow for the study of genetic basis underlying adaptation to the environmental stress factor. Adaptive evolution studies on D. salina strains resulted in increased carotenoid productions of lutein and beta carotene [Fu W. *et al*., 2019] [7]. Thus ALE is being used as an innovative and effective tool for the microalgal strain improvement (Table 5).

Table 5: Key factors affecting carotenoid accumulation in microalgae

Factor	Effect on Carotenoids
Light intensity	Elevated light enhances carotenoid synthesis as a photoprotective response; excessive light may cause photoinhibition
Temperature	Moderate heat stress stimulates carotenoid accumulation; extreme temperatures reduce growth and pigment yield
Nitrogen limitation	Nitrogen deprivation redirects carbon flux toward carotenoid biosynthesis as a storage and stress response.
Salinity stress	Moderate salinity enhances carotenoid production via osmotic adaptation; severe stress inhibits growth
Oxidative stress	Induces
Metal ions	Certain metals (Fe, Mn, Cu) serve as cofactors and enhance pigment synthesis; excess can be toxic.

Induction of stress

Modern biotechnology tools have led to microorganisms from diverse lineages being used to produce bio-based feedstocks and many bioactive compounds. However, the commercial success of these compounds in their respective industries warrants further investigations into improving their production through strain development. The photosynthetic microalgae have the potential to be called as "cell factories" [Fu W *et al*., 2019] [7], due to their ability to synthesize carotenoids. Various culture-stimulating and

environmental factors are required to effectively stimulate and accumulate these carotenoids for increased microalgal yield per unit volume. These significant factors include temperature and light regulation, along with salts, metal ions, nanoparticles, and other chemical substances in culture medium. These factors directly affect the level and activity of the biosynthetic enzymes in variable intensities.

Stress conditions such as nutrition and other abiotic factors lead to molecular evolution and adaptive changes, thereby channeling metabolic fluxes into enhanced biosynthesis of lipids and carotenoid production in microalgae. Under extreme environmental conditions such as limited nutrients, microalgae generate several secondary metabolites to maintain their growth rate. It has been observed that levels of intracellular reactive oxygen species (ROS), increase with stress conditions and lead to carbon metabolic flux to change from glycolysis to the oxidative pentose phosphate pathway, resulting in acute accumulation of intracellular equivalents of excessive reduction (NADPH). The elevated levels of ROS lead to damage to biological macromolecules such as DNA, proteins and lipids, and to counter this, microalgae synthesize additional carotenoids to scavenge ROS under stress conditions [Shi *et al*., 2020] [23].

A two-stage cultivation process is a potent strategy which balances both cell growth and metabolite accumulation, whereby in the first stage, optimum growth conditions are provided to obtain maximum biomass production, and in the second stage, accumulation of carotenoids and lipids occur under various stress conditions [Li Q *et al*., 2021] [16]. Using a two-stage heterotrophy/ photoinduction strategy for the culture of *Scenedesmus incrassatulus*, the lutein productivity was found to be increased by 60% as compared to the autotrophic fed-batch culture [Sun *et al*., 2018] [25].

Temperature affects the growth and development of the organisms along with the concentration of the enzyme thereby controlling the carotenoid level in microalgae [Gammone *et al*., 2015] [8]. For example, the ß-carotene content of *Rhodotorula* sp. increases at low temperatures. The intensity of light too has a stimulating effect on carotenoids synthesis as well as accumulation in fungi and algae. With intense activity of the enzymes involved in carotenoid biosynthesis, the growth of microorganisms improves. For example, *Dunaliella* sp. is known for increased β-carotene synthesis under various abiotic stress conditions, including high salinity, intense light and more nitrogen consumption [Wu Z Y *et al*., 2009] [28].

Salt stress treatment is a profitable and easy approach to affect the microalgal biochemical components that leads to carotenoid accumulation, especially for seawater culture of microalgae. The gene expression, physiological characteristics and metabolic pathways are all affected by salt stress. The morphological changes, such as the thickening of cell walls, an increase of cell volume, etc., leads to a series of downstream and in-depth signalling changes in both genetic and metabolic pathways. Widely used methods to increase carotenoid accumulation are factors such as light induction, nutrient starvation and addition of chemicals coupled with salt stress. A 13 % increase in the dry weight of β carotene in *D. salina* was observed under high light and temperature, salt stress, and nutrition-deprived conditions [Rammuni *et al*., 2019] [21].

Most microalgae have a positive correlation between carbon/nitrogen ratio and carotenoid accumulation. In microalgae, *Nephroselmismis* sp. carotenoid lycopene showed three times increased productivity with 2.4 times more antioxidant activity due to high nitrogen [Cordero BF *et al*., 2012] [6]. The presence of metal ions such as potassium, magnesium, calcium, zinc, iron and sodium, which are active groups of few enzymes, are essential for microbial growth and increased carotenoid production, as observed in *H. pluvialis* with increased astaxanthin production in iron-rich medium [Li J *et al*., 2011] [15]. Oxidative stress is created by chemicals, salts, or photooxidation, which induces microalgae to accumulate carotenoids [Hu *et al*., 2008] [10]. *C. zofingiensis* accumulated canthaxanthin when exposed to ferrous salt-based oxidative stress while ß-carotene accumulation was observed in *D. salina* [Rammuni *et al*., 2019] [21]. Photooxidation generated by high light irradiance (UV), produces free active oxygen molecules which generate oxidative stress, thereby eliciting carotenogenesis process which leads to astaxanthin synthesis and accumulation [Hu *et al*., 2008] [10]. (Table 6)

Table 6: Molecular and Biotechnological Strategies for Enhancement of Carotenoids

Strategy	Process
Random mutagenesis	Chemical or UV induced random mutations to increase pigment yield.
Directed mutagenesis	Targeted site specific mutations in carotenogenic genes.
Adaptive laboratory evolution (ALE)	Gradual adaptation under stress conditions to select high yield strains.
CRISPR Cas9 genome editing	Precise gene knock in/knock out to optimize biosynthetic flux.
Transcriptional engineering (TE)	Overexpression/regulation of transcription factors controlling carotenoid metabolism. Increased carotenoid pools by upregulation of stress responsive transcriptional factors.

Induction of Mutation and Adaptive Evolution

Various mutagenic techniques such as UV rays or different radiation are used to enhance carotenoid production, as in *H. pluvialis* which on exposure to radiation accumulates lutein and astaxanthin [Cordero *et al*., 2012] [6] while *D. bardawil* has increased β-carotene content. Gamma radiation exposure also increased lipid content in *Scenedemus dimorphus* mutant strain [Choi *et al*., 2014] [5]. Other mutagens like, N-nitro-N-nitrosoguanidine also resulted in the successful production of *H. pluvialis* mutant strain with high astaxanthin content [Li Q *et al*., 2021] [16].

Adaptive laboratory evolution (ALE) is used extensively for developing novel phenotypic and biological functions and strain improvement in microalgae, leading to increased carotenoid and lipid accumulation [Fu *et al*., 2019] [7]. ALE method in *D. salina* led to increased lutein and carotene content. It also developed a high tolerance to carbon dioxide along with enhanced carotenoids and chlorophyll accumulation [Fu *et al*., 2019] [7].

Another approach to enhance carotenogenesis and increase microalgal biomass is by heterotrophic and mixotrophic types of microalgal cultivation Heterotrophic cultivation is done in the absence of light, wherein carbon sources such as acetate, glycerol, and glucose are used, which leads to enhanced carotenoid production and biomass by utilizing organic carbon in media. In a mixotrophic cultivation system, microalgae perform photosynthesis to fix carbon as external source to accumulate carotenoids [Zhan *et al*., 2017] [29]. *H. pluvialis* can accumulate astaxanthin in both

heterotrophic and mixotrophic conditions, whereas *D. salina* accumulates β-carotene only in the mixotrophic cultivation system [Goswami *et al*., 2021] [9].

Further methods for carotenoid accumulation are mutual cultivation or cohesive cultivation, or coculture cultivation techniques, where in one or more different organisms shown to have mutual benefits are grown simultaneously in the same reactor or same culture [Sun *et al*., 2018] [25]. For example, bacteria and microalgae, when grown together, accumulate carotenoid in microalgae. However, this process requires careful selection of organisms due to producing toxic metabolites.

Carotenoid Enhancement through Genetic Modifications

Carotenoid accumulation in microalgae is also achieved by genetic engineering techniques to obtain desired end products. Genetic engineering of genes to manipulate metabolic pathways involves up and down-regulation of factors involved in transcription and translation, which leads to desired pigment enhancement [Saini *et al*., 2020] [22]. The host algae and its target cellular compartment (nuclear or organelle) determine DNA delivery methodology and cell wall/membrane permeabilisation. Eukaryotic microalgae have cell walls that require aggressive delivery methods compared to cyanobacteria which take up environmental DNA without manipulative methods. Thus, the transformation of microalgae can either be in the mitochondrial, chloroplast or in the nuclear genome. DNA delivery leads to a stable chromosomal integration or an extrachromosomal plasmid replication of foreign transgenic expression elements [Wannathong *et al*., 2016] [27]. Hence, to achieve a desired metabolic trait, both nuclear and plastid genomes can be engineered [Chen *et al*., 2022] [4]. One of the first examples of carotenogenic pathway manipulation in eukaryotic microalgae was the genetically engineered *C. reinhardtii,* which was inserted with carotene ketolase cDNA, bkt1 gene from *H. pluvialis.* This gene, involved in the synthesis of asthaxanthin, led to the formation of ketocarotenoids in the transgenic strain of *C. reinhardtii* [Perozeni *et al*., 2020] [20]. The model algae *C. reinhardtii* has a well-annotated genome, and tools for its genetic engineering [Sproles *et al*., 2021] [24]. Genome editing and knock down technology-RNA interference (RNAi) have been established in *D. salina* [Jia *et al*., 2009] [12] and *C. reinhardtii* [Kim and Cerutti, 2009] [13]. DNA-free mutagenesis CRISPR-Cas9 RNP-mediated knock down of zeaxanthin epoxidase gene (zep) in *C. reinhardtii* led to an increase of zeaxanthin content by almost 56% [Baek *et al*., 2018] [2]. A similar genetic manipulation of *Porphyridium* sp. using CRISPR/Cas9 RNP enhanced phycoerythrin content by 63.30% in a chlorophyll synthase-deficient mutant [Jeon *et al*., 2021] [11].

Another strategy is transcriptional engineering (TE), using engineering regulators such as transcription factors (TFs) led to simultaneous modification of metabolic pathway components [Bajhaiya *et al*., 2017] [3]. Transcriptome studies in *M. neglectum and Nannochloropsis* sp showed increased lipid accumulation along with considerable up-regulation of Lysophosphatidic acid acyltransferase and Glycerol-3-phosphate dehydrogenase, which indicated a positive correlation between cellular lipid accumulation and transcription of genes [Lv *et al*., 2013; Chen *et al*., 2022] [17, 4].

Further RNA interference technology has been utilized for carotenoid profile alteration of *C. reinhardtii.* The *pds* gene, when targeted, resulted in a drastic reduction of its mRNA content, but the carotenoid content did not alter significantly [Vila *et al*., 2008] [26]. In another analogous approach, when the phytoene synthase gene (*psy*) was transformed in *C. reinhardtii* nucleus, increased carotenoid accumulation was observed. Also, more lutein was observed in the transformed strains of *D. salina* and *C. zofingiensis* by the overexpression of *psy* [Cordero *et al*., 2012] [6] *H. pluvialis*, when engineered with a mutant *pds* gene (nuclear transformation), accumulated up to 26% more astaxanthin.

Another method involves the transport of the desired metabolite away from the site of synthesis (metabolic sink) to prevent feedback inhibition. In *H. pluvialis* and *D. salina*, astaxanthin and β-carotene biosynthesis is paused with inhibiting lipid accumulation, as for these carotenoids, lipid serves as the metabolic sink [Zhekisheva *et al*., 2005] [30]. Recent advancements in development and optimization of novel genetic elements and transformation processes have paved way for manipulation of the algal gene/enzyme to produce increased targeted engineered bioproducts by altering their metabolic pathway (Table 7).

Table 7: Metabolic engineering in carotenoid biosynthesis pathway in microalgae

Enzyme/Gene	Function	Source strain-type of modification	Host strain-phenotypic change
Carotenoid hydroxylase (CRTR-B)	Catalyzes conversion of canthaxanthin to astaxanthin	*Haematococcus pluvialis*	*Synechocystis sp.* PCC6803- carotenoid biosynthesis
β carotene ketolase (BKT)	1.Catalyzes conversion of echinenone to canthaxanthin 2.Catalyzes zeaxanthin transformation to adonixanthin and adonixathin into astaxanthin	*Haematococcus pluvialis*-Nuclear overexpression	*Chlamydomonas reinhardtii*- 4 keto-leutin synthesis
			Synechocystis sp. PCC6803- carotenoid biosynthesis
β carotene ketolase (CrBKT)			*Agrobacterium tumefaciens*-2 to 3- and 2-8-fold increase in Astaxanthin, Echinenone, and Canthaxanthin
Phytoene desaturase (PDS)	Catalyzes first dehygrogenation reactions of phytoene to form phytofluene and zeta carotene	*Haematococcus pluvialis*-Endogenous w/mutation Nuclear overexpression-	*Chlamydomonas reinhardtii*- Increase of several carotenoids
			Chlorella zofingiensis - 32.1% increase of total carotenoids
			Haematococcus pluvialis - 26% increase of astaxanthin
		Dunaliella salina Endogenous	*Agrobacterium tumefaciens*- 78% decrease in β-carotene, lutein, and lycopene.

		Chlamydomonas reinhardtii- Endogenous (Glass bead method)	29% decrease in β-carotene
Phytoene synthase (PSY)	Catalyze the rate limiting step of synthesis of phytoene from geranyl pyrophosphate	1. *Dunaliella salina* - Nuclear overexpression	*Chlamydomonas reinhardtii* - 2.6fold increase in lutein and Violaxanthin
		2. *Chlorella zofingiensis*- Nuclear overexpression	*Chlamydomonas reinhardtii* 2.2-fold increase in lutein and Violaxanthin
		3. Phaeodactylm tricornutum - Microparticle bombardment method	*Phaeodactylum tricornutum*- 1.45-fold increase in Fucoxanthin

Source: Sproles, A. E. *et al*., (2021) [24]; Gimpel J. A. *et al*., (2015) [56]

Future Prospects and Challenges

The global demand for natural carotenoids is steadily increasing, owing to their diverse applications across various industries such as pharmaceutical, nutraceuticals, food and poultry. Although chemical synthesis is a cost effective option, the growing preference for a sustainable, bio-based alternative has led to microalgae being the most potential and sustainable feed stock for the commercial production of carotenoids. Further the productivity of microalgal based carotenoids has significantly improved as a result of the advancements in cultivation strategies, metabolic engineering, and systems biology approaches. However certain challenges remain before a full-scale industrial adoption can be realized.

One of the major limitations is the trade-off between the biomass generation and the metabolite accumulation. The two-stage cultivation processes, which decouple the growth from stress-induced carotenoid biosynthesis, have shown promise, but further optimization is definitely required for strain-specific responses under diverse environmental conditions. The hybrid systems integrating open ponds and photobioreactors may provide a practical balance between scalability, contamination control, and cost-effectiveness of the culture technique. However the large-scale energy requirement and water usage would still limit their widespread deployment.

Mutagenesis, adaptive laboratory evolution (ALE), and genetic engineering methods including transcriptional engineering and CRISPR/Cas-based genome editing have proven to enhance metabolic flux towards carotenoid biosynthesis. Despite these advancements certain barriers such as the regulatory restrictions pertaining to genetically modified microalgae, technological difficulties in stable transformation, and the large-scale production of altered strains continue to be major obstacles.

Henceforth the future research should focus on integrating the multidisciplinary approaches that would merge biotechnology, process engineering, and environmental science for a sustainable, stable natural carotenoid yield. Various omics-based platforms can be applied to find out the regulatory networks in microalgae. Development of certain low-cost extraction and purification techniques of carotenoids would be an added advantage to improve the cost effectiveness of the culture techniques. Further, the future studies should explore more species of microalgae capable of tolerating extreme environmental factors such as high salinity, light, or temperature, thereby leading to accumulation of high carotenoid levels. With the advent of genetically modified microalgae, more regulations and policies need to be drafted and implemented for shaping market adoption by the consumers.

Thus, to unlock the full biotechnological potential of microalgae as "green biofactories, it is imperative to integrate the systems biology, and synthetic biology with eco-innovative cultivation strategies.

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Author's contributions

PKN and MY conceptualized the review and wrote the initial draft. PKN assisted in the literature search while MY revised the manuscript. Both the authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no conflict of interest.

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