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ORIGINAL PAPERS

Valuable pigments from microalgae: phycobiliproteins, primary carotenoids, and fucoxanthin

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Abstract

Phycobiliproteins, carotenoids and fucoxanthin are photosynthetic pigments extracted from microalgae and cyanobacteria with great potential biotechnological applications, as healthy food colorants and cosmetics. Phycocyanin possesses a brilliant blue color, with fluorescent properties making it useful as a reagent for immunological essays. The most important source of phycocyanin is the cyanobacterium *Arthrospira platensis,* however, recently, the Rhodophyta *Galdieria sulphuraria* has also been identified as such. The main obstacle to the commercialization of phycocyanin is represented by its chemical instability, strongly reducing its shelf-life. Moreover, the high level of purity needed for pharmaceutical applications requires several steps which increase both the production time and cost. Microalgae (*Chlorella*, *Dunaliella*, *Nannochloropsis*, *Scenedesmus*) produce several light harvesting carotenoids, and are able to manage with oxidative stress, due to their free radical scavenging properties, which makes them suitable for use as source of natural antioxidants. Many studies focused on the selection of the most promising strains producing valuable carotenoids and on their extraction and purification. Among carotenoids produced by marine microalgae, fucoxanthin is the most abundant, representing more than 10% of total carotenoids. Despite the abundance and diversity of fucoxanthin producing microalgae only a few species have been studied for commercial production, the most relevant being *Phaeodactylum tricornutum*. Due to its antioxidant activity, fucoxanthin can bring various potential benefits to the prevention and treatment of lifestyle-related diseases. In this review, we update the main results achieved in the production, extraction, purification, and commercialization of these important pigments, motivating the cultivation of microalgae as a source of natural pigments.

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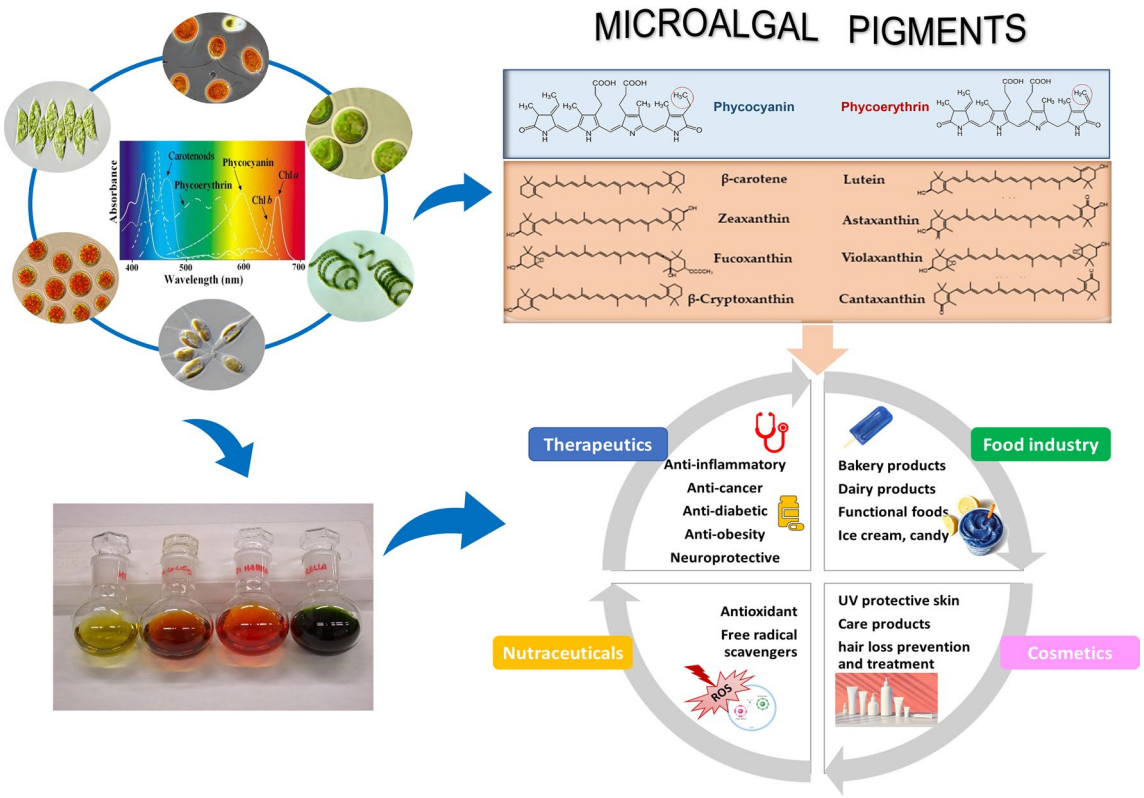
Graphical abstract



Keywords Phycocyanin · Primary carotenoids · Fucoxanthin · Microalgae

Abbreviations

A_{max}	Absorption maximum
APC	Allophycocyanin
CAGR	Compound annual growth rate
DMAPP	Dimethylallyl diphosphate
EFSA	European food safety authority
FCP	Fucoxanthin-chlorophyll a/c-protein complex
FDA	Food and drug administration
Fx	Fucoxanthin
GGPP	Geranylgeranyl pyrophosphate
IPP	Isopentenyl diphosphate
MEP	Methylerythritol phosphate
PBPs	Phycobiliproteins
PBR	Photobioreactor
PC	Phycocyanin
PE	Phycoerythrin

1 Phycobiliproteins

Phycobiliproteins (PBPs) are brilliantly colored protein-pigments of the photosynthetic light-harvesting antenna complexes produced by some algae, such as Rhodophyta, Cryptomonads, Glaucophytes and, particularly, by Cyanobacteria. The biological importance of these compounds in Cyanobacteria is evidenced by the great amount of PBPs they produce, which can represent up to 50–60% of the total soluble proteins in some organisms [1], such as *Arthrospira platensis* or *A. maxima*. In fact, PBPs are the principal accessory pigments of cyanobacteria. In these organisms, PBPs are also nitrogen storage compounds, and are decomposed when nitrogen starvation occurs [2, 3]. PBPs are generally classified based on their absorption maximum (A_{max}) in the visible region of the spectrum. The most common PBPs are phycocyanin (PC, A_{max} = 610–625 nm, blue),

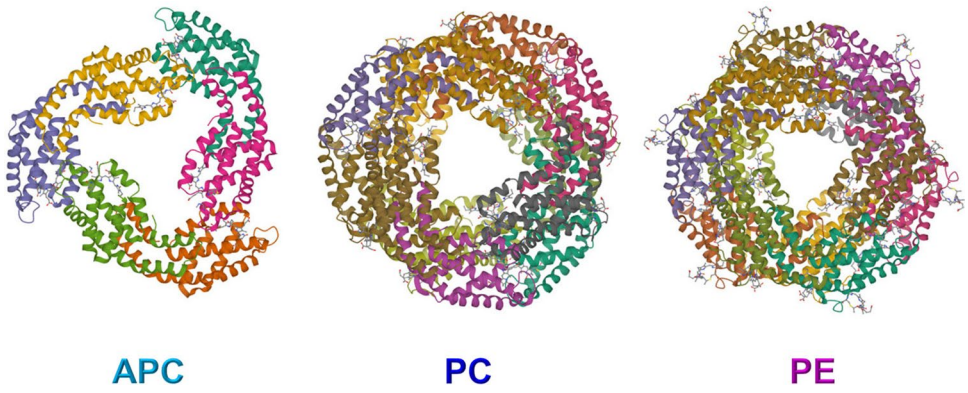


Fig. 1 Crystal structure of [4, 5] allophycocyanin (APC, *Synechococcus elongatus* PCC 7942), [5, 6] phycocyanin (PC, *Synechococcus elongatus* PCC 7942) and [7, 8] phycoerythrin (PE, *Palmaria palmata*)

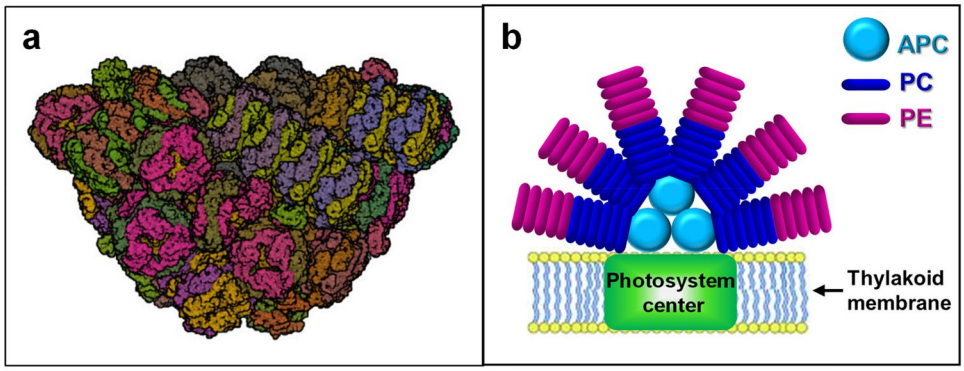


Fig. 2 The phycobilisome: (**a**) cryo electron microscopy image (*Porphyridium purpureum*) [10, 11] and (**b**) general structure scheme. *APC* allophycocyanin, *PC* phycocyanin, *PE* phycoerythrin

allophycocyanin (APC, $A_{max} = 650–660$ nm, blue green) and phycoerythrin (PE, $A_{max} = 490–570$ nm, red). Their structure is shown in Fig. 1 [4–8]. The main components of these pigments are protein subunits α and β, which form the "monomeric" unit (αβ). Various units self-assemble into the PBPs, which, in vivo, are organized on the thylakoid membrane to form larger protein complexes, the phycobilisomes [9] (Fig. 2a [10, 11]). The number and type of PBPs composing the phycobilisome is variable both in individual organisms of a species (due to environmental acclimation of the organism) and among species [12]. The general phycobilisome structure is sketched in Fig. 2b. The protein's brilliant color is due to the prosthetic groups: covalently bound tetrapyrrole derivatives (such as phycocyanobilin (Fig. 3a) or phycoerythrobilin, (Fig. 3b)), protected by the protein backbone and blocked in a geometry (extended conformation) suitable to efficiently funnel light energy (from PE to PC and, finally, to APC) towards the chlorophyll photosystem center [9]. In the phycobilisome, the pigment with the highest energy state, PE, always occupies the outermost position of the complex. Inwards follows PC and, finally, next to the photosynthetic chlorophyll center, the pigment with the lowest energy state, APC, as sketched in Fig. 2b.

When extracted and not engaged in the energy transfer processes, these molecules exhibit a fluorescence characterized by a very high quantum yield. Thanks to their

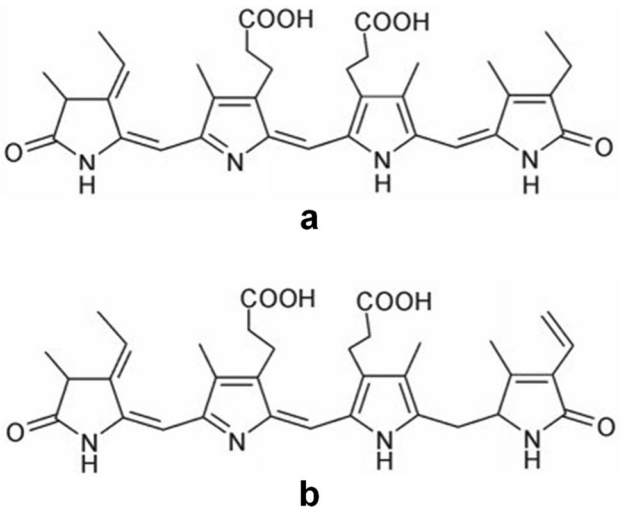


Fig. 3 Structure of (**a**) phycocyanobilin and (**b**) phycoerythrobilin

remarkable characteristics, PBPs have several biotechnological applications in food, cosmetic, pharmaceutical, and biomedical sectors. Among PBPs, PC has by far the largest market. The increasing utilization of these water-soluble pigments is boosted by their safe and healthy nature, as it is evidenced by the numerous papers published in the last years [1, 3, 13–21].

PBPs are used as natural cosmetic dyes or as fluorescent probes (APC and PE in particular) in flow cytometry and immunoassays. *A. platensis* (*Spirulina*) blue aqueous extracts, containing PC and APC, are approved by EFSA (Regulation (EU) No. 1333/2008 and No. 231/2012) as foodstuff coloring. The US FDA classifies PC (21CFR73.1530) as a natural food color additive. PC and PBPs therapeutic activities as antioxidant, anti-inflammatory, neuroprotective, anti-cancer and immunomodulatory have been reported [1, 3, 13, 14, 18, 19, 21, 23–27].

The commercial value of a PBP is strongly dependent on its purity grade, which is usually evaluated by the ratio between the value of PBP absorption maximum in the visible region of the spectrum and the absorbance value at 280 nm, which is related to the total amount of proteins detectable in the product. PBP purity greater than 0.7 is considered as food grade, greater than 1.5 as cosmetic grade, greater than 3.9 as reactive grade and greater than 4.0 as analytical grade. Usually, a purity greater than 3.0 is required to be used as fluorescent agent, while analytical grade PC is required for biomedical and therapeutic applications [28, 29]. Despite the increasing demand of safe natural products, the widespread use of PBPs is still limited by the costs of large-scale biomass production [30–32] as well as of extraction and purification methods.

1.1 Extraction and purification methods

Both extraction and purification are still too expensive, rather complex, and time-consuming, while industry needs simple, cheap and easily scalable, procedure to achieve a sustainable production of PBPs [3, 14, 22, 29, 33–35]. In addition, climate change due to the increase in emissions of carbon dioxide and other greenhouse gases, and the worldwide growing environmental deterioration, due to heavy pollution, are requiring industries to urgently align with current guidelines and regulations towards a progressive reduction of carbon dioxide emissions as well as energy consumption, boosting a "green revolution" based on the adoption of sustainable processes. Thus, there is a high industrial and commercial interest for sustainable, environmentally friendly, green chemistry extraction/purification processes to provide PBPs (particularly PC) of various purity grades on large scale while using a minimum number of steps, due to increasing world demand of safe, non-toxic, healthy bio-products.

Tan et al. [17] recently reviewed the research trends of PBPs using a bibliometric approach. They found that the principal issues addressed in the documents were the optimization of microalgae culture parameters and PBPs extraction methods. Moreover, bioactivity properties and extraction of PBPs turned out to be the future research priorities.

Indeed, a quick bibliography survey can easily show the growing interest on the extraction and purification of these compounds, that is proved by the growing number of published documents over the last years. As an example, a survey made on 1st August 2022, based on the Scopus database (combining the terms phycobiliprotein, phycocyanin, phycoerythrin, allophycocyanin, extraction and purification, and eliminating spurious documents) evidenced that in 2021 the number of published documents [83] was more than four time higher that ten years before [19]. In Fig. 4 is reported the number of documents per year from 2000 to 2021 retrieved in the survey, while in Fig. 5 is reported the number of documents retrieved in the same period, using the same database, in relation to the extraction (Fig. 5a) and purification (Fig. 5b) method adopted. Most often various extraction and, particularly, various purification methodologies are combined and used in sequence to achieve a better result.

The literature survey evidenced that ultrasound-assisted extraction was the most used PBPs extraction method (Fig. 5a). Ultrasound-assisted extraction is considered a green process [36]. It requires short operation time and low solvent consumption, allowing to achieve high yields, but the increase of temperature in the PBPs samples, which are temperature-sensitive compounds, must be controlled and avoided to preserve PBPs' functionalities [3, 34]. Ultrasound-assisted extraction is, in principle, suitable to large scale PBPs extraction, but a relatively low purity degree is generally achieved [37–39]. Other green methodologies applied to PBPs extraction are high pressure processes, pulsed electric field-assisted and microwaves-assisted extraction. Although it is reported that high pressure processes (such as hydrostatic pressure processing, in which

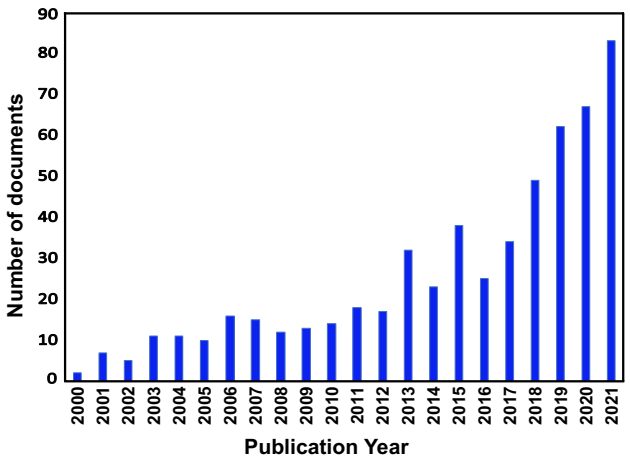


Fig. 4 Documents published from 2000 to 2021 retrieved from the Scopus database combining the terms phycobiliprotein, phycocyanin, phycoerythrin, allophycocyanin, extraction and purification, and eliminating spurious documents

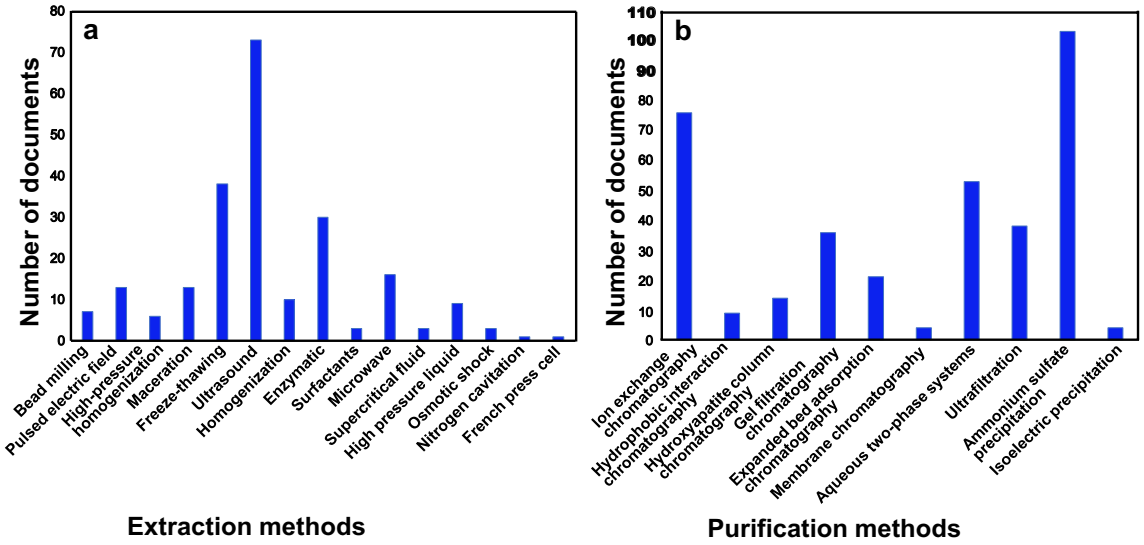


Fig. 5 Documents published from 2000 to 2021 according to (**a**) the extraction and (**b**) purification method adopted (Scopus database)

pressures around 300–600 MPa are applied, and high-pressure homogenization, in which lower pressures, usually up to 400 MPa, are applied [40]) may exhibit low selectivity [34], good results have been reported for PC extraction [41]. In addition to being considered eco-friendly, this methodology is also suitable to large scale production of PBPs. Other two processes considered green are pulsed electric field-assisted and microwaves-assisted extraction. However, the increase in temperature of the samples, caused by both methods, and the possible electrode corrosion and metal leaking in pulsed electric field-assisted extraction, are drawbacks limiting the application of these methods to the extraction of PBPs [34, 36]. Enzymatic-assisted extraction and repeated freeze–thawing are other two popular methods (Fig. 5a). Enzymatic-assisted extraction can ensure high yield, but it is not particularly suitable to large scale production of PBPs because of the high cost of the commercially available enzymes [36]. On the contrary, repeated freeze-thawing, though time consuming, it is particularly attractive thanks to its simplicity, reproducibility, and moderate cost. Therefore, it is commonly used to treat small quantity of biomass. In fact, repeated freeze–thawing efficiently lyses cells and ensures a higher purity respect to other approaches [3, 34]. However, it cannot be considered a general method since the cells of some cyanobacterial or algal species are too stiff to be broken by freeze–thawing and require a different approach. Indeed, a single preferrable PBPs extraction method does not exist, instead it must be chosen and optimised for each species. In addition, the type of biomass (fresh, spray-dried, freeze-dried, oven-dried, etc.) used as pigment source must be considered when choosing the optimal extraction method.

As concerns purification methods, Fig. 5b shows how ammonium sulfate precipitation (or salting out) was the most exploited one. Indeed, ammonium sulfate salting out is widely used in protein purification procedures [42], including PBPs [43], and is most often applied as pre-purification step, to obtain highly purified PBPs [44] (see Table 1). The highest PBPs purity is usually achieved by column chromatography, particularly ion exchange chromatography, or exploiting liquid (especially aqueous) two-phase systems [1, 35]. However, column chromatography is difficult to scale up, while aqueous two-phase systems are more suitable for large scale PBPs production [18]. Very recently membrane chromatography has been advantageously exploited to obtain analytical grade PBPs [29, 45, 46]. This methodology is interesting because fundamentally "green", cost-effective, environmentally friendly, and suitable to be used on an industrial scale [47]. Table 1 shows a summary of PBP extraction and purification procedures available in the literature.

1.2 Main environmental factors affecting the productivity of phycocyanin

As previously mentioned, currently, among the PBPs, PC occupies the largest market area as it is widely used in the food sector. Nowadays, commercial production of PC relies almost exclusively on *Arthrospira* (Spirulina) biomass. *Arthrospira* extracts were approved for use in candy, chewing gum and other types of confectionery in the US in 2013 and 2014 [104] while EU approved *Arthrospira* extracts in 2013 as food coloring [105]. The decision was based upon the fact that *Arthrospira* has been consumed for centuries and its consumption is considered safe worldwide [106].

Table 1 Procedures applied to extract and purify phycobiliproteins from cyanobacterial and algal species

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthronema africanum	Fresh	Freeze-thawing	Phosphate buffer pH 6.7	1. One rivanol treatment				[48] Minkova et al. (2007)
				2. $(NH_4)_2SO_4$ precipitation				
				3. Gel filtration chromatography				
				4. $(NH_4)_2SO_4$ precipitation	APC	2.41	35.0	
				1. Two rivanol treatments				
				2. $(NH_4)_2SO_4$ precipitation				
				3. Gel filtration chromatography				
				4. $(NH_4)_2SO_4$ precipitation	PC	4.52	55.0	
Arthrospira maxima	Fresh	Glass beads	$CaCl_2$ 100 mM	1. One aqueous two-phase extraction step	PC	2.10	99.5	[49] Rito-Palomares et al. (2001)
				1. Two aqueous two-phase extraction steps	PC	2.40	86.8	
				1. Two aqueous two-phase extraction steps				
				2. Ultrafiltration	PC	3.10	61.1	
				1. Two aqueous two-phase extraction steps				
				2. Ultrafiltration				
				3. $(NH4)_2SO_4$ precipitation	PC	3.80	29.5	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthrospira maxima	–	Freeze-thawing	Water	1. One vortex fluidic device-intensified aqueous two phase extraction	PC	1.3	93.0	[50] Luo et al. (2016)
				1. Four vortex fluidic device-intensified aqueous two phase extraction	PC	2.3	78.0	
				1. One vortex fluidic device-intensified aqueous two phase extraction 2. Ultrafiltration	PC	2.92	–	
Arthrospira. maxima	Fresh	Freeze-thawing	PBS: phosphate-buffered saline pH 7.0	1. Microfiltration 2. Ultrafiltration	PC	3.72	–	[30] García-López et al. (2020)
Arthrospira maxima	Dry powder	Maceration	Phosphate buffer pH 6.0	1. Ultrafiltration 2. Ion exchange chromatography 3. Gel filtration chromatography	PC	> 4.00?	–	[51] Park et al. (2022)
Arthrospira maxima	Dried	Maceration	Water	1. Ultrafiltration	PC	1.16	96.9	[52] Nisticò et al. (2022)
Arthrospira platensis	Fresh	a. Enzymatic (Lysozyme) b. Homogenization	Phosphate buffer pH 7.0 + EDTA	1. $(NH_4)_2SO_4$ precipitation	PC; APC	1.26; 0.52	–	[53] Boussiba et al. (1979)
				1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography	PC; APC	2.80; 4.12	–	
				1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	PC	4.15	–	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthrospira platensis	Fresh	High pressure homogenization	Water	1. Chitosan affinity precipitation				[54] Patil et al. (2006)
				2. Activated charcoal adsorption				
				3. Aqueous two-phase extraction	PC	5.10	66.0	
				1. Chitosan affinity precipitation				
				2. Activated charcoal adsorption				
				3. Aqueous two-phase extraction				
				4. Ion exchange chromatography	PC	6.69	–	
Arthrospira platensis	Dry powder	Maceration	$(NH_4)_2SO_4$ solution	1. Expanded bed adsorption chromatography				[55] Niu et al. (2007)
				2. Ion exchange chromatography	PC	3.64	8.7	
Arthrospira platensis	Fresh	High pressure homogenization	Water	1. Three aqueous two-phase extraction steps	PC; APC	4.02; 0.75	78.6; 51.7	[56] Patil et al. (2008)
				1. Three aqueous two-phase extraction steps				
				2. Ultrafiltration	PC; APC	4.02; 1.50	–; –	
Arthrospira platensis	Air dried	Bead milling	Water	1. Two $(NH_4)_2SO_4$ precipitation steps				[57] Moraes et al. (2009)
				2. Ion exchange chromatography	PC	4.00	–	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/ solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthrospira platensis	Fresh	Freeze-thawing	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[58] Su et al. (2010)
				2. Hydroxyapatite chromatography	APC	2.00	70.0	
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Hydroxyapatite chromatography				
				3. Ion exchange chromatography	APC	5.00	43.0	
Arthrospira platensis	Fresh	Freeze-thawing	Acetate buffer pH 5.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[59] Yan et al. (2011)
				2. Ion exchange chromatography	PC; APC	5.59; 5.19	67.0; 80.0	
Arthrospira platensis	Dry powder	Maceration	Phosphate buffer pH 6.8	1. Chitosan affinity precipitation				[60] Liao et al. (2011)
				2. Activated charcoal adsorption				
				3. Ion exchange chromatography	PC	4.30	42.3	
Arthrospira platensis	Freeze-dried powder	Maceration	Phosphate buffer pH 7.0	1. Expanded bed adsorption chromatography				[61] Bermejo et al. (2012)
				2. Ion exchange chromatography	PC	> 4.00	59.0	
Arthrospira platensis	Dry powder	Freeze-thawing	Water	1. One aqueous two-phase extraction step				[62] Zhao et al. (2014)
				2. Ultrafiltration	PC	1.31	89.5	
				1. Three aqueous two-phase extraction steps				
				2. Ultrafiltration	PC	2.11		
Arthrospira platensis	Air dried	Frozen + milled	Water	1. Ion exchange chromatography in Expanded bed mode	PC	1.60	79.0	[63] Moraes et al. (2015)
				1. Ion exchange chromatography in Fixed bed mode	PC	1.70	62.0	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthrospira platensis	Dry powder	Supercritical fluid	CO_2 + 10% ethanol	- None	PC	–	90.74	[24] Deniz et al. (2016)
Arthrospira platensis	Dry powder	Freeze-thawing	Phosphate buffer pH 6.5	1. One aqueous two-phase system step	PC	5.21	98.5	[64] Wang et al. (2017)
				1. Two aqueous two-phase system steps	PC	6.71	≈ 95.0	
Arthrospira platensis	Fresh	Ultrasound	TRIS-SO_4 buffer	1. Microfiltration				[28] Sala et al. (2018)
				2. Ultrafiltration	PC	1.60	93.4	
Arthrospira platensis	Freeze-dried	Maceration	NaCl 100 mM	1. Two membrane chromatography steps	PC	4.20	67.0	[45] Lauceri et al. (2018)
Arthrospira platensis	a. Fresh b. Freeze-dried	c. Freeze-thawing d. Freeze-thawing + ultrasound	NaCl 100 mM	1. Two membrane chromatography steps	PC	4.50^{ac}-4.20^{ad}	77.0^{ac}-76.0^{ad}	[29] Lauceri et al. (2019)
						4.30^{bc}-4.20^{bd}	81.0^{bc}-79.0^{bd}	
				1. Three membrane chromatography steps	APC	3.70^{ac}-3.60^{ad}	47.0^{ac}-38.0^{ad}	
						3.40^{bc}-3.30^{bd}	46.0^{bc}-36.0^{bd}	
Arthrospira platensis	Spray-dried	–	Phosphate buffer pH 7.3	1. Two $(NH_4)_2SO_4$ precipitation steps				[65] Böcker et al. (2020)
				2. Ultrafiltration	PC	3.1	–	
Arthrospira platensis	Spray-dried	Homogenization	Water	1. Ionic liquid-based aqueous two-phase systems	PC	–	83.26	[66] Choi et al. (2021)
Arthrospira platensis	Fresh	Freeze-thawing + homogenization	1.5% $CaCl_2$ (wt/vol)	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	–	–	[67] Ilter et al. (2021)
Arthrospira platensis	Fresh	a. High-shear homogenization b. Freeze-thawing c. Freeze-thawing + mixing d. Freeze-thawing + homogenization	Water	1. Anion exchange 3D-printed discs	PC	$4.20^{a, b, c, d}$	$41.0^{a, b, c, d}$	[68] Scorza et al. (2021)

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Arthrospira platensis	Dry powder	Freeze-thawing + ultrasound	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[69] Wan et al. (2021)
				2. Ultrafiltration				
				3. Hydroxyapatite chromatography				
				4. Gel filtration chromatography	PC	3.9	–	
Arthrospira platensis	Dry powder	a. Freeze-thawing b. Enzymatic (Lysozyme) c. Ultrasound d. Pulsed electric field	Phosphate buffer pH 6.8	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	2.47[a] 1.66[b] 2.15[c] 2.13[d]	– – – –	[70] Berrouane et al. (2022)
Arthrospira platensis	Air dried and powdered	High pressure	Phosphate buffer pH 7.0	- None	PC	3.59	100	[41] Carlos et al. (2022)
				1. Ion exchange chromatography	PC	4.74	35	
				- None	APC	1.72	100	
				1. Ion exchange chromatography	APC	2.19	38	
Arthrospira platensis	Freeze-dried	Maceration	Phosphate buffer pH 6.8	1. $(NH4)_2SO_4$ precipitation	PC	1.31	–	[71] Huo et al. (2022)
Spirulina (Arthrospira) sp.	Freeze-dried	Ultrasound + Freeze-thawing	Phosphate buffer pH 7.0 + NaN_3	1. Three $(NH_4)_2SO_4$ precipitation steps				[72] Patel et al. (2005)
				2. Ion exchange chromatography	PC	4.42	45.6	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Spirulina (Arthrospira) sp.	Dried	Maceration	Phosphate buffer pH 7.0	1. Two microfiltration steps				[73] Chaiklahan et al. (2018)
				2. Ultrafiltration	PC	1.22	100	
				1. Two microfiltration steps				
				2. Ultrafiltration				
				3. Activated charcoal column chromatography	PC	1.34	88.9	
				1. Two microfiltration steps				
				2. Ultrafiltration				
				3. Gel filtration chromatography	PC	2.81	51.9	
				1. Two microfiltration steps				
				2. Ultrafiltration				
				3. Ion exchange chromatography	PC	3.29	48.2	
				1. Two microfiltration steps				
				2. Ultrafiltration				
				3. Gel filtration chromatography				
				4. Ion exchange chromatography	PC	3.74–3.82	21.8–25.3	
Spirulina (Arthrospira) sp.	Dry powder	Enzymatic (Lysozyme)	Phosphate buffer pH 7.0	1. One $(NH_4)_2SO_4$ precipitation step	PC	2.01	75.0	[27] Mahendran et al. (2022)
				1. One $(NH_4)_2SO_4$ precipitation step				
				2. Ion exchange chromatography	PC	5.02	12.0	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Corallina elongata	Freeze-dried	Homogenization	PBS: phosphate-buffered saline pH 7.0	1. Hydroxyapatite chromatography				[74] Rossano et al. (2003)
				2. Gel filtration chromatography	PE	6.67	20.3	
Galdieria sulphuraria	Fresh	Freeze-thawing + homogenization	Phosphate buffer pH 7.2	1. Two $(NH4)_2SO_4$ precipitation steps	PC	1.10	53.0	[75] Sorensen et al. (2013)
				1. Ion exchange chromatography				
				2. One $(NH4)_2SO_4$ precipitation step	PC	3.50	21.0	
				1. $(NH4)_2SO_4$ precipitation step				
				2. Ion exchange chromatography	PC	3.50	31.0	
				1. One $(NH4)_2SO_4$ precipitation step				
				2. One aqueous two-phase extraction step				
				3. Ultrafiltration	PC	4.50	42.0	
				1 One $(NH4)_2SO_4$ precipitation step				
				2. One aqueous two-phase extraction step				
				3. Ion exchange chromatography	PC	4.50	39.0	
Galdieria sulphuraria	Dry powder	Freeze-thawing + ultrasound	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[69] Wan et al. (2021)
				2. Ultrafiltration				
				3. Hydroxyapatite chromatography				
				4. Gel filtration chromatography	PC	3.6	–	
Galdieria sulphuraria	Freeze-dried	Glass beads	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	0.71	–	[76] Avci et al. (2022)
Galdieria phlegrea	Fresh	High pressure homogenization	Phosphate buffer pH 7.2	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	–	–	[77] Carfagna et al. (2018)
Galdieria phlegrea	Fresh	High pressure homogenization	Acetate buffer pH 5.5	1. Gel filtration chromatography	PC	> 5.00	–	[78] Imbimbo et al. (2019)

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Galdieria phlegrea	Fresh	High pressure homogenization	–	1. Ultrafiltration	PC	5.00	80.0	[79] Ferraro et al. (2020)
Gracilaria corticata	Fresh	Homogenization in liquid N_2	Phosphate buffer pH 7.0	1. Native polyacrylamide gel electrophoresis	R-PE	4.20	44.3	[80] Sathuvan et al. (2022)
Grateloupia turuturu	Freeze-dried	Homogenization in liquid N_2	Water	1. Ultrafiltration	R-PE	1.07	100.0	[81] Denis et al. (2009)
Halomicronema sp. A32DM	Fresh	Freeze-thawing	TrisCl buffer pH 8.1	1. Two $(NH_4)_2SO_4$ precipitation steps	PE	1.46	–	[23] Madamwar et al. (2015)
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Gel filtration chromatography	PE	3.73	–	
Lyngbya sp.	Freeze-dried	Ultrasound + freeze-thawing	Phosphate buffer pH 7.0 + NaN_3	1. Three $(NH_4)_2SO_4$ precipitation steps				[72] Patel et al. (2005)
				2. Ion exchange chromatography	PC	4.59	36.8	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Lyngbya sp.	Fresh	Freeze-thawing	Phosphate buffer pH 7.2	1. Two $(NH_4)_2SO_4$ precipitation steps				[82] Sonani et al. (2014)
				2. Gel filtration chromatography				
				3. Ion exchange chromatography	PE	6.75	76.2	
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Triton X-100 precipitation				
				3. Gel filtration chromatography				
				4. Ion exchange chromatography	PC	5.53	60.2	
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Triton X-100 precipitation				
				3. One $(NH_4)_2SO_4$ precipitation step				
				4. Gel filtration chromatography				
				5. Ion exchange chromatography	APC	5.43	71.9	
Lyngbya sp.	Fresh	Freeze-thawing	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[83] Ghosh et al. (2020)
				2. Ion exchange chromatography				
				3. Gel filtration chromatography				
				4. One $(NH_4)_2SO_4$ precipitation step	R-PE	5.17	–	
Nostoc sp.	Fresh	Ultrasound	Phosphate buffer pH 7.0	1. Ion exchange chromatography				[84] Johnson et al. (2014)
				2. Aqueous two-phase extraction	PC	3.55	–	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Phormidium fragile	Fresh	Homogenization in liquid N_2	Tris–HCl pH 8.1	1. Two $(NH_4)_2SO_4$ precipitation steps				[85] Soni et al. (2008)
				2. Hydrophobic interaction chromatography	PC	4.52	62.0	
Phormidium sp.	Freeze-dried	Ultrasound + freeze-thawing	Phosphate buffer pH 7.0 + NaN_3	1. Three $(NH_4)_2SO_4$ precipitation steps				[72] Patel et al. (2005)
				2. Ion exchange chromatography	PC	4.43	35.2	
Phormidium sp.	Fresh	Freeze-thawing	TrisCl buffer pH 8.1	1. Two $(NH_4)_2SO_4$ precipitation steps	PE	2.77	–	[23] Madamwar et al. (2015)
Phormidium sp.	a. Fresh b. Freeze-dried	c. Ultrasound d. Glass beads e. Freeze-thawing f. Homogenization (mortar-pestle)	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[76] Avci et al. (2022)
				1. Two $(NH_4)_2SO_4$ precipitation steps	PC	2.11[bf]	–	
				2. Gel filtration chromatography	PE	4.26	–	
				1. Two $(NH_4)_2SO_4$ precipitation steps	PC	1.75[be]		
					PC	1.11[ae]	–	
					PC	0.70[ac]	–	
				1. Three $(NH_4)_2SO_4$ precipitation steps				
				2. Ion exchange chromatography	PC	2.33[bf]	–	
					PC	2.17[be]	–	
Phormidium sp	Fresh	Homogenization	Acetate buffer pH 5.1 + NaCl 50 mM	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	4.25	95.3	[86] Roy et al. (2022)
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Ion exchange chromatography	PC	5.56	56.4	
Plectonema sp	Fresh	Homogenization	Acetate buffer pH 5.1 + NaCl 50 mM	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	3.87	95.1	[86] Roy et al. (2022)
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Ion exchange chromatography	PC	5.63	32.4	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Pyropia yezoensis	Fresh	Freeze-thawing	Phosphate buffer pH 7.2–7.4	1. Two $(NH_4)_2SO_4$ precipitation steps				[46] Zang et al. (2020)
				2. Ultrafiltration				
				3. One membrane chromatography step	PC	5.80	14.7	
				1. Three $(NH_4)_2SO_4$ precipitation steps				
				2. Ultrafiltration				
				3. Two membrane chromatography step	PC	4.18	39.9	
				1. Three $(NH_4)_2SO_4$ precipitation steps				
				2. Ultrafiltration				
				3. Three membrane chromatography steps	R-PE	4.25	45.3	
Porphyra yezoensis	Dried	Freeze-thawing	Phosphate buffer pH 6.8	1. Two $(NH_4)_2SO_4$ precipitation steps	R-PE	2.93	75.59	[87] Xu et al. (2020)
				1. Two $(NH_4)_2SO_4$ precipitation steps				
				2. Deep eutectic solvents aqueous two-phase system	R-PE	3.82	69.99	
Porphyra yezoensis	–	–	–	Three-phase partitioning	R-PE	3.90	–	[88] Wang et al. (2022)
Porphyridium cruentum	Fresh	Freeze-thawing + osmotic shock	Acetate buffer pH 5.5	1. Expanded bed adsorption chromatography				[89] Bermejo et al. (2003)
				2. Ion exchange chromatography	B-PE	> 4.00	66.0	
Porphyridium cruentum	Fresh	Ultrasound	Water	1. Aqueous two-phase extraction	B-PE	3.20	90.0	[90] Benavides et al. (2006)
Porphyridium cruentum	Fresh	Ultrasound	Water	1. Isoelectric precipitation				[91] Hernandez-Mireles et al. (2006)
				2. Aqueous two-phase extraction	B-PE	4.10	72.0	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Porphyridium cruentum	Fresh	Bead milling	Phosphate buffer pH 7.4	1. Isoelectric precipitation	B-PE	1.76	65.0	[92] Ruiz-Ruiz et al. (2013)
				1. Isoelectric precipitation				
				2. Aqueous two-phase extraction				
				3. Ultrafiltration	B-PE	4.10	54.0	
Porphyridium cruentum	Fresh	Freeze-thawing + maceration	Acetate buffer pH 5.5	1. Expanded bed pulsing chromatography	B-PE	> 4.00	–	[93] Gonzalez-Ramirez et al. (2014)
Porphyridium cruentum	Fresh	Freeze-thawing + maceration	Water	1. Microfiltration				[94] Marcati et al. (2014)
				2. Two ultrafiltration steps	B-PE	2.30	48.0	
Porphyridium cruentum	Fresh	Freeze-thawing	Acetate buffer pH 5.5	1. Expanded bed chromatography (vortex flow reactor)	B-PE	> 4.00	78.1	[95] Ibañez-Gonzalez et al. (2016)
Porphyridium cruentum	Freeze-dried	Osmotic shock	Acetate buffer pH 6.0	1. Ultrafiltration	B-PE	1.70	98.1	[96] Tang et al. (2016)
				1. Ultrafiltration				
				2. Ion exchange chromatography	B-PE	5.10	68.5	
Porphyridium cruentum	a. Fresh b. Freeze-dried	c. Freeze-thawing d. Freeze-thawing + ultrasound	PBS: 10 mM phosphate, 100 mM NaCl, pH 7	1. Two membrane chromatography steps	B-PE	4.80^{ac}-4.80^{ad} 4.50^{bc}-4.50^{bd}	71.0^{ac}-69.0^{ad} 69.0^{bc}-62.0^{bd}	[29] Lauceri et al. (2019)
P. cruentum	Fresh	Maceration	20% $(NH_4)_2SO_4$	1. One three-phase partitioning step	B-PE	2.12	78.8	[97] Huang et al. (2022)
				1. Two three-phase partitioning steps	B-PE	1.75	91.0	
P. purpureum	Fresh	Maceration	20% $(NH_4)_2SO_4$	1. One three-phase partitioning step	B-PE	1.83	69.1	[97] Huang et al. (2022)
				1. Two three-phase partitioning steps	B-PE	1.59	88.9	
Rhodomonas sp.	Fresh	Freeze-thawing	Phosphate buffer pH 6.9	1. One $(NH_4)_2SO_4$ precipitation step	B-PE	1.69	81.4	[98] Pu et al. (2022)
				1. One $(NH_4)_2SO_4$ precipitation step				
				2. Hydrophobic interaction chromatography	B-PE	13.66	78.6	

Table 1 (continued)

Species	Biomass form	Cell lysis method	Extracting solvent/solution	Crucial purification consecutive treatments	Purified PBP	Purity (A_{PBP}/A_{280}) *	Y (%)	Refs.
Synechochoccus sp.	Fresh	Freeze-thawing	Carbonate buffer pH 10.5	1. Hydrophobic interaction chromatography				[99] Abalde et al. (1998)
				2. Ion exchange chromatography	PC	4.85	76.6	
Synechococcus sp.	Fresh	Triton-X 100 + ultrasound	Tris–HCl pH 8.0	1. Two $(NH_4)_2SO_4$ precipitation steps				[100] Sonani et al. (2017)
				2. Ion exchange chromatography	PC	4.03	–	
Synechococcus sp.	Fresh	a. Freeze-thawing b. Homogenization	PBS: phosphate-buffered saline	1. Foam fractionation	PC	0.75[b]	48.8[b]	[101] Antecka et al. (2022)
				1. One aqueous two-phase system step	PC	2.00[a]	85.0[a]	
					PC	1.31[b]	97.8[b]	
				1. Ultrafiltration	PC	2.15[a]	92.0[a]	
					PC	2.98[a]	49.0[a]	
Synechocystis aquatilis	Freeze-dried	Maceration	Phosphate buffer pH 7.0	1. Ion exchange chromatography in Expanded bed mode	PC	2.61	90.0	[102] Ramos et al. (2011)
				1. Ion exchange chromatography in Expanded bed mode				
				2. Ion exchange chromatography	PC	> 4.00	69.0	
Synechocystis sp.	Freeze-dried	Glass beads	Phosphate buffer pH 7.0	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	1.95	–	[76] Avci et al. (2022)
				1. Three $(NH_4)_2SO_4$ precipitation steps				
				2. Ion exchange chromatography	PC	4.84	–	
Synechocystis sp.	Fresh	High pressure homogenization	PBS: phosphate-buffered saline pH 7.8	1. Two $(NH_4)_2SO_4$ precipitation steps	PC	2.90	84.0	[103] Puzorjov et al. (2022)

*A_{280} = absorbance at 280 nm; A_{PBP} = absorbance at PBP maximum (PBP = phycobiliprotein, i.e., PC or APC or PE)

Therefore, information on production, extraction and purification of PC come mostly from *Arthrospira* cultures.

1.2.1 Light intensity and spectra

Both light intensity and wavelength can strongly modify the productivity and concentration of PC in the biomass. In general, acclimation to low light, achieved either by exposing cells to low intensity incident light or by increasing the density of cultures, entails a raise in both chlorophyll and PC contents [31]. In *A. platensis* cultures, during the logarithmic growth phase, the percentage of chlorophyll *a* (Chl *a*) doubled from 0.8% to 1.6% of dry weight, while the percentage of PC increased from an initial value of 3% up to 12–14% depending on the strain and light spectra at which cultures were grown [32].

Considering the high market value of PC, artificial light is often used for the production. Fluorescent lamps and light emitting diodes (LEDs) are used for the microalgal cultivation, with different spectrum and energy conversion efficiency. Fluorescence tubes have the advantage to deliver light irradiance at a 360 degree angle and are usually characterized by much larger emission bands compared to LEDs. However, due to small size, light weight, durability efficiency of energy conversion and longer operating life, LEDs have attracted the interest of algal biomass producers. LEDs have several advantages that have promoted their diffusion as the light source for the purpose of microalgae growth, and recent studies on the growth of *A. platensis* were conducted using fluorescent lamps [32, 107].

The cyanobacterium *A. platensis* grown under illumination with lights of different colors (white, orange and blue) shows absorption peaks in the blue and red part of the spectrum (440 and 680 nm), due to Chl *a*, and in the orange part (620 nm) due to PC (Fig. 6). The ratio between the PC peak at 620 nm and that of Chl *a* at 680 nm is higher for cells grown under blue light than under the orange and white light, indicating an increase in PC content under blue light.

It has been proposed that blue light creates an unbalance between the two photosystems, with an excess of energy at the PSI side and a deficiency at the PSII side of the photosynthetic electron transport chain of *A. platensis* [32, 108]. It is well known that cyanobacteria invest much more of their chlorophyll *a* in PSI than in PSII [109, 110]. This unbalance between the two photosystems is compensated for by the light-harvesting phycobilisomes (PBSs) which are mainly associated to PSII [111]. In this way cyanobacteria maintain a balanced excitation distribution between the two photosystems enabling the production of both ATP and NADPH necessary for growth. Under blue light, the PBSs do not absorb blue photons efficiently, as their short wavelength of 450 nm does not coincide with the absorption spectrum of PC (peaking at 620 nm). Therefore, under blue light, PBSs hardly transfer any energy to PSII. On the other hand, Chl *a,* which is more abundant in PSI, can transfer energy to PSI efficiently. Moreover, in cyanobacteria β-carotene, which absorbs blue light efficiently, is more abundant in PSI than in PSII, thus further contributing to photosynthesis light harvesting by PSI. Therefore, under blue light, PSII experiences a severe shortage of photons in comparison to PSI, with a strong limitation in the linear electron transport. Consequently, cell acclimate to blue light by enhancing the production of PBSs normally serving the PSII, so to restore the balance between the two photosystems. However, this strategy is ineffective towards growth since PBSs do not absorb blue light. These facts strongly support the findings that cultures grown under blue light have a much lower growth rate compared to orange and white lights, but a significantly higher PC content [32]

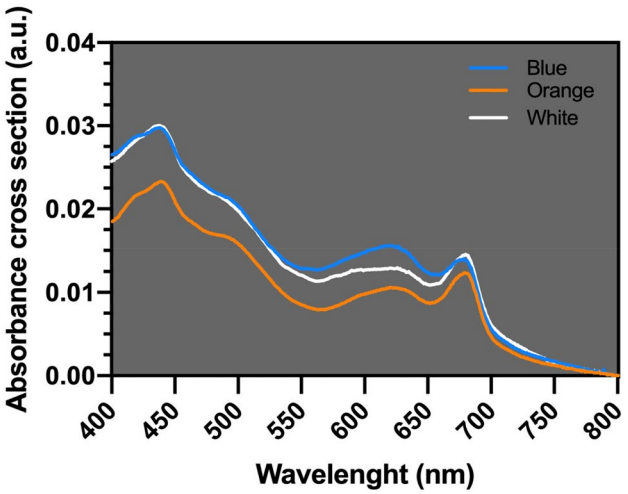


Fig. 6 In-vivo light absorption spectra of *A. platensis* cells grown under white, orange and blue lights, adapted from [32]

There is a consensus that blue light increases PC concentration in *Arthrospira* cells, while it is less effective in promoting growth which was much higher when culture was grown under white or orange lights [32, 112]. Therefore, a two-step strategy to produce *Arthrospira* biomass enriched in PC could be employed, starting by growing the culture under orange or white light, and once the culture has reached the stationary phase, shifting the light to blue to further enhance PC content. As synthesis of PC requires high amount of nitrogen, and the exposure to blue light can stimulate protein synthesis [113, 114], it is important to avoid nitrogen deprivation during the accumulation step, which may occur at the end of the growth phase. The lack of nitrogen in this step may even cause a reduction of the PC concentration, since PC would be used by cells as source of nitrogen [2]. Therefore, it is advisable to use a two-step process to improve the economic feasibility of the process [115].

1.2.2 Influence of nitrogen source on productivity of phycocyanin

PC is an intracellular nitrogen source, and it can be used in prolonged nitrogen-limited conditions [2, 32]. Thus, the content of PC can be strongly affected by the nature of the nitrogen source and its concentration in the culture medium. Depletion of nitrate in the culture medium leads to the reduction of PC while an excess of nitrate could lead to its inhibition [116]. According to [116], the optimal nitrate concentration for high PC concentration is in the range between 1200 and 1600 mg/l. Addition of glutamate and succinic acid to Zarrouk medium enhanced the PC concentration by about 20% [116]. The reason for this increase of PC content is that glutamate and succinyl-coenzyme A are among the intermediate metabolites in the biosynthesis of tetrapyrroles including phycobilin in cyanobacteria cells [116]. The type of nitrogen source plays an important role in the production and concentration of PC in the biomass. Studies carried out in *A. platensis* using different sources of nitrogen ($NaNO_3$, KNO_3, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, and urea), have shown that the highest PC production was attained by using $(NH_4)_2SO_4$. Higher biomass productivity was also accompanied by high PC content (11.3%) [117].

Ammonium is a more reduced form of nitrogen than nitrate and this may account for the better culture performance, particularly in the cyanobacterium *Arthrospira* whose protein content is very high (60–70%). However, at a pH of above 9, typical of *Arthrospira* cultures, ammonia (NH_3) is the dominant chemical species, and its diffusion through the cell membrane results in ammonia accumulation inside the cell and consequent toxic effect [118]. Ammonia causes damage to the manganese (Mn) cluster of the oxygen-evolving complex of the PSII complex, causing a considerable increase of sensitivity of PSII to photodamage [119]. Chlorophyll fluorescence measurements showed that the PSII damage is related to the light intensity, and that the inhibition of the PSII performance by ammonia is not relieved neither by re-incubating the cells in the dark, nor by reducing the ammonia concentration [120]. *A. platensis* cultures with high biomass density and without pH regulation resulted less susceptible to ammonia inhibition, most likely through the faster assimilation of ammonia present in the medium [121]. Nonetheless, care is needed when ammonium salts are used as a nitrogen source in *Arthrospira* cultures, particularly when pH is uncontrolled as it may occur in large scale ponds. Moreover, at high pH a consistent amount of ammonia is lost by outgassing, contributing to the increase of greenhouse gas released in the atmosphere, and to an increase in costs. For these reasons, ammonium should be supplied in a fed-batch way to prevent accumulation. The most used nitrogen source remains $NaNO_3$ as it can be supplied in a large amount to the culture (2.5 g/l in Zarrouk medium) without incurring in toxicity problems. Similar problems can be foreseen for the use of digestate for biomass production of cyanobacteria. This waste represents an excellent source of nitrogen and phosphorus as well as other minor nutrients. The amount of ammoniacal nitrogen in digestates ranges between 500 and 2000 mg/l, therefore it is inevitable to operate a dilution with fresh water. At the present the use of digestate in EU countries seems to be restricted to the production of biomass for use as feed. The legislation however doesn't specifically allow or prohibit the use of slurry to grow algae as it is permitted for land plants used in the feed chain [122].

1.2.3 Effect of high dissolved oxygen concentrations and light–dark cycle

Large raceway ponds equipped with paddle wheels are usually characterized by low turbulence, and in *Arthrospira* cultures, faster mixing achieved by increasing to rotation of the paddle wheels or by other means can cause breakage of filaments, and foam formation, and a consistent increase in production costs. Oxygen removal is therefore usually limited and O_2 concentration of 300–400% of air saturation are very frequent particularly in the afternoon [123]. Mass transfer coefficient can vary greatly in each section of the reactor: K*la* is higher in proximity of the paddle wheel (about 164 h^{-1}) while in the channel and bends it drops dramatically (below 1 h^{-1}). This is a clear indication that cultures are poorly mixed, not allowing for an adequate stripping of oxygen [124]. Oxygen stripping can be improved by placing a sump where air is injected. However, care is needed with filamentous cyanobacteria such as *Arthrospira,* as the injection of air under pressure may result a fragmentation and flotation of the trichomes.

The negative effect of oxygen on culture productivity of mass cultures has been overlooked for many years. Torzillo et al. [125] reported evidence that, in *Arthrospira* cultures, a concentration of oxygen above 30 mg L^{-1} had a negative effect on both growth and biomass protein content. However, the problem of oxygen toxicity becomes evident when cultivating microalgae in closed systems [126]. Only a few studies have addressed the potential role of singlet oxygen as a marker for resilience to excess light environments, and to find out whether singlet oxygen resilience correlates with a strain's robustness in excess light environments.

Hiday and Belay [127] followed the daily time course in the photosynthetic pigments in cultures of *Arthrospira* grown in large scale ponds (0.5 ha). Larger variations in the PC content correlated with higher dissolved oxygen concentrations in the pond. PC content fluctuated according to an opposite trend to that of the oxygen concentration. It was evident that the lowest level of PC coincided with that of the peak of oxygen [127].

The concentration of PC can also change during the day as the illumination conditions vary, decreasing as light increases. Changes in PC are particularly evident in low density cultures, particularly in summer. The PC content is usually higher in the morning particularly in low density cultures. Usually, in *Arthrospira* cultures exposed to high light, there is an accumulation of carbohydrates during the day. Because of nocturnal respiration of carbohydrates, the concentration of PC can result significantly higher in the morning [127, 128]. Therefore, the time of biomass harvesting can affect the PC content in the biomass.

1.2.4 Effect of temperature

Because of seasonal and diurnal fluctuations, light and temperature represent the major biological limitations for biomass production of microalgae. However, as pointed out by Borowitzka [129], temperature plays the most important role on productivity, and therefore it should probably be the first parameter for strain selection. In the case of *A. platensis,* laboratory experiments have shown that the maximum biomass yield is obtained when *Arthrospira* is grown at the optimal temperature of 35 °C [130–133]. However, outdoors, in open ponds, culture temperature can fluctuate from 15 °C in the early morning hours to about 35 °C in the middle of day, causing a significant reduction in productivity and changes of the biomass composition [127]. Using different strains acclimated to the different temperatures at different times of the year can be useful to extend the cultivation period and attain higher productivities year-round. This strategy has been successfully tested with *Arthrospira* at Earthrise Farm in California, USA [134].

Studies carried out on *Synechococcus* sp. PCC 7002, whose optimal temperature for growth is 38 °C, reported that when the strain was grown at 15 °C there was a loss of blue color by the cell in a similar manner to nutrient starvation [135]. The amount of PBP and chlorophyll decreased while there was a strong accumulation of glycogen. By contrast, when the cells were grown with urea as a nitrogen source, cells grew normally without any sign of chlorosis. The authors hypothesized that the defect in nitrogen assimilation was most likely due to an inefficiency of transport of nitrate across the cytoplasmic membrane.

Summing up, the PC production by cyanobacteria (and by *Arthrospira* in particular) strongly depends on maintaining the optimal conditions of temperature and light intensity at which cells are exposed, two environmental factors that can strongly change along the year and during the day. The culture system can affect the temperature profile and light uptake. The control of temperature is more easily achieved in a closed system, and more difficult to implement in open ponds. In this system, it is frequently observed that temperature does not rise fast enough in the morning in comparison to light which goes up very fast, and this desynchronization between the raise in light and temperature may causes cell photoinhibition [136, 137].

1.3 Strain selection

Surprisingly, while the cultivation of *Arthrospira* for industrial purposes, dates several decades back, the progress made in the selection of newer more productive strains is minimal. Indeed, any enhancement in strain performance would reflect immediately in industrial applications. Factors discouraging from planning a systematic strain selection activity are the necessity to have a large collection of strains which requires considerable manpower for its maintenance, and the too large variation in PC content determined by environmental factors which makes more complicate the evaluation of differences between strains. Moreover, it is worth to point out that because of the high morphological plasticity of *Arthrospira* strains, the selected laboratory strains once transferred in mass cultured may perform significantly different compared to the original laboratory strain. Indeed, outdoors, strains are subject to strong variability in the environmental factors, particularly light, temperature and oxygen exposure, and to hydrodynamic stress caused by mixing, which can strongly modify the morphology of trichomes and their biochemical composition. Prolonged cultivation of the same strain can generate phenotypes with improved performance, with higher resilience to temperature and light stress. This may justify the reluctance of the grower to change their strain, together with the necessity to guarantee a constant production and consistent biochemical characteristic of the product. In addition, employing higher PC content strains for industrial production must prove to be economically advantageous, compared to conventional strains. In other words, the increase in PC content should not be achieved at the expense of a reduced biomass production. Therefore, biomass productivity of the strain, is thus an important aspect that should not be overlooked during strain selection. Moreover, it often happens that the producer of the pigment is usually not the grower, and producers are interested in cheaper biomass rather than high concentration of the pigment in the biomass (Vonshak, personal communication).

Strain selection of a NaCl tolerant mutant of *A. platensis,* showed that the PC production was 12.2% as compared to its wildtype counterpart when cultivated in a nitrate and bicarbonate sufficient medium (40 and 60 mM, respectively) at pH 9.0 under phototrophic conditions [138]. Among 13 cyanobacteria species investigated, *Arthrospira* sp., *Pseudanabaena* sp. and *Synechococcus elongatus* might be the promising candidates for PC, PE and APC sources, respectively, for commercial production purposes

Table 2 Average PC content of different *Arthrospira platensis* strains cultivated under controlled laboratory and outdoor conditions

Strains	PFD (μmol m^{-2} s^{-1})		Culture density g (DW) L^{-1}	PC content (% DW)	Depositary culture collection	Origin	References
A. platensis M2	White	180	2.8	10.0	CNR-Italy	Mombolo Lake (Ciad)	[32]
	Orange	180	3.2	7.1			
	Blue	180	2.3	13.2			
A. maxima	White	180	3.9	10.3		Texcoco lake (Mexico)	[114]
	Orange	180	3.4	6.3			[114]
	Blue	180	1.6	14.7			[114]
A. platensis	White	35	0.6	7.2	Hiri company (Tamanrasset, South of Algeria)	–	[141]
A. platensis	Sunlight (Dry Powder)	Ambient temperature		8.7	Cyanothec Corporation Haway, USA		[141]
A. platensis	Sunlight Dihé	Ambient temperature		1.8	–	Lake Ciad	[141]
A. platensis	Sunlight	Open pond (0.5 ha)	Culture density 0.7 OD	min 11.0 max 14.0	Earthrise Nutritional LLC's Calipatria, CA	–	[127]
A. platensis M2	Sunlight	Vertical plates (3 m2)	3–5	min 4.3 max 6.3	CNR-Italy	Lago Mombolo (CIAD)	[142]
A. platensis	Sunlight	Tubular reactor (1 m2)	0.6–1.2	min 3.7 max 4.8	CNR-Italy	Lago Mombolo (CIAD)	Torzillo (unpublished)

[139]. Interestingly, the specific growth rate and biomass productivity of *Synechocystis* sp. were significantly higher than the other cyanobacteria. This was attributed to a higher surface to volume ratio (*S*/*V*) that allows this cyanobacterium to uptake more nutrients [140].

A comparison of the biochemical composition of different *A. platensis* strains from different geographical areas (Algeria, Chad, USA) was carried out. The *Arthrospira* strain from USA showed the highest PC content (% of dry weight) compared to the other strains [141].

In Table 2 shows the PC content found in different *Arthrospira* strains. To make the comparison among the different strains easier, the culture conditions are specified. The geographic origin of the strain and depositary institution is also indicated.

1.4 Production of C-PC with *Galdieria sulphuraria*: an alternative to Arthrospira

Galdieria sulphuraria is a red (Rhodophyta) polyextremophilic microalga which can tolerate very low pH (as low as 0.2), temperature up to 57 °C [77, 143, 144], and high osmotic pressure, up to 400 g L^{-1} of sugar, and 2–3 M of salt. Similarly, to *Arthrospira,* it contains only chlorophyll *a*, and accessory photosynthetic pigments are represented by blue PBPs, PC and APC. This organism can growth heterotrophically (in the dark), in which organic substrates represent the source of both carbon and electrons, as well as autotrophically (in the light) in which reducing power is generated via water splitting by PSII, while the source of carbon is CO_2. It can also grow mixotrophically, that is, a combination of heterotrophy and autotrophy [145–148]. Their rather unique ability to cope with very harsh growth conditions strongly reduce the risk of contamination even in heterotrophy, making this organism very attractive to produce PC and proteins (up to 72%), which are also richer in essential amino acids compared to those from *Arthrospira* and *Chlorella* [146]. Since this organism, contrary to *Arthrospira,* does not have a record history of its use as food and feed, it is considered a potential novel food by the EU regulation, therefore it needs to go through the procedure of admission as novel food before the approval [148]. Autotrophic cultivation of *Galdieria* requires a careful choice of the cell density and of the light supply to avoid risk of photoinhibition [146]. This phenomenon can occur after a strong dilution of a dense culture where most cells are acclimated to low light and thus may suffer of the suddenly raise in the specific light supply. Although this problem can be easily managed in laboratory by temporarily reducing the light supply, it may represent a problem when working with large scale bioreactors under solar light. The risk of photoinhibition is less

Table 3 Phycocyanin content of *Galdieria sulphuraria* and *Cyanidioschyzon merolae* biomass

Strain	Growth regime	PFD (μmol m^{-2} s^{-1})	Culture density (g L^{-1})	PC content (% DW)	Depositary culture collection	Origin	References
G. sulphuraria ACUF 054	Autotrophic	511	4.5	9.5	University of Naples (Italy)	Mt. Lawu (Indonesia)	[146]
	Mixotrophic	511	8.1	10.1			
	Heterotrophic	Glucose	4.5	0.3			[148]
		Glycerol	4.6	0.6			
Cyanidioschyzon merolae	Autotrophic	100	1.0	30	National Institute for Environmental Studies (NIES, Japan) n. 1332	–	[152]

relevant with mixotrophic cultures, particularly when they are grown according to an "oxygen balanced" regime, in which the supply of substrate, and thus the oxygen uptake is balanced with photosynthesis [145]. This culture mode allows to maintain the reactor closed and thus reduces the risk of contamination which may occur through the injection of air in the reactor.

Under heterotrophic conditions, PC content is very low, ranging from 0.3 to 0.6% DW, in cultures grown on glucose and glycerol, respectively. The PC content resulted 40-fold higher and 20-fold higher in cultures grown under autotrophically [147]. These results indicated that heterotrophy inhibits pigment synthesis particularly in cells grown on glucose. [148]. Heterotrophic cells grown on glucose became yellowish while those grown on glycerol still maintained the green color. The pigmentation was restored in glucose grown cells when it is totally consumed. PC content ranging between 0.8 and 1.2% of dry weight was reported by [149] in *G. sulphuraria* strain 074G under heterotrophy conditions in carbon-limited cultures and nitrogen sufficient cultures, while the PC content dropped to 0.1% in nitrogen deficient cells. Therefore, despite the much higher volumetric productivity attained with heterotrophic cultures, the PC content in this biomass is too low for economic extraction, with limitation of the commercial applications. Indeed, according to EU regulations, the concentration of pigment in the source determines whether the extract can be considered as a *food extract* with coloring property (> 3% of dry weight) or just a *natural food additive* (< 3% dry weight) [150]. Moreover, while food extracts are considered *food ingredients* and are used in clean label food products, the natural additives require an "E" number. Therefore, due to PC content, PC extracts obtained from heterotrophic *Galdieria* biomass fall into the additives categories, while PC extracts from autotrophic and mixotrophic cultures would enter in the first category, not requiring any labelling.

A strategy to conjugate the high biomass accumulation with high level of PC in *G. sulphuraria* was proposed [151]. It consists of sequential production of biomass in heterotrophy followed by dilution and exposure of cells to light to induce synthesis of PC. By this way it was possible to raise the PC content up to 13.88%, that is, higher than that attained by growing cells autotrophically or mixotrophically. Although this strategy may be appealing, the economic comparison between autotrophic, mixotrophic, and sequential heterotrophic/autotrophic needs to be assessed. The sequential heterotrophic/autotrophic strategy entails the need to strongly dilute the culture before exposure to light which increases the cost of harvesting, particularly with unicellular microalgae (Table 3).

Another thermoacidophile red microalga, *Cyanidioschyzon merolae*, belonging to the order Cyanidiophyceae of the phylum Rhodophyta, has been proposed for PC production [152]. Similarly to *G. sulphuraria,* it thrives at pH between 0.5 and 3, and tolerates temperatures as high as 56 °C. Indeed, expressed sequence tags and high-throughput genomic sequence reads covering > 70% of the *G. sulphuraria* genome were compared to the genome of *Cyanidioschyzon merolae*, and more than 30% of the *Galdieria* sequences did not relate to any of the *Cyanidioschyzon* genes [153]. Contrary to *Galdieria* which has a large metabolic flexibility, *C. merolae* is an obligate photoautotrophic organism, which restricts its cultivation to autotrophic conditions (light and CO_2). Interestingly, it lacks a cell wall, which makes it possible to extract PC with high degree of purity (up to 9.9) just using osmotic shock and centrifugation. The PC of *C. merolae* resulted stable to pH 4–5 up to 80 °C. Because of the high degree of purity of PC produced using this species, without applying time consuming and expensive purification procedures, its employment may be evaluated for production of analytical/reagent grade PC. However, the effective performance of this species needs to be assessed in mass cultures, where hydrodynamic stress could strongly hamper its cultivation.

1.5 Attempts to enhance phycocyanin stability

PC molecule proved to be very sensitive to environmental factors such as temperature, pH, and light, which can cause blue color to fade up to a total loss of color. This fact strongly hampers its utilization in food and cosmetics. Therefore, several efforts have been devoted to finding out the optimal conditions preventing PC degradation and thus increase its shelf-life. Different stabilization techniques have been proposed, by using preservatives as well as formulation processes. Food grade PC is usually used to assess its degradation, which is commonly determined by UV–vis spectrophotometry at 620 nm. The stability of the PC molecule is also influenced by its purity. A comparison between reactive grade PC and food grade, determined a higher thermostability in food grade PC [154]. However, it is worth noting that a hard purification procedure can affect the pigment stability. In general, gentle extraction and purification procedures are expected to better preserve PC stability. Most of the studies on PC stability have been carried out on PC extracted from *Arthrospira* (*Spirulina*) *platensis*, which is nowadays the major source of PC utilized worldwide.

1.5.1 Effect of temperature and use of preservatives to enhance the thermal stability

Temperature influences the stability of PC [[155] and references therein]. The stability of total PBPs from 13 isolated cyanobacteria after 24 h of incubation at − 20 °C resulted variable. The loss of PBP content ranged between 4.88% and 15.94%. The highest stability of total PBPs was observed in *Pseudanabaena* sp. (reduced by 4.88%), whereas PBP content from *Desertifilum* sp. extracts decreased the most by 15.94% over 24 h [156]. These findings may indicate that the stability of PBPs may be related to cyanobacteria species and/or strains. Recently, heterologous expression of phycocyanin derived from the thermophilic cyanobacterium *Thermosynechococcus elongatus*, was applied in the mesophilic cyanobacterium *Synechocystis* sp. 6803 to produce thermostable PC [103]. Importantly, the yields of PC in the *Synechocystis* mutant strains resulted comparable to that of native PC in wild-type *Synechocystis*, while the thermostability properties of PC matched those from *T. elongatus* [103].

Very often, experiments on the effect of temperature are associated to the use of preservatives to improve the pigment stability. Incubation of PC from *Arthrospira* biomass at temperatures between 47 and 64 °C, in a pH range of 5.5–6.0, caused a reduction in the concentration by 50% [157]. Adding 20–40% glucose or sucrose and heating to 60 °C for 15 min, the C_R (relative concentration) value of PC at pH 7.0 was maintained at around 62–70%, and the half-life increased from 19 min to 30–44 min. The addition of 2.5% sodium chloride had an even better preservative effect on PC at pH 7.0 since 76% of the PC concentration was retained [157].

PC was found to be stable at temperature as high as 45 °C, but the stability decreased proportionally between 45 and 70 °C. Sodium chloride (20% w/w) was an effective stabilizing agent for PC, and its efficacy was higher at higher concentrations used [158].

Consistently to other studies, at temperature of 74 °C the degradation of PC was increased ($t½$ 9.7 min, pH 6.0) [159]. However, stability of PC can vary significantly within the species studied. A different thermostability was found between the extracts of the cyanobacterium *A. platensis* and the polyextremophile *G. sulphuraria* (Rhodophyta) incubated at temperatures between 25 and 55 °C. In this Rhodophyta the PC absorbance remained stable (95% of initial value) until 55 °C and decreased steadily within 55 and 75 °C, down to 39% of the initial value. In the case of *A. platensis*, PC extracts started to degrade already at 45 °C losing 18% of absorbance at 620 nm, while at 75 °C the absorbance was reduced to only 20% of initial value [146]. Similar findings were also reported by [77], and by [160] who found that PC from *G. sulphuraria* was more stable than that from *A. platensis* under all conditions, especially in the range of 50–65 °C, and in neutral environment of pH 7. After 30 min incubation at 60 °C and pH 7, the color preservation rate of PC from *G. sulphuraria* and *A. platensis* were 86.66% and 60.83%, respectively.

The higher thermostability showed by the PC extracted from *G. sulphuraria* is likely the result of a long adaptation process of the organism to the extreme environment (temperature up to 57 °C) where it still thrives. In conclusion, temperatures lower than 45 °C are generally considered optimal to preserve PC stability.

1.5.2 Effect of pH

The pH of the PC extract is another environmental factor that can destabilize the pigment. The pH of the solution can also modify the color of the PC solution. At neutral pH, the PC color is perceived as blue, while at acidic pH it is green. The optimal pH range for PC was found to be between 5.0 and 6.0 [158]. Usually, acid compounds are added to beverages to provide tartness and tangy taste to balance the sweetness of sugar added to beverages. It was reported that *Arthrospira* extracts lost 60% of the absorbance at 620 nm moving from pH 5.5 to 3.5, while within the same range of pH, *Galdieria* extract maintained 100% of the absorbance. Thereafter, the stability declined in a linear manner reaching about 30% of the initial value at pH 1.5. [146] As said above, this significantly greater tolerance to high acidity showed by *G. sulphuraria* PC extract is probably the result of a

long-term adaptation of this organism to extremely acidic environments, with pH as low as 0.2.

1.5.3 Effect of light

Several studies have assessed the degradation kinetics of aqueous solutions of PC evaluating temperature or light as accelerating factors using a first order kinetic model, and both environmental factors have been studied separately to evaluate the effect [158, 161]. Pérez-Rico et al. [162], have developed an empirical model able to predict the effect of temperature and light combined in the degradation ratio of this pigment at selected storage condition. They reported that exposure of PC to a photon flux density of 50 and 100 µmol m^{-2} s^{-1} resulted in a decrease in the concentration according to a dose-dependent pattern. The light-induced degradation resulted dependent on pH; at pH 6, the pigment degradation was lower compared to pH 5 and pH 7.0. The final protein concentration declined by 20% after continuous exposure to a photon flux density of 100 µmol m^{-2} s^{-1} for 36 h, independently of the pH of the solution. Therefore, to preserve PC, the best storage condition is in the dark.

1.5.4 Uses of preservatives

Since degradation of the protein structure of PC strongly affects its color and bioactivity, preservatives are added to ensure a longer shelf life of the product [[155] and references therein]. Both the chemical composition and the preservative concentration are relevant aspects since the resulting mixture must not affect the human health. The most used preservatives are mono or di-saccharide (glucose, fructose, saccharose, lactose, maltose) and the polyol sorbitol [71, 154], organic acids (citric, ascorbic, benzoic), and sodium chloride and calcium chloride among inorganic salts. Among the acidic preservatives, citric acid performs better. Indeed, with citric acid, 67% of the PC remained stable after 45 days compared to less 3% for the control [163]. This result is explained by both the decrease of pH of the solution caused by the addition of citric acid, and by its chelating property.

1.6 Market

The commercial value of PC is strongly dependent on its purity grade. The commercial price of PC as a colorant in food and cosmetics industries (the largest market) is about 0.35 US$ and 135 US$ per gram respectively, and can reach 4600 US$ per gram for therapeutic and diagnostic applications [169].

Figure 7 reports the production volume (tons) forecasted within the period 2020–2027 [22]. By 2027 the total volume of PC production is expected to raise 7.2-fold in Middle East & African countries, to a maximum near ninefold for

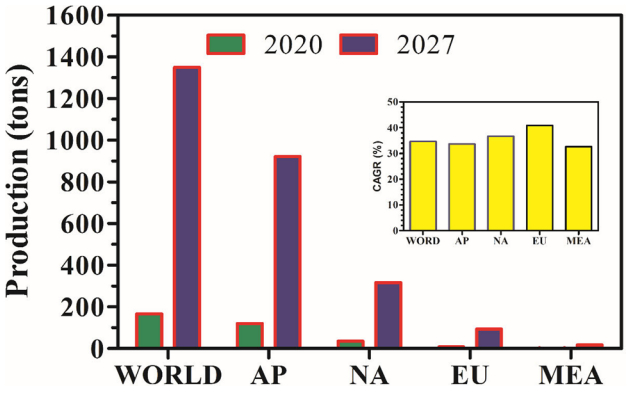


Fig. 7 Production volume of phycocyanin by countries. AP, Asia–Pacific; NA, North America; EU, European Union; MEA, Middle East & Africa. Insert shows the CAGR (%) calculated within the period 2000–2027. Data source [21] adapted

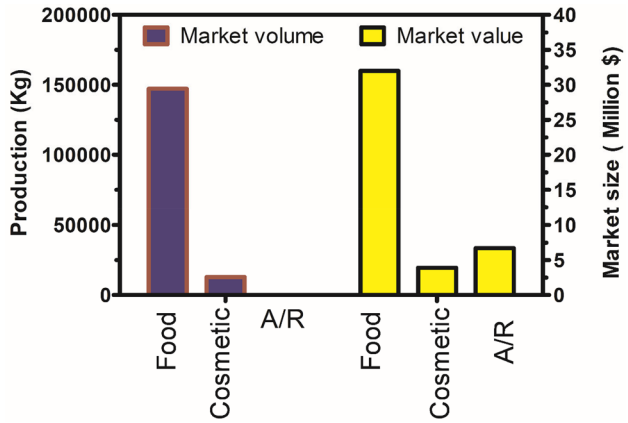


Fig. 8 Comparison of the different PC sectors and their respective market value. Due to its very low production size (4.4 kg), the analytical/reactive grade market volume cannot be appreciated (A/R = analytical/reactive grade). Data source [21], adapted

European countries. During the same period, the calculated CAGR (compound annual growth rate) is expected to range within about 33% for Middle East & Africa countries to near 41% for European Union (Fig. 7 insert).

The increasing demand of powder is due to its use as alternative for synthetic color in food and beverages, being PC preferred by consumers attracted by organic and natural ingredient-based food products, and by regulations which are posing increasing constraints to the use of synthetic colors. Western Europe is the biggest consumer of this product (around 33%) and that 80% of PC produced is used in the food industry. Pharmaceutical, is another promising sector which will likely see a sizeable increase by 2030. In 2020, PC turnover accounted for more than $12 million, and it's expected to rise to 36 million $ with a CAGR of 10%. Other than the generally recognized

antioxidant properties attributed to PC, its use for cancer diagnosis generates further demand in pharmaceutical industry. By 2030 the turnover of the global PC market is expected to exceed $400 Million with an estimate CAGR of 9.6% within 2021–2030 [164].

Figure 8 shows the share of PC among food, cosmetic and analytical/reactive recorded in 2020. It can be seen, the largest part of PC is destinated to food, while the part used for pharmaceutical purposes, is incomparably lower (only 4.4 kg). Yet, when the comparison is made based on market value, the analytical-reactive grade surpasses that of cosmetic, owing to its much higher price.

The key players of the PC market include Earthrise Nutritionals LLC, Bluetec Naturals Co., DDW Inc., DIC Corporation, Japan Algae Co. Ltd., Phyco-Biotech Laboratories, Cyanotech corp, Parry Nutraceuticals Qingdao ZolanBio Co. Ltd., Sigma-Aldrich Corporation, and Yunnan Green A Biological Project Co. Ltd, BueBiotech int. GmbH, Algosource.

1.6.1 Concluding remarks

PBPs are valuable, safe and healthy bio-compounds with many bio-technological applications and an increasing global market. However, their large-scale production is still restrained by the complexity and high cost of the available extraction and purification methods. In fact, PBPs extraction and purification procedures usually involve numerous steps, which reduce product yield and increase the costs, impairing the exploitation at a large scale. Most of the proposed procedures are often time-consuming, and can affect the quality of the final product, since PBPs are photo- and temperature-sensitive.

A few published works have addressed these critical issues. For example, proposed procedures are characterized by a minimum number of steps so as to reduce the production time and cost. Generally, in those studies, the ordinary approach characterized by two distinct phases (extraction followed by purification) was avoided. For example, stirred fluidized bed chromatography [63, 165], aqueous two-phase systems [166], glycerol-based natural deep eutectic solvents [167], three-dimensional printed anion exchange monoliths [168] and three phase partitioning processes [88, 97] have been successful applied directly on cyanobacteria or algae biomass, obtaining crude extracts of commercial interest, having good purity grade. Recently, an innovative extraction/purification method, based on ultrasound cell lysis in ammonium sulphate solution, was exploited to obtain PC from fresh *A. platensis* biomass [169]. In this process PBPs extraction was decoupled from biomass cell lysis, and the pigment recovered in a subsequent extraction step, obtaining a crude PC extract of elevated purity grade and concentration. However, the economic feasibility of this procedure should be proved on an industrial scale. New PC microalgal strains need to be isolated and selected from nature. An example is represented by the Rhodophyta *G. sulphuraria* which has recently attracted the attention of researchers owing to the large metabolic flexibility, and its capacity to grow under prohibitive conditions for the majority of other PC producers. However, the production cost with other PC producers, such as *Arthrospira* need to be addressed.

2 Primary carotenoids in microalgae

2.1 Metabolic pathways of carotenoids synthesis in microalgae

Carotenoids exist in a large variety of colors ranging from yellow to orange, red and purple. They are composed of eight isoprene units with a 40-carbon skeleton, generally consisting of a polyene chain with nine conjugated double bonds and an end group at both ends of the polyene chain. The five-carbon (C5) ubiquitous precursor metabolites isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are necessary to produce carotenoids. Two distinct routes, the MEP (methylerythritol phosphate) pathway and the MVA (mevalonate) pathway, contribute in the synthesis of these compounds in biological systems [170].

The biosynthetic pathway of carotenoids is reported in Fig. 9. Isopentenyl pyrophosphate (IPP, C5) and its isomer dimethylallyl diphosphate (DMAPP, C5) are converted at a ratio of 3:1 to geranylgeranyl pyrophosphate (GGPP, C20) via the methylerythritol phosphate pathway (MEP) in the chloroplast. In a further reaction, phytoene synthase condenses two molecules of GGPPS into (15Z)-phytoene (C40), which is then desaturated and isomerized into lycopene via phytoene desaturases, ζ-carotene isomerase, ζ-carotene desaturase, and carotene isomerase [171].

The initial step of carotenogenesis, which enables the creation of both α-carotene and β-carotene in algae, involves lycopene. A crucial branching point is the determination of the proportion of lutein coming from α-carotenoids, and β-carotene, coming from β-carotenoids. Zeaxanthin, which can derive from β-carotene, is epoxidized to violaxanthin, via antheraxanthin (xanthophyll cycle of violaxanthin). The other xanthophyll derived from violaxanthin are: (i) neoxanthin, by a violaxanthin de-epoxidase-like enzyme recently reported [172], which can be converted to dinoxanthin, which, in turns, generates vaucheriaxanthin and peridin, (ii) diadinoxanthin, that can be de-epoxidated to diatoxanthin (diadinoxanthin cycle). Both diadinoxanthin and dinoxanthin can be converted into fucoxanthin, by an enzymatic pathway still unclear.

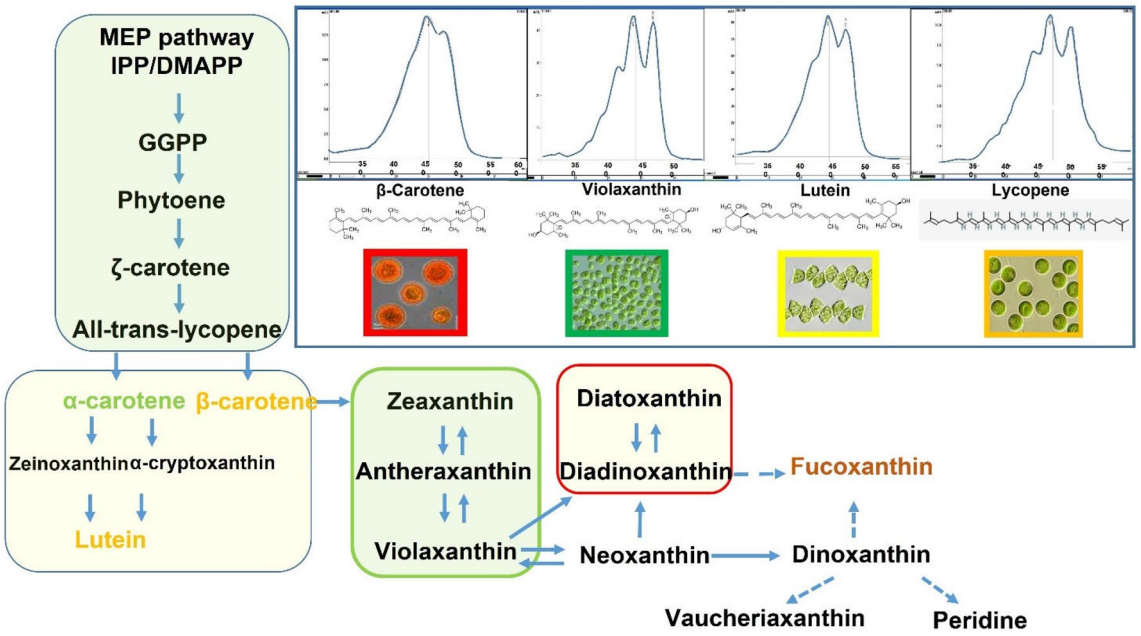


Fig. 9 Scheme of carotenoid synthesis pathways in microalgae. The microalgae *Dunaliella* sp. (red border, diameter: 9–11 µm), *Nannochloropsis* sp. (green border, diameter: 2–4 µm), *Scenedesmus* (yellow border, 11–18 µm long, 3.5–7 µm wide) and *Chlorella* sp. (orange border, diameter *2–10 µm*) are presented as principal producers of β-carotene, violaxanthin, lutein, and lycopene, respectively. Dashed lines indicate still unclear pathways

Carotenoids may be divided into two classes based on their chemical composition: carotenes and xanthophylls. While xanthophylls, which include β-cryptoxanthin, lutein, zeaxanthin, astaxanthin, fucoxanthin, neoxanthin, and peridinin, are carotenoids with oxygen atoms, carotenes, such as lycopene, α-carotene, β-carotene and γ-carotene, lack oxygen.

Carotenoids are divided into primary and secondary. The major carotenoids, such as certain xanthophylls and ß-carotene, are often found in the chloroplast and are directly involved in photosynthesis due to their function in the collection of light energy.

Under stress conditions (nutrient deficiency, high light, salt stress, high/low temperature), the photosynthetic system would not be able to effectively use the absorbed light energy, and the dissipation process might be activated. Additionally, a consistent supply of secondary carotenoids can be developed to serve as a free radical and reactive oxygen species quencher.

2.2 Main microalgal strains high producer of carotenoids

When microalgal cells receive a signal of high reductive state level inside the cells, specific actions are started to dissipate the accumulated electrons. These methods entail the synthesis of antioxidant carotenoids such as lutein, the xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin, as well as loroxanthin and fucoxanthin, which are largely found in the marine microalgae *Phaeodactylum* and *Isochrysis* [173–175].

The xanthophyll cycle is activated by the thylakoid lumen acidification, with the activation of the enzyme violaxanthin-de-epoxidase, leading to the synthesis of zeaxanthin, via antheraxanthin [176, 177]. In diatoms and dinoflagellate, such as *Phaeodactylum* and *Isochrysis,* respectively [173] an additional xanthophyll cycle can be found, consisting in diadinoxanthin, which can be de-epoxidized to diatoxanthin [178]. Thanks to the induction of these cycles, the reduction of the singlet oxygen inside the cell is activated, and the cellular damage can be avoided or reduced. The carotenoids production of some of the most studied and productive microalgal strains is reported in Table 4.

Haematococcus pluvialis, Chlorella species, *Dunaliella salina, Scenedesmus* species, *Botryococcus braunii, Nannochloropsis* species, *Coelastrella striolata, Chlorococcum* species, and some diatoms are known for producing β-carotene, lutein, canthaxanthin, astaxanthin, and fucoxanthin among the microalgae [171, 185, 195, 196].

The green microalga *Chlorella vulgaris* is well-known for its use as supplemental nutrition and for having a high protein and chlorophyll content in its biomass. A significant quantity of lutein, β -carotene, and zeaxanthin (1.18 mg g^{-1},

Table 4 Microalgal carotenoid contents and productivity, adapted from [186]

Carotenoid	Content	Productivity/yield	Microalgae	Refs.
Lutein			*Chlorella sorokiniana* FZU60	[179]
			Chlorella vulgaris UTEX 265	[180]
			Chlorella vulgaris CS-	[181]
	7.4–42.0 mg g^{-1} DW	3.4–20.0 mg L^{-1} d^{-1}	*Scenedesmus* sp.	[182]
			Chlorella sp. GY-H4	[183]
			Chlorella sorokiniana MB-1-M12	[184]
	6.49 mg g^{-1} DW		*Coelastrella sp.*	[185]
	2.9 mg g^{-1} DW		*Scenedesmus bijugatus*	[185]
Violaxanthin	3.7 mg g^{-1} DW	–	*Chlorella ellipsoidea* *Chlorella vulgaris*	[186] [187]
β-Carotene	30–130 mg g^{-1} DW	–	*Dunaliella salina*	[188]
		–	*Nannochloropsis oceanica* CCNM 1081	[189]
	9 mg g^{-1} DW		*Chlorella zofingiensis*	[185]
Zeaxanthin	13.15 mg g^{-1} DW	0.72 mg L^{-1} d^{-1}	*Coelastrella* sp. M60	[190]
	4.26–27 mg g-1 DW		*Chlorella ellipsoidea*	[191]
	11.20 mg g^{-1} DW		*Chlorella saccharophila*	[192]
	30.2 mg g^{-1} DW		*Nannochloropsis oceanica* strain CCNM 1081	[189]
	97.4% of total carotenoids		*Porphyridium cruentum*	[193]
Diadinoxanthin	19% of total carotenoids	–	*Phaeodactylum tricornutum*	[194]
Diatoxanthin	17% of total carotenoids	–	*Phaeodactylum tricornutum*	[194]

0.31 mg g^{-1}, and 0.24 mg g^{-1}, respectively) with good bioavailability have also been recorded for this microalga [197]. Interesting results have been reported in *Chlorella* strains for the production of lutein, as primary carotenoids, under heterotrophic conditions [198]. In particular, up to 5.3 mg g^{-1} of lutein has been extracted from heterotrophic culture, and around 7.5 mg g^{-1} in mixotrophic cultivation [199, 200]. Lutein production and choice of culture medium may depend on the strain and the cultivation conditions: for *C. protothecoides* and *C. pyrenoidosa*, under heterotrophic conditions, glucose is preferred, while under phototrophic conditions the highest lutein increase is obtained with acetate.

Dunaliella salina, a green unicellular microalga that primarily produces β-carotene, may also be regarded as a strong carotenoid producer. In this microalga, the pigment begins to build up in lipid structures on one side of the cell, and eventually it fills the entire cell.

It is possible to improve the synthesis of carotenoids in the green microalga *Asterarcys quadricellulare*, a potential good producer of carotenoids, by regulating the medium composition, pH, salinity, light quality, intensity, and duration [201]. The amount of β-carotene, lutein, astaxanthin, and canthaxanthin it can generate per dry gram of biomass is 47.0, 28.7, 15.5 and 14.0, respectively.

Scenedesmus can produce significant levels of lutein, which turns cells from green to yellow, and is a significant source of antioxidant carotenoids. A strain of *S. obliquus* isolated from Kapulukaya Reservoir (Kırıkkale, Turkey) has been found to produce a considerable amount of carotenoids, mainly found as -trans forms of lutein, β-carotene, α carotene, zeaxanthin, cis-neoxanthin, 9-or 9'-cis-α-carotene, 13-or 13'-cis-lutein, 13-or 13'-cis-β-Carotene, 9-or 9'-cis-lutein, cis-lutein, 9-or 9'-cis-β-Carotene, high amount of carotenoids, among them, the most abundant were all-trans isomers of lutein and β-carotene, being 83.74% (2.52 mg g^{-1}) in total carotenoids [202]. These results are interesting since they demonstrate that there is a large variety of microalgae that may be considered a valuable source of natural chemicals.

2.3 Main environmental factors affecting the production of primary carotenoids

The over-reduction of the photosynthetic chain and the production of free radicals are caused by variables that have an impact on growth and photosynthetic efficiency. Optimal nutrient composition, and optimal values of light intensity, pH, salinity and temperature, are some of the parameters that vary according to the strain, specie, and origin. Among these parameters, a crucial one is light intensity. The photosynthetic system has various physiological restrictions that prevent it from utilizing light irradiation above a certain level of intensity. Despite being a useful method for accumulating antioxidant compounds, inducing the synthesis of carotenoids through exposure to stress conditions like high light, nutrient limitation-starvation, excessive low or high

temperature, or salinity stress is not suitable in terms of biomass productivity, as in these circumstances growth is severely impacted.

2.3.1 Light intensity

The microalga *Chlamydomonas reinhardtii*, which is regarded as a model organism for physiological and biochemical investigations on photosynthesis, has been used extensively in research in this respect. In this microalga, zeaxanthin production was triggered within 10 min of exposure to 800 mol photons m^{-2} s^{-1}, while a partial induction of the cycle was seen at 300–350 mol photons m^{-2} s^{-1} [177, 203, 204].

With the activation of the other xanthophyll cycle, the synthesis of diatoxanthin by the de-epoxidation of diadinoxanthin, microalgal cells promptly react to excessive light intensities. In *P. tricornutum*, the conversion of diadinoxanthin to diatoxanthin is very quick, as it can happen in outdoor cultures exposed to direct sunlight in as little as 15 min.

Lutein is among the powerful antioxidant carotenoid reported to be enhanced in high light conditions. As a main carotenoid, this pigment is naturally present in most photosynthetic cells, and under conditions of photo-oxidative stress, its production may rise. Under conditions of 1900 mol photons m^{-2} s^{-1} and 35 °C, the microalga *Scenedesmus almeriensis* in a tubular photobioreactor outdoors produced extremely high levels of lutein (around 5 mg m^{-2} d^{-1}) [205]. The simultaneous presence of high light and heat was the cause of this increase. In fact, this microalgal strain typically thrives best at temperatures between 25 and 28 °C.

Another key carotenoid that often rises in content with increased light exposure is β-carotene, which is naturally present in microalgal cells but may be over-synthesized in certain conditions.

Among the microalgae with a reputation for being a good producer of this molecule, surely *Dunaliella salina* is one of the most promising. In a lab setting, with a light intensity of 200–1200 mol photons m^{-2} s^{-1}, at 30 °C, a production rate of 13.5 mg $^{-1}$ d^{-1} has been reported [206].

Studies on a strain of *Tetraselmis* sp. showed that under a light intensity of 170 µmol photons m^{-2} s^{-1}, the lutein and β-carotene content increased to 3.17 and 3.21 mg g^{-1} of dry weight, respectively [207]. In addition to the light stress, this strain was subjected to the quite high temperature of 30 °C, revealing a thermotolerance that is a suitable characteristic for cultivation under extreme conditions.

2.3.2 Nutrient limitation or starvation

Sulfur is essential for maintaining protein synthesis, hence its lack or restriction results in a sharp decrease in protein content, which in turn affects photosynthetic activity and slows development. In closed photobioreactors, the drop in photosynthetic oxygen evolution and the presence of oxygen respiration may result in the formation of extremely low oxygen contents or even anaerobic conditions, a very strong reductive environment [208]. The microalga *C. reinhardtii* has been the subject of many investigations for its capacity to produce hydrogen in the absence of sulfur. Interestingly, and the xanthophyll cycle was strongly induced with a significant increment in antheraxanthin, zeaxanthin, and violaxanthin levels. The amounts of the other xanthophylls, lutein and β-carotene, also rose [177]. Additionally, anaerobiosis also induced the same reaction over a longer period but in a complete medium (5 h, instead of some minutes). Antheraxanthin and zeaxanthin dropped after aerobic conditions had been achieved, along with lutein and β-carotene, suggesting the occurrence of a recovery.

The xanthophyll cycle is induced in *Nannochloropsis gaditana* by a sulfur restriction or deprivation, with an increase in violaxanthin and zeaxanthin as was seen in the case of nitrogen [209].

Since nitrogen is an essential component of proteins and enzymes, the lack of this nutrient in the culture medium strongly affects both the growth and biomass composition. *Dunaliella*'s ability to produce β-carotene under conditions of intense light exposure is increased by nitrogen deprivation. The combination of nitrogen starvation and exposure to the light intensity of 200 mol photons m^{-2} s^{-1} induced an increment in β-carotene to reach levels of 2.7% of the biomass [210].

Under both severe light stress and nitrogen deficiency, the increase of this pigment has been linked to the production of total fatty acids [211]. The production of this carotenoid and the fatty acid biosynthetic pathway are strongly related, which may be explained by the fact that β-carotene is stored in lipid globules. This element is quite intriguing and demonstrates how the metabolic pathways in microalgae are closely linked to provide an effective and efficient response to stressors.

The xanthophyll cycle is induced in *Nannochloropsis gaditana* by nitrogen restriction or deprivation, which results in an increase in violaxanthin and zeaxanthin. In [209] the cells underwent sulfur limitation or starvation after cultivation on complete medium to produce a sizeable quantity of biomass. In this instance, xanthophyll pigments were enhanced in the biomass. Given that this pigment participates in the formation of zeaxanthin via antheraxanthin, the violaxanthin rise was less pronounced. The high induction of carotenoids synthesis is signaled by its increase, which points to violaxanthin's de-novo synthesis.

Phosphorus has a crucial role in the cellular composition of DNA, RNA, and phospholipids as well as the control of enzyme activity, metabolic pathways, and ATP synthesis. It can be provided as polyphosphate or orthophosphate, and its

inclusion in the medium composition ensures that photosynthetic activity will be effective [212].

The xanthophyll cycle is induced in *Nannochloropsis gaditana* by phosphorus restriction or deprivation, with an increase in violaxanthin and zeaxanthin, as shown in the case of nitrogen [209].

2.3.3 Salt stress

Salt stress is one of the most significant abiotic variables influencing the development and productivity of microalgae. Some microalgae have developed saline environment adaptation methods, and in these unfavorable environments, they may control the creation of osmoregulatory chemicals that can assist the cells in dealing with stress. Particularly, osmotic stress can cause a rise in the concentration of various polar lipids, such as glycolipids and phospholipids, in chloroplasts and cellular membranes. As previously mentioned, several carotenoids depend on lipid production for their placement, and frequently, lipid production is closely related to the synthesis of carotenoids.

In fact, an excess of the ions Na^+ and Cl^- reduces the osmotic potential of the environment, which in turn reduces water intake, resulting in a wide range of bioenergetic and biochemical alterations in photosynthetic species under salt stress. The effects of salt stress on microalgal cells result in modifications and effects at several levels, including changes in membrane permeability, ion homeostasis blockade, increased catabolism of lipids and biopolymers, and altered yield of energy-producing activities.

Salt stress can be used as a strategy to encourage the synthesis of carotenoids, since like other stressors it may have a significant influence on the growth rate of microalgal cultures and also lower photosynthetic activity. An increase in NaCl concentration inhibited the growth of *Chlorella zofingiensis* while the quantity of carotenoids was enhanced [213].

The effect on lutein production in *C. vulgaris* has been reported by [214]. The results showed that using different levels of salinity stress, the highest increase of lutein content (11.36 mg g^{-1}) was induced by a salt concentration of 35 ppt, the highest tested. The influence of high light intensity and salt stress was investigated also in *Scenedesmus sp*. by [215]. The authors reported the production of 6.45 mg g^{-1} of lutein, with NaCl 156 mmol L^{-1}, under exposure to 160 µmol photons m^{-2} s^{-1}. In a strain of *Scenedesmus* grown on 10 g L^{-1} of NaCl the increase of carotenoids content was observed, particularly the xanthophyll component [216].

According to [217] salt stress in the range of 0.34 M to 0.51 M caused a decline in the growth rate in *C. vulgaris*. This decline may be related to an imbalance in ion homeostasis, which reduces photosynthetic activity, affects light utilization and the metabolism of carbohydrates, which is involved in osmotic regulation, and leads to the accumulation of reactive oxygen species. As with nitrogen, sulfur, or phosphorus restriction, this is thought to be a catalyst for the induction of carotenoids synthesis, therefore in this instance, the poor biomass productivity can also be linked to a high carotenoids production.

2.4 Extraction of carotenoids from microalgal cells

Conventional solvent extraction techniques using organic solvents are used to obtain carotenoids from microalgae. The polarity, solubility, and chemical stability of the carotenoids to be extracted determine whether conventional extraction should be carried out using organic or aqueous solvents.

Non-polar solvents (n-hexane, dichloromethane, dimethyl ether, diethyl ether) or polar solvents (acetone, methanol, ethanol, biphasic combinations of various organic solvents) can be utilized according to the polarity of the target carotenoid,

For the recovery of carotenoids from microalgae, the use of green solvents (environmentally safe and non-toxic solvents) like ethanol, limonene, and biphasic mixes of water and organic solvents has been examined. Due to the high cost of manufacturing and extensive solvent use, the economic feasibility of carotenoid extraction from microalgal species is currently low.

Thus, interest in the use of unconventional extraction techniques has grown recently. These unconventional extraction techniques have quick extraction times, little solvent usage, improved recovery, and more selectivity, among other benefits [218–226]. The most used extraction methods are briefly described.

2.4.1 Microwave-assisted extraction (MAE)

Due to the presence of algaenan and sporopollenin in their cell walls, breaking down microalgal cells can be a challenging task [224]. Additionally, the efficiency of traditional cell disruption and extraction methods is poor. When microwave radiation is used at a frequency close to 2.45 GHz, polar molecules vibrate, resulting in inter- and intramolecular friction.

Two marine microalgae, *Dunaliella tertiolecta* (Chlorophyta) and *Cylindrotheca closterium* (Bacillariophyta), were subjected to microwave irradiation for the extraction of pigments. [219] examined the effectiveness of this method in comparison to traditional methods. All techniques used on *D. tertiolecta* produced quick pigment extraction. The optimum extraction method for *C. closterium* pigments was determined to be MAE, bringing benefits in speed, repeatability, homogenous heating, and high extraction yields.

2.4.2 Ultrasound-assisted extraction (UAE)

UAE is based on ultrasound cavitation. Ultrasound waves of high strength and low frequency are used to perform ultrasonic extraction. Low intensity-high frequency (100 kHz–1 MHz) and high intensity-low frequency (> 1 MHz) ultrasound can be distinguished from one another (20–100 kHz). UAE is affordable, it dramatically shortens the extraction time, and increases extraction yields.

4.66 mg of β-carotene per g of dry weight was extracted using ultrasonic assistance from the microalga *Arthrospira platensis* [38]. For enhanced extraction, several parameters (amplitude, duty cycle, sonication period, and depth of horn immersed in solution) were tuned.

The ideal parameters for the greatest amount of β-carotene extraction from this alga were 80% amplitude, 33% duty cycle, 0.5 cm of horn depth in the solution, and 10 min of ultrasonication. The extraction of lutein, β -carotene, and α-carotene from *Chlorella vulgaris* has also been carried out using UAE [227]. The highest extraction levels were, respectively, 4.844 ± 0.780, 0.258 ± 0.020, and 0.275 ± 0.040 mg g^{-1} of dry weight biomass.

2.4.3 Subcritical fluid extraction

Subcritical fluid extraction uses liquefied subcritical fluids as the extraction solvent. Compared to supercritical fluid extraction, subcritical fluid extraction operates at comparatively modest temperatures and pressures. Moreover, using ethanol modified subcritical 1,1,1,2-tetrafluoroethane, the carotenoids and chlorophyll-a can be efficiently extracted [170].

2.4.4 Electrotechnologies-assisted extraction

Electrotechnologies including pulsed electric field (PEF), moderate electric field (MEF), and high-voltage electric discharges (HVED), are other non-thermal and environmentally friendly methods of extraction which target intracellular chemicals from bio-suspensions.

Carotenoids extracted from *Chlorella vulgaris* with pulses of milliseconds (5 kV/cm–40 ms) or microseconds (20 kV/cm–75 s) were extracted with an 80% greater efficiency. Up to 73% of carotenoid extraction was achieved in the microalgae *Heterochlorella luteoviridis* when MEF and ethanol were used as the solvent (180 V, 75 mL/100 mL of ethanol solution) [228].

2.4.5 Pressurized liquid extraction (PLE)

The primary benefit of employing PLE is that it enables quick extraction while consuming less solvent. In PLE, solvents are extracted under pressure and temperature conditions that are always below their critical points.

Carotenoids have been extracted using PLE from freeze-dried macro- and microalgal biomass. Pressurized liquid extractions produced extraordinary levels of extraction of fucoxanthin in *Phaeodactylum tricornutum*, up to 26.1 mg g^{-1} DW [229]. In the instance of *Neochloris oleoabundans*, pressurized liquid extraction was used to recover the bioactive carotenoids lutein, carotenoid monoesters, and violaxanthin [230].

In comparison to maceration, soxhlet extraction, and ultrasound-assisted extraction, pressurized liquid extraction (PLE) demonstrated greater extraction efficiency for the extraction of carotenoids and chlorophylls from the green microalga *Chlorella vulgaris* (UAE) [231].

2.4.6 High pressure homogenization (HPH) treatment and enzyme-assisted extraction

Since microalgal cells are not easy to disrupt, recovering carotenoids can be aided by a physical or enzymatic pretreatment before extraction. One such approach is HPH, which uses high intensity fluid stress to cause cell breakdown (50–400 MPa). It has important advantages over conventional physical milling methods, including simplicity of use, commercial viability, repeatability, and high throughput [170].

2.4.7 Supercritical fluid extraction (SFE)

SFE includes extraction utilizing supercritical fluids, or fluids at a temperature and pressure above their critical limit. Due to their low viscosity and high diffusivity, supercritical fluids have better solvating and transport qualities than liquids.

SFE was used to produce the maximum carotenoid output in *Scenedesmus obliquus* at 250 bar and 60 °C [232].

2.5 Purification of carotenoids

To ensure that the carotenoids maintain their qualities, when they reach the consumer, further methods are required after the process of extracting the carotenoids. These strategies include purifying the compounds of interest, removing any leftover cell debris, and preserving the molecules [233]. Carotenoids obtained from microalgae are as pure as those found in other natural sources. The Willstatter technique is the foundation of the traditional approach for purifying carotenoids [180].

To prevent product degradation, this purification method uses salts (NaOH or KOH) as saponification agents at low temperatures (below 60 °C), followed by organic solvents

Table 5 Properties and applications of the main carotenoids produced by microalgae

Carotenoid	General properties and applications	References
β-carotene	Anti-oxidant property Provitamin A function In colorectal cancer In the prevention of acute and chronic coronary syndromes Photoprotection of skin against UV light Prevent night blindness Prevents liver fibrosis	[185, 188, 189]
Lutein	Anti-oxidant property Prevention of acute and chronic coronary syndromes and stroke Maintenance of a normal visual function Prevention of cataracts and macular degeneration Prevention of retinitis Prevention of gastric infection by H. Pylori Anti-cancer activity	[179–181] [182–185]
Violaxanthin	Anti-inflammatory activity	[186, 187]
Zeaxanthin	Anti-oxidant property	[185, 190, 191]
Lycopene	Anti-oxidant property Anti-neuroinflammation	[239, 240]

(such as hexane or blends of ethanol, water, and dichloromethane) that will later be removed in order to obtain the desired carotenoid products. This time-consuming method of purification is presently being superseded by several chromatographic methods that have been compiled and thoroughly discussed in numerous publications [234, 235].

After the carotenoids have been extracted and purified, air, light, or heat may cause the final products to degrade [180]. The stability of standard solutions of carotenoids, such as lutein, lycopene, zeaxanthin, α and β-carotene, β-cryptoxanthin, and zeaxanthin, was examined after the extraction. With the exception of lycopene, these studies found that the carotenoid standard solutions (0.05–5 g mL^{-1}) did not degrade and could be kept at − 70 °C for 6 months [236–238].

2.6 Application of some most representative primary carotenoids

Carotenoids are in very high demand in the healthcare and pharmaceutical industries because they are pro-vitamin A and have potent antioxidant properties (Table 5).

As an example, regular consumption of 9-(Z) neoxanthin may reduce the chance of developing lung cancer [241].

Violaxanthin and its derivatives have been shown to have potent lipid peroxidation inhibition characteristics and are reported in the literature to have antioxidant effects [242]. Additionally, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical tests were used to demonstrate the radical scavenger properties of violaxanthin derived from the microalgae *Eustigmatos cf. polyphem* [176].

In ophthalmological areas, zeaxanthin plays a large and vital function. It shields the visual organ from near UV rays and reduces the risk of age-related macular degeneration, and is naturally found in the central macula of the eye [243]. Its potential as a natural dye indicates its use in food, as antioxidant in cosmetics, and in the coloring of fish and fowl [244]. Zeaxanthin has been reported to be beneficial in treating cardiovascular issues and may help diabetic individuals from developing pancreatic and lung cancer [245]. This xanthophyll is helpful in adjuvant therapy because it can lead melanoma cells to apoptosis.

Antioxidant and photo-protective qualities are present in lycopene. It exhibits anti-cancerous properties in human colon, breast, prostate, liver, and lymphocyte cell lines [246]. It combats high blood pressure issues and early arthrosclerosis by enhancing endothelial function and reducing oxidative stress. Lycopene maintains blood cholesterol levels by acting as a hypolipidemic in a manner similar to statins [247]. Daily dosages of lycopene strengthen the skeletal system, improve intercellular connections of gap junctions, and maintain glucose homeostasis. According to [246], lycopene-induced tumor metastasis was inhibited as a result of regulated cell cycle progressions.

An in vivo study in high cholesterol-fed rats found that algal lycopene obtained from *Chlorella marina* displayed stronger antioxidant and anti-inflammatory effects compared to the lovastatin and trans-lycopene derived from tomatoes.

Although, at the industrial level, marigold is the primary source of lutein production, many microalgal strains are able to produce a considerable amount of lutein [248]. A crucial aspect to be considered is that, while lutein from marigolds is esterified, it is found in free forms in microalgae, which are more easily absorbed than esterified forms [249], with various benefits for human health: antioxidant, light-filtering and anti-inflammatory activity, eye protection, potential therapeutic benefits for many chronic diseases, lower risk of cancer.

The antioxidant lutein can protect eyes, brain, and skin from damage. Like zeaxanthin, it shields the eyes from harmful UV and blue light, earning the moniker "eye vitamin." It protects against age-related macular degeneration and cataract by lowering the amount of plasma factor D. It is also effective as a chemotherapeutic agent [250]. It is a chemical that actively fights prostate, breast, and colon cancers. Consuming lutein can also reduce the risk of developing lung cancer and early atherosclerosis. It lessens the consequences of neurodegenerative diseases brought on by inflammation. It is extensively used in chicken as a feather

Table 6 Major companies involved in carotenoid production at commercial level

Company	Carotenoid (microalgal species)	Web Site	Country
Aquacarotene Ltd	β-carotene (*D. salina*)	https://www.investogain.com.au	Australia
Cognis Nutrition and Health	β-carotene (*D. salina*)	https://www.nutraingredients.com	Australia
Nikken Sohonsha Corporation	β-carotene (*D. salina*)	https://www.nikken-kosakusho.co.jp	Japan
Tianjin Norland Biotech Co., Ltd	β-carotene (*Chlorella* sp., *Spirulina)*	https://www.norlandbiotech.com	China
Seambiotic	β-carotene (*Dunaliella* sp.)	https://www.bloomberg.com	Israel
Sun Chlorella Corporation	Lutein, β-carotene (*Chlorella* sp.)	http://lab-sunchlorella.com	Japan
Maypro Industries Inc	Lutein, β-carotene (*Chlorella* sp.)	https://www.maypro.com	USA, Taiwan
Chlorella Manufacturing Co., Ltd	Lutein β-carotene (*Chlorella* sp.)	https://www.taiwanchlorella.com	Taiwan
Far East Microalgae Ind Co., Ltd	Lutein, β-carotene (*Chlorella* sp.)	http://www.femico.com.tw/eng/about	Taiwan
Roquette Klotze GmbH and Co. KG	Lutein, β-carotene (*Chlorella* sp.)	https://www.algomed.de/en	German

colorant, a yolk color enhancer, and as an additive in baby food, drugs, and cosmetics.

It is widely known that β-carotene has anti-inflammatory, antioxidant, immune, dermo-, hepato-, and retino-protective properties [251]. This pigment is widely used in the cosmetic and food coloring industries [241]. It has been claimed that this pigment plays a protective function from malignancies of the breast, colon, lungs, liver, and skin.

Apoptosis, cell differentiation, and proliferation may all be maintained by β-carotene, which can also improve gap junction intercellular communications and lessen the detrimental effects of H_2O_2 on them [244]. Its frequent inclusion in the diet guards against liver fibrosis and night blindness.

2.7 Market

The global market for carotenoids was estimated to be worth $1.24 billion in 2016 and $1.53 billion in 2021 [252]. Their worldwide economic value is determined by consumer demand and the cost of extraction.

Feed, food, and beverage pharmaceutical, cosmetic, aquaculture, and nutraceutical industries are significant carotenoid market segments [253]. At global level, a market of 1.5 billion USD was predicted; however, due to strong demand, it is anticipated to increase to 2 billion USD by 2022, with a compound annual growth rate (CAGR) of 5.7%. Due to the variety of uses, β-carotene, lutein, fucoxanthin, and zeaxanthin dominate the market. Currently, the main commercial source of lutein is marigold flowers, however, microalgae biomass contains 3 to 6 times as much lutein per unit of dry matter. As reported by [170], β-carotene accounts for 246.2 million USD (17% of total), lutein for 225 million USD (15.6% of total), lycopene for 107 million USD (7.4% of total), according to a report by BCC Research in the context of the global carotenoid market.

Because of its demand, it has been estimated that by 2022, the market value of lutein will increase to 357.7 million USD, from 263.8 million US dollars in 2017 (6.3% CAGR from 2016). Instead, the price of β-carotene on the market has been evaluated around 300–1500 USD/kg, depending on demand and carotenoid purity [254].

By 2023, the feed sector will reach the biggest market share within the global carotenoid market, due to fewer restrictive regulations and also considering the growth in the production of meat, poultry, and dairy products that will hasten the expansion of the feed business.

Concerning the carotenoid production on a large scale in European countries, Ambati et al. [252] explained that the expansion of the European carotenoid market may benefit from the investment and participation of well-known cosmetic companies as Hindustan Unilever, L' Oreal, Henkel, and Beiersdorf.

The bottleneck for a sustainable and economically advantageous carotenoids production with microalgae consists in the lack of raw materials, effective instruments, and techniques for the upstream (extraction) and downstream (purification) processes all contribute to high monetary value [255]. Moreover, the market for carotenoids is constrained

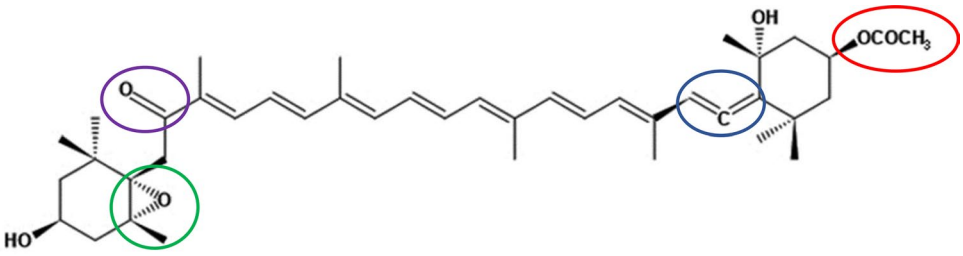


Fig. 10 Chemical structure of the Fx molecule. Red, blue, green, and purple circles, respectively, represent an acetyl group, an allenic bond, an epoxide group, and a conjugated carbonyl group

by laws and tight regulations relating to health care, the environment, and animals. However, due to the increasing demand for natural goods and the depletion of conventional resources, major corporations are investing heavily in carotenoid manufacturing to increase their competitiveness in the carotenoid market. In Table 6 some of the main companies involved in carotenoids commercialization and production are reported.

3 Microalgae as fucoxanthin source

More than one billion people around the world will be living with obesity by 2030 [256]. The reasons for this disastrous trend are attributed to the global tendency towards the reduction in exercise and physical activity and the increased dietary intake of fats, sugars and calories. To prevent life-style-related diseases, like metabolic syndrome (MS), researchers are focusing on many functional ingredients in foods which may be useful for the prevention and treatment of life-style-related diseases [257], among these Fucoxanthin (hereinafter shortened as Fx), a characteristic carotenoid present in edible brown seaweeds and several species of microalgae. Fx is the major carotenoid pigment in marine ecosystem, representing more than 10% of total carotenoid production [258]. This yellow-orange pigment is produced by an algae group composed of brown and golden-brown macro and microalgae such as diatoms, haptophytes, dinophytes and brown seaweeds. Fx masks the color of chlorophyll and gives the characteristic brown color to the algae. In diatoms, it was proven that Fx enables the absorption of photons in a wide blue-green spectral range of visible light, which is the most available range in a water column [259]. Indeed, there is a lack of red light availability for chlorophylls at certain depths as only blue light can reach further to the cells. Therefore, along with light harvesting by chlorophylls, Fx increases the efficiency in using different light wavelength for carbon fixation.

It is widely reported in literature that Fx has various potential benefits for human health [260]. This paragraph aims to give an overall view, although not exhaustive, on microalgae-derived Fx in terms of the main species as sources of Fx, their cultivation strategies and conditions favoring Fx accumulation, the bioactivities of the pigment, and its potential application in farma-, nutra-, and cosmeceutical industries.

3.1 Structural characteristics of fucoxanthin

Fx, whose molecular structure was fully described in [261], is part of the carotenoid family under the denomination of xanthophylls, whose chemical structure is distinct from the second carotenoid family, the carotenes, as only the former contain oxygen. Moreover, the oxygenic functional groups consisting of epoxy, hydroxyl, carboxyl, and carbonyl groups, exceptionally express the superiority of Fx over other carotenoids. This particularity, along with an epoxide group and a conjugated carbonyl group, confer anti-oxidant properties to xanthophylls, which makes them very demanded in cosmetics and pharmacology [262]. The diverse health-promoting effects [263–266] of Fx are attributed to its unique chemical structure, presenting an acetyl group, an allenic bond, and a conjugated carbonyl, along with 5,6-monoepoxide (Fig. 10).

As other carotenoids, Fx is highly unsaturated, thus very reactive and sensitive to oxidation, readily vulnerable to a high number of factors, including light, heat, oxygen, enzymes, unsaturated lipids, and pro-oxidant molecules. Fx possesses four isomers, all-trans Fx, which is the most represented isomer in the natural environment, 9'-cis Fx, 13-cis Fx and 13'-cis Fx (Fig. 11). The trans-form of Fx is closely related to Fucoxanthin's pharmacological activities, as evidenced by several studies. Degradation of all-trans isomers of Fx, with subsequent increase in 13-cis and 13′-cis isomers, was observed when the pigment is exposed to light and air or to high temperature [267]. According to Nakazawa et al. [268], cis forms of Fx demonstrated to have better antiproliferative effects against leukemia cells colon cancer cells. In a study conducted by Kawee-ai et al. [267] the antioxidant activity decreased with the increase of cis-isomers formation.

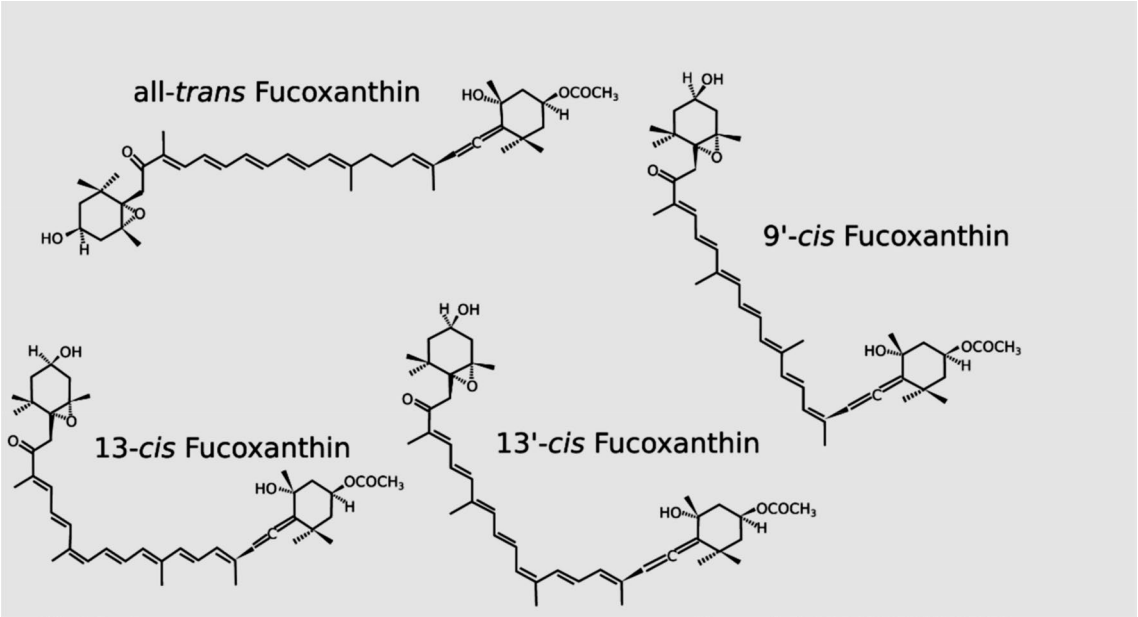


Fig. 11 Isomers of Fx, all-trans Fx, 9'-cis Fx, 13-cis Fx, 13'-cis Fx

3.2 Producing species

Fucoxanthin is found in the Haptophyta, the Fx-containing Dinophyta, the Chrysophyta and in the Bacillariophyta. The latter two belong to a group referred to as the Heterokontophyta.

Currently Fx is produced mainly from the waste parts of brown seaweeds (macroalgae) such as *Laminaria japonica*, *Eisenia bicyclis*, *Undaria pinnatifida*, and *Hizikia fusiformis*. However, these macroalgae are mostly harvested for food in Asia and they contain very low concentrations of Fx [269], with content varying from 0.02 to 5 mg g^{-1} in fresh biomass and 0.01–2.1 mg g^{-1} in dried biomass [270]. Microalgae can be considered as a more promising source of Fx for commercial production, as the concentration of Fx in microalgae is much higher than in macroalgae, they are fast-growing microorganisms and the techniques of industrial production of microalgae are developing. Despite the abundance and diversity of Fx producing microalgae, only a few species were studied for commercial production of Fx [271, 272], diatoms and Haptophytes being the most promising groups [269].

The microalgae that have been characterized for Fx synthesis in the last decade are summarized in Table 7. The Fx content of most species ranges from 1 to 10 mg g^{-1} of dry biomass, but some elite species accumulate more than 20 mg g^{-1} of Fx. Of particular interest among them, *Tisochrysis lutea* accumulates up to 80 mg g^{-1} Fx in dry biomass, one the highest known [273]. The marine microalga *Pavlova* spp. (Haptophyta) is considered another good candidate: it has a satisfactory Fx content (20.9 mg g^{-1} in pilot scale) and a very thin cell wall, a favorable characteristic for Fx extraction [274]. *Phaeodactylum tricornutum* is a model diatom due to its high growth rate, ease of cultivation, availability of genome sequence and genetic manipulation, which has been used to enhance the synthesis of Fx [275]. Different strains of *P. tricornutum* exhibit various performances in Fx production with a Fx yield ranging from 2 to 60 mg g^{-1} under different culture conditions [276]. *Cylindrotheca closterium* is considered as a suitable candidate for Fx production due to its high Fx content (up to 25.5 mg g^{-1}), resistance to grazers, relatively high growth rate and rapid sedimentation under static conditions [277]. Aside from the marine species, Fx can also be produced by freshwater species like *Sellaphora minima* (7.5 mg g^{-1}), *Nitzschia palea* (5.5 mg g^{-1}) [278] and *Mallomonas* sp. (26.6 mg g^{-1}) [269].

3.3 Biological function and biosynthesis in microalgae

As a primary carotenoid, Fx is intricately organized with chlorophyll, antenna proteins and diadinoxanthin and diatoxanthin, forming a Fucoxanthin-chlorophyll a/c-protein (FCP) complex, a light harvesting complex which captures photons and transfers them to photosynthetic reaction centers [285, 286]. FCP complexes are embedded in the thylakoid membrane and associated with both photosystems II and I. Due to their particularity of absorbing in the blue-green spectrum, the light-harvesting complexes are capable of a wider light spectrum absorption thanks to Fx, between 390

Table 7 Microalgae species producing Fucoxanthin. Fx contents of the widely brown seaweed species commercially used are also reported for comparison. Fx amount is expressed on the basis of algal dry weight

Species		Fresh/dried (F/D)	Fucoxanthin (mg g^{-1})	References
Microalgae[a]				
Chaetoceros calcitrans	Diatom	D	2.1–5.2	[279]
Chaetoceros calcitrans	Diatom	D	17.5	[270]
Chaetoceros gracilis	Diatom	D	2.2	[279]
Cylindrotheca closterium	Diatom	D	5.2	[279]
Cylindrotheca closterium	Diatom	D	25.5	[279]
Thalassiosira weissflogii	Diatom	D	9.0	[279]
Nitzschia laevis	Diatom	D	12	[270]
Anaphora capitellata	Diatom	D	41.8	[270]
Phaeodactylum tricornutum	Diatom	D	7–22	[270]
Phaeodactylum tricornutum	Diatom	D	3.1	[280]
Phaeodactylum tricornutum	Diatom	D	2–60	[276]
Odontella aurita	Diatom	D	16.2–18.5	[270, 279]
Tisochrysis lutea	Haptophyte	D	6.7–79	[270]
Tisochrysis lutea	Haptophyte	D	4.9	[281]
Tisochrysis lutea	Haptophyte	D	18.23	[273]
Diacronema lutheri	Haptophyte	D	20.9	[270]
Diacronema lutheri	Haptophyte	D	3.3	[282]
Pavlova spp.	Haptophyte	D	2.06–12.9–20.9	[274]
Nitzschia palea	Diatom	D	5.5	[278]
Sellaphora minima	Diatom	D	7.5	[278]
Mallomonas sp.	Chrysophyte	D	26.6	[269]
Macroalgae (seaweeds)[b]				
Laminaria japonica (kombu)		F	0.19	[283]
Undaria pinnatifida (wakame)		D	7.3	[283]
Eisenia bicyclis		F	0.26	[279]
Hizikia fusiformis (syn. Sargassum fusiforme)		D	0.01	[283]
Sargassum horneri (akamoku)		D	1.4–4.5	[284]
Fucus vesiculosus		D	0.7	[284]

[a]see also [276]; [b]For a more exhaustive discussion see [284] https://doi.org/10.3390/foods11152235

and 580 nm [259]. This ability is useful and advantageous for algal cells when there is a lack of red light availability for chlorophylls at certain depths, where only blue light can reach further to the cells [259]. Several studies reported that diatoms contain different Fx molecules that according to their photon absorption capacity are classified as "Fx-blue" (λ_{max} 463 nm), "Fx-green" (λ_{max} 492 nm) and "Fx-red" (λ_{max} 500–550 nm) [270].

Fx biosynthesis is part of the xanthophyll cycle, closely related to their photoprotection role. Biosynthesis of Fx is mainly regulated by light. The diatoms respond to variations in the quantity and quality of light, modulating the metabolic pathway of the photosynthetic pigments and thus changing their concentration. These changes in pigmentation lead to variations in photosynthetic responses as well as in microalgae growth rate [287].

The biosynthesis of Fx in microalgae is currently still being studied. A hypothetical Fx biosynthetic pathway proposed in *P. tricornutum* is reported in detail by Wang et al. [276]. The biosynthesis process model is comprised of the 2-C-methyl-erythritol 4-phosphate (MEP) pathway, along with formation of geranylgeranyl diphosphate (GGPP) and carotenoid synthesis. Xanthophyll cycle-involved pigments (zeaxanthin, antheraxanthin, and violaxanthin) are synthesized after the formation of β-carotene. The cycle is mediated by light intensity, i.e., under low light, zeaxanthin is converted into violaxanthin, via antheraxanthin, while under high light the process may be reverted by violaxanthin de-epoxidase (VDE). The

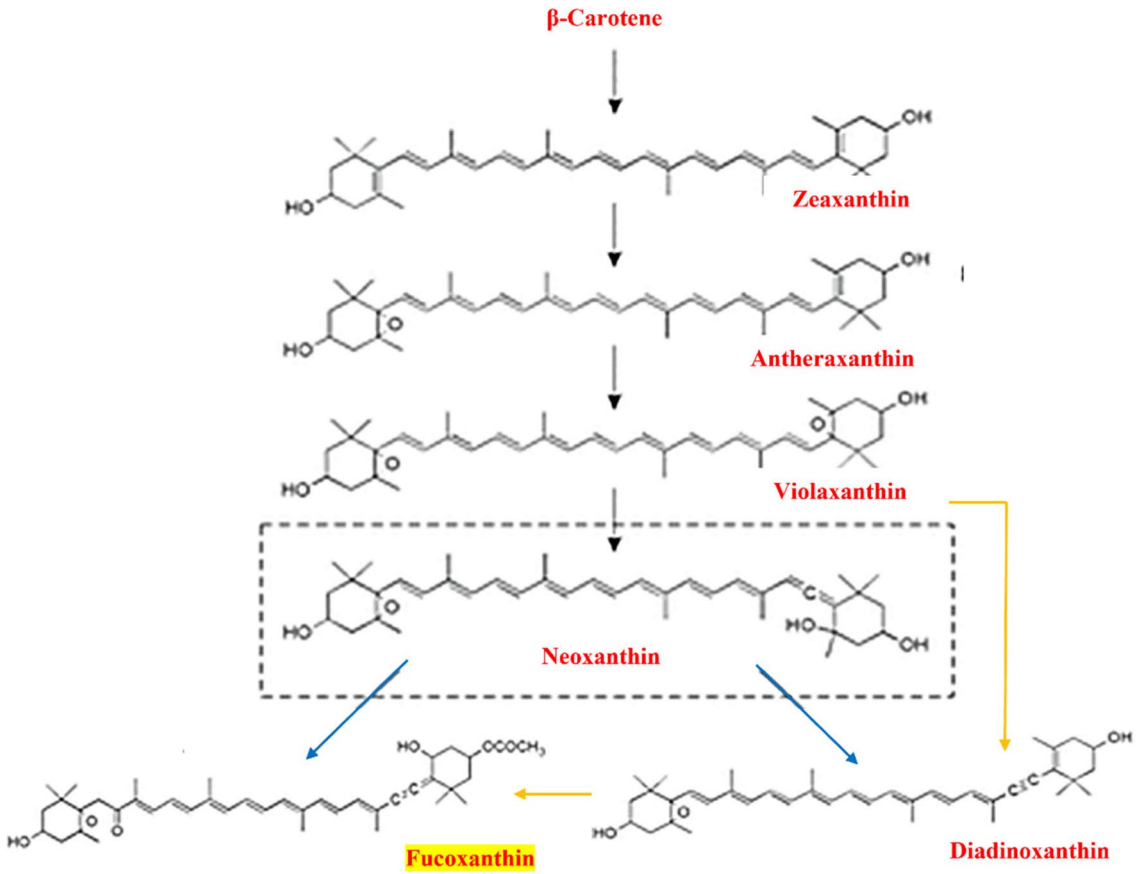


Fig. 12 Proposed carotenoid biosynthesis pathway in *P. tricornutum* from β-carotene to the end products diadinoxanthin and Fx. neoxanthin as the branch point is boxes

transforming steps between violaxanthin and Fx have not yet been completely elucidated. Figure 12, shows two possible hypotheses. According to Lohr & Wilhelm [288], Fx is synthesized from violaxanthin via diadinoxanthin; on the other hand, Dambek et al. argue that violaxanthin is converted to neoxanthin, at which stage the metabolic flux diverges to produce Fx and diadinoxanthin separately [289]. In contrast with extraplastidial accumulation typical of other carotenoids, the overproduction of Fx taking place inside the plastids can require a concurrent increase in the other constituents of the FCP, and also a strategy to regulate Fx sequestration and/or degradation after its de-novo synthesis [276].

3.4 Effect of culture conditions and environmental factors on growth and fucoxanthin production

Different factors, including strains, cultivation systems, nutrient availability, light intensity and quality and harvest stage affect growth rate and pigment accumulation in microalgae [290]. Although the main carotenoids of commercial interest (astaxanthin and β-carotene) accumulate in microalgae under stress conditions, for Fx-producing microalgae the conditions that favor Fx synthesis appear quite different. In general, the biomass productivity and the Fx content are the two parameters directly responsible for Fx production in microalgae.

One of the most important parameters for Fx production and productivity is the light. Various studies have demonstrated the influence of light wavelength, intensity and the light-darkness cycle on the accumulation of Fx. Low light intensity (less than 100 µmol m^{-2} s^{-1}) promotes the Fx synthesis [291, 292], while high light intensity (starting from 150 µmol m^{-2} s^{-1}) can damage the photosystems, therefore, activates the production of photo-protective pigments (diadinoxanthin and diatoxanthin) [293]. The increase of Fx in the low light regime is ascribed to the compensation of low light irradiance, since Fx is a part of the light-harvesting antenna that promotes the photon capture for photosynthesis. Moreover, as more photons are available, cells do not need to capture more photons than necessary due to photon saturation and hence do not produce more chlorophylls and Fx.

As biomass production increases under high light an improved Fx productivity results from a compromise

between high and low light intensity. This statement is confirmed by the study of Gao et al. [292] in cultures of *Tisochrysis. lutea* that obtained the highest Fx productivity (9.81 mg L^{-1} d^{-1}) at 300 µmol m^{-2} s^{-1}, while the highest Fx content (5.24 mg g^{-1}) was achieved at 150 µmol m^{-2} s^{-1}. Similar trend was shown in cultures of *Isochrysis zhangjiajiensis* as reported by Li et al. [294]. Fx content in *P. tricornutum* is lower under high light intensity (9.9 mg g^{-1} at 210 µmol m^{-2} s^{-1}) than under low light intensity (42.8 mg g^{-1} at 100 µmol m^{-2} s^{-1}) [295].

Fx absorbs blue-green light and thus has shown, in diatoms, to be accumulated under blue light even at high intensity, while red light favors the production of other pigments such as diadinoxanthin [295]. Furthermore, in the diatom *Cylindrotheca closterium,* red light and green light decreased the Fx content because of the lack of blue light [277]. In *T. lutea*, the highest Fx content was found in blue-green light while the highest Fx productivity was found in red-blue-green light (which is closer to white light) [292]. The accumulation of Fx in blue-green light was expected, as this spectrum is prevalent in the natural habitat of brown algae [259]. Indeed, the absorbance spectrum of Fx ranges from 450 to 540 nm in solution even thought is range is wider (390–580 nm), once the Fx is bound to a FCP.

In conclusion, high light intensity and extra-long lightening are favorable for biomass but adverse for Fx accumulation due to the photoprotection function of Fx; the preference of blue light, with regards to Fx accumulation, can arise from the distinctive absorption of blue-green light by Fx. This conflict needs to be solved to ensure concurrent biomass and Fx accumulation and to achieve a successful Fx production.

Nutrients play an important role in microalgae growth as they are required to synthesize bio-compounds essential for algal cells. Nitrate, phosphate, iron, and silicate (for diatoms) have been identified in various studies as nutrients that have an impact on cells growth and on metabolite accumulation [296]. Nitrogen is a major macronutrient required by microalgae to grow as it is essential in protein synthesis. The importance of nitrogen sources in producing Fx has been widely illustrated. For instance, two phases, nitrogen supply and nitrogen starvation were studied in batch cultivation of *T. lutea*. Under nitrogen supply phase the Fx content almost doubled and then decreased significantly under nitrogen deprivation phase [292]. The enrichment of nitrate in medium (ten-fold higher than f/2) improves the Fx content of *P. tricornutum* to 59.2 mg g^{-1} in seven days [295]. Sufficient nitrogen supply stimulates the biosynthesis of Fx (6.01 mg L^{-1} d^{-1}) in *Odontella aurita*, more than fourfold higher than it does in nitrogen-deplete condition [297].

Compared to nitrogen, phosphorus is the main limiting nutrient for microalgae in the environment. However, the impact of phosphorus concentration was less conspicuous than nitrogen concentration on the growth and Fx accumulation of microalgae as shown in the work of Sun et al. [298].

In diatoms, another important nutrient is silicate which is required for cell growth as the cell wall contains silicon. High silicate level promotes the accumulation of both biomass and Fx in diatoms. The study of Mao et al. [299] showed that a high silicate concentration in medium promotes Fx production in *Nitzschia laevis* but at a low level it suppresses the Fx production. Higher silicate concentration avoids the adverse impact of high light (> 250 µmol m^{-2} s^{-1}) on Fx accumulation in *P. tricornutum* [300]. Available evidence supports the understanding that rich nutrients favor Fx accumulation and protect microalgae from stress while deplete nutrients lead to reduced Fx in microalgae.

Optimum salinity is highly dependent on the microalgal species. The marine *Isochrysis galbana* showed a greater growth and Fx content (12 mg g^{-1}) under a salinity of 35‰ than 20‰ [301]. Meanwhile, the growth (1.49 g L^{-1} d^{-1}) and Fx content (7.4 mg g^{-1}) of *P. tricornutum* were in favor of a 20‰ salinity level. *C. fusiformis*, grew better under 30‰ by producing the highest biomass concentration whereas the greatest Fx content (5.8 mg g^{-1}) was generated under a salinity of 10‰ [302].

The responses of Fx synthesis to changes in temperature and pH have been poorly discussed as in most of the studies microalgae are grown in a temperature and pH-controlled environment, at values generally considered optimum for growth. Like salinity, each Fx-producing microalga has its optimum temperature to yield the maximum growth and Fx content. An optimization study has shown that the optimum temperature for *T. lutea* to generate the greatest Fx content was 25 °C [303]. Depending on cultivated species, the optimal temperature can vary in the mesophilic range [292, 302].

The effect of trophic modes, including photoautotrophy, heterotrophy and mixotrophy have been studies in several Fx-producing microalgae. As photoautotroph microorganisms, all the species may be cultivated under autotrophic conditions by capturing light and incorporating CO_2 [276] [Table 1]. However, several species as *N. laevis* and *Cyclotella cryptica* are able to be grown heterotrophically in the dark using organic C sources, even showing an increased Fx content than autotrophic culture [304, 305].

By taking advantage of both photoautotrophic and heterotrophic modes, mixotrophic cultures are more effective in utilizing resources, achieving high biomass production and, as consequence of cell's self-shading, even an enhanced Fx production. Alkhamis et al. [306] reported more than twice of Fx content in the mixotrophic culture of *T. lutea* compared with the phototrophic culture (11.5 vs 4.8 mg g^{-1}). In a mixotrophic culture of the diatom *N. laevis*, the Fx content reached 15.6 mg g^{-1} while in heterotrophic culture it reached 10 mg g^{-1} [305]. Mixotrophic cultures of *P. tricornutum*

have been widely investigated and have shown promising results for both biomass and Fx production [287, 307]. Yang et al. [174, 286] reported a maximum Fx content of 16.3 mg g^{-1}, the highest level of ever reported so far.

The above results highlighted that ideal culture conditions to achieve desirable growth and Fx content are strongly specie-specific and would have to be optimized individually. Moreover, there is evidence that the mixotrophic cultivation strategy for *P. tricornutum* could be more suitable for both biomass and Fx accumulation. At an industrial scale, one limit of the mixotrophy culture could be the cost of the organic carbon sources, although in a circular economy perspective, waste products from industrial processes could be used.

3.5 Microalgae cultivation for fucoxanthin production

Fucoxanthin-producing microalgae are capable of growing in photoautotrophic and some strains also in mixotrophic conditions, both indoor and outdoor, in open and closed systems, in batch, semicontinuous, or continuous mode. The main advantage of using microalgae as a source of Fx is that they can be grown in close photobioreactors (PBRs) providing an available, stable and sustainable supply of biomass all year round. Besides, as microalgae can grow in PBRs, their growing conditions can be optimized for maximizing the production of a specific compound. Many PBR designs have been proposed [308], however, mass cultivation of Haptophytes and diatoms was widely performed in tubular, flat panel, vertical columns and bags.

The production of Fx from *O. aurita* is very attractive, with the maximum yield of 7.96 mg L^{-1} d^{-1} achieved indoors in a bubble column PBR. A comparable yield obtained in the scale-up flat plate PBR confirmed the technical feasibility and scalability of *O. aurita*-based Fx production on a large scale [271]. In the same microalga cultivated in a 240 L well-controlled PBR internally illuminated by LEDs lamps, red lighting was more suitable for cell growth and Fx production than blue light and white light. However, the biomass and Fx productivities were further promoted by optimizing the ratios of red and blue light, reaching the highest Fx productivity of 9.41 mg L^{-1} d^{-1} with a mixed ratio of red light and blue light at 8:2. These findings demonstrated a Fx over-production in *O. aurita* by using pilot scale PBRs under optimum light spectrum and also demonstrated its promising feasibility in commercial Fx production [309]. Blue light-emitting diode at 100 µmol m^{-2} s^{-1} and 18/6 L/D cycle induced maximum Fx productivity in *C. closterium* and minimized energy consumption. The Fx content and productivity in 20-L bag PBRs with continuous illumination reached 25.5 mg g^{-1} and 1.4 mg L^{-1} d^{-1}, respectively. In *P. tricornutum*, Fx productivity in flat panel PBRs with *f*/2 medium was found to be 0.44–0.72 mg L^{-1} d^{-1} [295], which is significantly lower than the productivity of *C. closterium*.

Whereas many studies related to Fx production by microalgae have been done under indoor conditions, less data are available on outdoor cultivation where the fluctuating light intensities has a direct impact on biomass and bioproducts production. Outdoor pilot-scale cultivation has been only performed for few Fx producer species. P. *tricornutum* is the species widely cultivated in outdoor installations and pilot scale to produce biofuels, Fx and other valuable products. Cultures were grown outdoor on a batch mode in 4 bubble column PBRs, with working volume of 200 L each. Results indicate a Fx content of 8.6 mg g^{-1}, leading to an estimated annual yield of 0.18 tons of Fx [310].

Gao et al. [311] grow *T. lutea* and *P. tricornutum* in outdoor flat panel PBRs 40 L in volume (Green Wall Panel®-III; [312] and biomass productivities of approximately 130 mg L^{-1} d^{-1} and 158 mg $L^{-1}d^{-1}$ as well as Fx productivity of 2.1 mg L^{-1} d^{-1} and 1.7 mg L^{-1} d^{-1} were attained, when a biomass concentration of 0.4 g L^{-1} was maintained in the semi-continuous cultivation [311]. Using 40-L GWP®-III (Green Wall Panel) PBRs, *P. tricornutum* Utex 640 was cultivated outdoors under different nitrogen regimes, and growth and fatty acid content and productivity were evaluated. The GWP-III used in this work was entirely made of stainless steel, and as in the previous designs, the external metal structure encloses a plastic culture chamber. Nitrogen replete cultures achieved the highest productivity of biomass (about 18 g m^{-2} d^{-1}) and of valuable bioproducts such as EPA- eicosapentaenoic acid (about 0.35 g m^{-2} d^{-1}) [313]. In the same location, same season, and with the same strain, Benavides et al. [313] attained land areal productivities of 11.7 and 13.1 g m^{-2} d^{-1} in circular ponds and a horizontal tubular photobioreactor, respectively, adopting a daily dilution regime. Noteworthy is the fact that the highest pool of the xanthophyll diadino/diatoxanthin was found in the PBR grown cultures, while the highest amount of the main carotenoid Fx was found in the pond cultures, most likely as a result of extensive low-light acclimation of this culture [313].

Outdoor cultivation of *Pavlova* sp. OPMS 30,543 was performed in acrylic pipe PBRs (5-mm thickness and different outer diameters), a plastic bag (0.1-mm thickness, 450-mm outer diameter), a 200-L polycarbonate open tank, and a 500-L raceway pond. Under optimized culture conditions and using the 60-mm diameter acrylic pipe PBR, a Fx content of about 21 mg g^{-1} and Fx productivity of 4.9 mg L^{-1} d^{-1} was obtained. This study demonstrated the usefulness of *Pavlova* sp. OPMS 30543 for Fx production in outdoor cultivation [274].

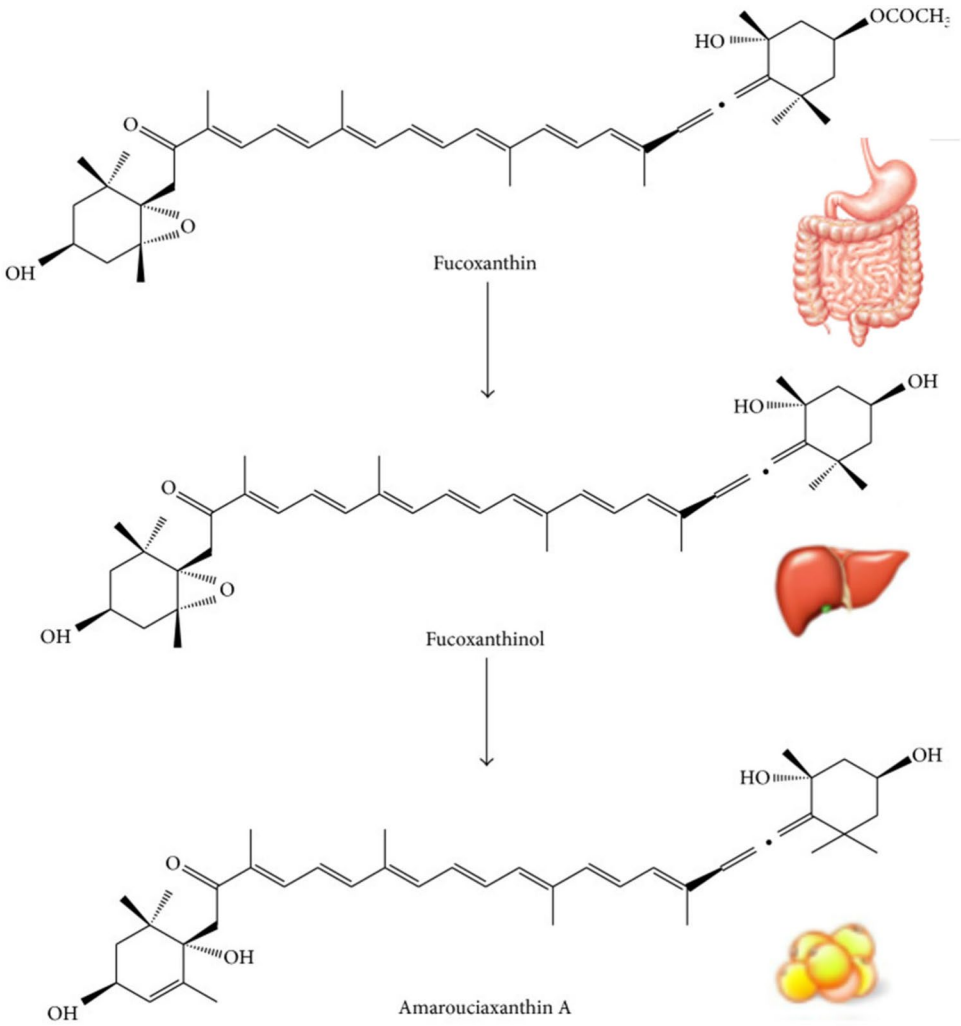


Fig. 13 Metabolism of Fucoxanthin

T. lutea and *P. tricornutum* were also cultivated at industrial scale in a 5000 L Camargue tubular PBRs [314] at Microphyt production plant (Baillargues, France) and under greenhouse. This PBR design ensures a co-circulation of the liquid medium and CO_2-enriched air in order to optimize gas exchanges and are developed to minimize mechanical stress, which guarantees the preservation of the most fragile microalgal specie. Fx content was 19 mg g^{-1} and 13.3 mg g^{-1} in *T. lutea* and *P. tricornutum*, respectively [315].

The first commercial report on the production of microalgal Fx was in 2018 by Algatechnologies Ltd., a microalgal biotechnology company located in Israel, which introduced a registered patent of natural 3% Fx oleoresin with the trademark Fucovital® from *P. tricornutum* grown in an industrial-scale plant based on vertically arranged, fully controlled tubular PBRs exposed to sunlight [316].

Shear stress, an important factor in microalgal cultivation, especially for large-scale installations, is diverse and complex in different types of PBRs, as it sources from mixing device, air-bubbling and pumping. Generally, Fx producers (diatoms and haptophytes) are more susceptible to shear stress than green algae and cyanobacteria and therefore shear stress, should be carefully minimized in a specific PBR to avoid the detrimental effect on Fx productivity. It can be concluded that a PBR characterized by a low shear stress, high surface area to volume ratio as well as effective temperature and biofilm control capacity, favors outdoor microalgal Fx production.

3.6 Metabolism and potential benefits of fucoxanthin in human health

Elucidating the mechanisms of absorption and the metabolism of Fx is important to understand its physiological effects. Both absorption and metabolism are closely related to the molecule's bioavailability. In mammals, Fucoxanthinol and Amarouciaxanthin A are known as the major Fx metabolites (Fig. 13). Once ingested, dietary Fx is hydrolyzed to Fucoxanthinol in the gastrointestinal tract by digestive enzymes such as lipase and cholesterol

esterase. Once deacetylated, Fx becomes a non-polar molecule of greater bioavailability ready to be incorporated into chylomicrons, making it possible for Fucoxanthinol to reach different systemic organs [317, 318]. In murine liver cells, Fucoxanthinol was converted into Amarouciaxanthin A which was predominantly shown in liver microsomes of mice and in HepG2 cells [319]. The in vitro study by Hashimoto et al. [320] demonstrated that Fucoxanthinol and Amarouciaxanthin A were detected in many tissues (plasma, erythrocytes, liver, kidneys, heart, spleen for Fucoxanthinol, and adipose tissue for Amarouciaxanthin A), while Fx was found in traces, therefore almost completely metabolized. Fx metabolites accumulate in abdominal adipose tissue at a higher rate than in plasma and other tissues, suggesting that adipose tissue is a main target of Fx metabolites.

The use of Fx has risen in the two last decades due to its potential bioactivities. Valuable properties of Fx such as anti-oxidant, anti-cancer, anti-obesity, anti-diabetic, anti-inflammatory, anti-angiogenic, anti-malarial, etc. have been shown in many studies. It can also be used as a protective agent for dermal, ophthalmic, bone, cerebrovascular, and cardiovascular disorders [260, 266, 319, 321]. Over the last few years, an increasing number of researches on Fx with pharmacological properties, either used in its purified form or extracted from marine algae, have been published [260, 318]. Here, a schematic view of the most relevant results regarding microalgae will be given.

3.6.1 Antioxidant activity

Fucoxanthin has shown strong antioxidant properties which can help reduce oxidative stress and prevent a variety of diseases [260, 322]. Compared to other carotenoids, Fx has the highest antioxidant activity under anoxic conditions [323] and the antioxidant properties of Fx and its metabolite Fucoxanthinol include scavenging of free radicals and quenching singlet oxygen in vitro [322].

The purified Fx from *O. aurita* was identified as all-trans-Fx, which exhibited strong antioxidant properties, with an effective concentration for 50% scavenging (EC50) of DPPH radical and ABTS radical being 0.14 and 0.03 mg mL^{-1}, respectively [271]. According to the study of Foo et al. [324] that used four antioxidant assays, *Chaetoceros calcitrans* and *I. galbana* displayed the highest antioxidant activity, followed by *Odontella sinensis* and *Skeletonema costatum* which showed moderate bioactivities. *P. tricornutum* and *Saccharina japonica* exhibited the lowest antioxidant activities amongst the algal species examined. Purified Fx obtained by *P. tricornutum* exhibited strong free-radical scavenging activity in vitro, obtaining values of 0.14 mg mL^{-1} using DPPH assay and 0.05 mg mL^{-1} with ABTS assay. Furthermore, it has been reported that the anticancer effects of Fx are closely related to its antioxidant activity or pro-oxidative development [325]. The antioxidant effects of Fx extracted from *P. tricornutum* do not significantly differ from that of astaxanthin and both showed a strong antioxidant effect compared to β-carotene [326].

3.6.2 Anti-obesity and antidiabetic activity

In general, nutritionally rich diets coupled with irrational eating habits could result in obesity and diabetes mellitus. Diabetes mellitus is usually caused by obesity because excessive energy intake and accumulation of lipids can elevate insulin resistance. Fx was proven to play an important role in reducing insulin resistance and blood glucose. The glycated hemoglobin (HbA1c) level is a risk indicator of glycemia and diabetic complications. The study of Woo et al. [327] suggested that supplementing 0.05% and 0.2% Fx significantly reduced the blood HbA1c and plasma insulin level compared with the control group. Studies on mice and rats have shown that Fx supplementation could play a beneficial role in preventing obesity through various pathways: by directly affecting weight loss and lipid metabolism via a reduction of plasmatic and hepatic triglyceride concentrations in the liver [319, 328], or reducing mRNA levels of the enzyme fatty acid synthase (FAS) and inducing uncoupling protein-1 (UCP1) in abdominal white adipose tissue (WAT) which leads to the oxidation of fatty acids and heat production [319, 329, 330]. Mayer et al. [331] in "in vivo" trials showed that *P. tricornutum* might be a promising marine source of novel food ingredients for the prevention of Metabolic Syndrome (MS) and obesity. Body weight, fat mass, inflammatory markers and insulinemia decreased in high-fat diet (HF) rats supplemented with 12% of *P. tricornutum* biomass versus the HF group without the microalga. Total plasmatic cholesterol, triacylglycerols and leptine diminished in HF-Phaeo rats, while HDL-cholesterol increased. In similar experiments in which rats were submitted to a standard diet or high-fat diet (HF), groups supplemented with 12% of *T. lutea* (HF-Tiso) or 12% of *D. lutheri* evidenced a difference against control groups, demonstrating these microalgae being effective for the prevention of obesity and weight gain and for the improvement of lipid and glucose metabolism [281, 282].

3.6.3 Anti-cancer activity

Numerous studies have shown the antitumor and cancer preventive properties of Fucoxanthin and Fucoxanthinol and this potential activity has also been explored against various cancer types [259, 332–334] The proposed mechanisms show how Fx and its metabolite Fucoxanthinol can inhibit tumor cell proliferation inducing cell cycle arrest and

apoptosis as well as inhibiting angiogenesis [332–334]. The inhibition of cell proliferation by Fx is due to cell growth arrest at G0/G1 or G1 phase of the cell cycle [335]. The apoptosis-inducing activity of Fucoxanthinol was found to be more potent than that of Fx. The study by Yamamoto et al. [336] showed that Fx and Fucoxanthinol induced cell cycle arrest during G1 phase and caspase-dependent apoptosis in primary effusion lymphoma cells. Smaller doses of Fx induce G1 cell cycle arrest in different cancer cells including colon carcinoma, hepatocarcinoma, gastric adenocarcinoma, Burkitt's and Hodgkin's lymphoma, and prostate cancer [335], see [Table 2]. The antiproliferative effect of Fx was confirmed "in vivo" by studies showing tumor growth arrest in the presence of Fx with several types of cancer [335] see [Table 3]. Oral or intraperitoneal administration of Fx inhibited the growth of lymphoma, melanoma and cervical cancer and caused apoptosis in sarcoma [336–339]. Fx extracted from *P. tricornutum* did not have anti-inflammatory properties. but it is able to induce apoptosis in different cancer [326].

The molecular mechanism that supports the observed G0/G1 phase arrest appears to be dependent on the cancer cell type but mainly involves cyclin D1 and/or D2 and CDK4 downregulation. Fx has been highlighted to induce autophagy and apoptosis in cancer cell lines and several pathways have been studied and identified to be involved in those cytotoxic effects [335].

3.6.4 Anti-inflammatory activity

Fucoxanthin has been shown to have marked anti-inflammatory effects in different "in vitro" experimental models. In this line, Fx suppressed the inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNFα), interleukin-(IL-)1β, IL-6, and inflammatory cytokines such as cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS). Fx and its derivatives, significantly inhibited NO production in lipopolysaccharide (LPD)-stimulated murine macrophage RWA 264.7 cells [337, 340]. Fx reduced the concentrations of NO, prostaglandin E2, TNF-α, IL-1β, and IL-6 through the inhibition of nuclear factor κB activation and the phosphorylation of mitogen-activated protein kinases [337]. In a study using Fx extracted from *P. tricornutum,* Neumann et al. [326] found no effects of Fx on the NO production of LPS-stimulated RAW 264.7 cells nor on the mRNA-expression of pro-inflammatory cytokines in human PBMCs. It is noteworthy that all mentioned studies utilized Fx extracted from seaweeds, therefore, it can be assumed that the carotenoid from *P. tricornutum* might have different effects.

3.6.5 Skin protective effect

Studies carried out "in vitro" and "in vivo" with Fx have demonstrated its positive effect in reducing the damage caused by UV. When tested in "in vitro" assay, Fx decreased the production of intracellular reactive oxygen species (ROS) caused by exposure of dermal fibroblasts to ultraviolet radiation, with an effect comparable to that of N-acetylcysteine (NAC) and other strong antioxidant compounds. "In vivo" results showed that Fx promotes synthesis of the structural protein filaggrin, which generates a protective barrier in case of burns induced by radiation [341]. Oral administration of Fx produced a suppression of transcription of the melanogenesis factor, by inhibiting dermal mRNA expression related to this disease [342]. In human fibroblast cells, Fx photoprotective role is also linked to its ability to remediate DNA damage and to its high antioxidant activity [343]. As a conclusion, administering Fx either as food supplement or a drug can reduce or even prevent the appearance of melanomas or otherwise mitigate the harmful effects of exposure to UV radiation.

3.6.6 Neuroprotective effect

Fucoxanthin extracted from the brown alga *Hizikia fusiformis*, widely consumed in Asia, has been demonstrated to inhibit the expression of N-myc, a proto-oncogene protein, as well as the proliferation of GOTO cells, a cell line responsible for human neuroblastoma. An amount of Fx equivalent to 1–10 g mL^{-1} is enough to inhibit the growth rate of such a cell line (GOTO) at 38%, decreasing the rate of progression of this neurodegenerative disease. Fx has been also demonstrated to be implied in the activation of the Nrf2 pathway and its related genes, such as those for the cytoprotective enzymes NQO1 and HO-1, showing that it may protect the brain against ischemia/reperfusion (I/R) injury. The study of Hu et al. indicated that treatment with Fx effectively reduced middle cerebral artery occlusion-induced cerebral I/R injury [344].

3.7 Safety and stability

Humans have been eating seaweed since ancient times and it seems that the Fucoxanthin in the form of eating seaweed does not provide a direct risk to human health. Relatively to the ingestion through food supplements and their relative dose, reference can be made to work of Beppu et al. [257] in which several doses of Fx administrated to living male and female mice. Both in the single dose study (1000 and 2000 mg kg^{-1}) and in a separate repeated dose

study (500 and 1000 mg kg^{-1} $d^{-1}$for 30 days) no increased mortality or abnormal growth were detected.

Although Fx is a suitable ingredient in medical and nutritional applications, its chemical stability and bioavailability can be reduced in real condition due to lipophilic and rich electron structure as well as poor water solubility and high melting point. Therefore, Fx is degraded during extraction, purification, storage, and utilization processes as a result of the acids, light, heat, oxygen, enzymes, metals, and pro-oxidant molecules. Fx stability is decreased in low and high pH values that caused degradation and *trans–cis* isomerization [345]. High temperatures, air and light exposure may induce the double bonds breakage, oxidation and isomerization in the Fx molecule and consequently pigment degradation [345, 346]. Among the potential solutions suggested for improving the stability of Fx against external factors, encapsulation and emulsion systems are promising approaches [291].

3.8 Current market and regulatory issues

In 2020, the global Fucoxanthin market was valued at approximately US$ 600 million, growing at a compound annual growth rate (CAGR) of 6% to reach US$ 780 million by the end of 2025. The current price for pure Fx ranges from USD 40,000 to 80,000 per kilogram, depending on quality and concentration [316].

Several Fx-based products are available in the market, many of which are slimming food supplements, mostly coming from seaweeds and rarely containing pure Fx. Brown seaweeds such as *Laminaria* sp., *Fucus* sp., *Sargassum* sp., *Hizikia fusiformis* and *Undaria pinnatifida* are the commercial sources of Fx for food [148]. They are usually sold in Asia as powder or oil in capsule or cachet, distributed by several major Asia companies, such as Yangling Ciyuan Biotech Co. and Agrochemi Co., and authorized in Europe by the Novel Food Regulation [148].

Fx from microalgae is utilized as supplement to whole feed or food, as it could enhance its functional and nutritional value. Several Fx products derived from microalgae are authorized for the market in the US, i.e., **Fucovital™** by Algatechnologies Ltd, Israel (from *P. tricornutum*; https://www.algatech.com/algatech-product/fucovital, accessed on 10 November 2022) which have obtained a "new dietary ingredient notification" (NDIN1048-2017) by the US-FDA; **NutriXanthin™** and **DermaXanthin™** by Alga-health, Israel, that could be used as ingredients for food supplements and cosmetics products (https://alga-health.com/products, accessed on 10 november 2022) and **BrainPhyt™** and **PhaeoSOL™** by Microphyt, France (https://microphyt.eu/, accessed on 10 november 2022). BrainPhyt™ is a microalgal extract with 2% Fx, advertised to improve cognitive performance (https://www.brainphyt.com/). PhaeoSOL™ obtained in 2019 the status of New Dietary Ingredient from the US-FDA. Fx as nutraceutical is approved by the Food Standards Australia and New Zealand (FSAN), and currently is being investigated by the United State Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA). As reported previously, two microalgal products have obtained approval as new dietary ingredient from the US-FDA. However, European regulation appears much more restrictive. Concerning food applications, the Novel Food Regulation in EU authorized the consumption of the Fx-producing seaweeds which, as used as food prior to 15th of May 1997, are listed as "not novel" in the EU Novel Foods Catalogue, whereas only one microalga, the diatom *O. aurita,* was authorized in 2005 (European Union, 2005; Regulation EU2015/2283; https://www.algae-novel-food.com) [148]. *P. tricornutum*, based on the lack of a safe history of use in the food chain and limited knowledge on its potential production of bioactive compounds with possible toxic effects, currently cannot be recommended for the list of qualified presumption of safety (QPS) [347]. On the other hand, Fx is not reported in the EU Register on nutrition and health claims (EU Register on nutrition and health claim;https://ec.europa.eu/food/safety/labelling_nutrition/claims/register/public/?event=register.home), nor in the European Pharmacopoeia (EP10), United States Pharmacopoeia National Formulary or Japanese Pharmacopoeia.

3.8.1 Concluding remarks

Except for the fundamental role in microalgal photosynthesis, Fucoxanthin has attracted more and more attentions from different industrial sectors. Towards the eventual commercialization of microalgal Fx, significant scientific and technical progresses have been made in last decade at each step of production, although several bottlenecks, such as screening of Fx-producers, conflict between biomass and Fx accumulation under high light condition, unclear steps in biosynthesis pathway and limited evaluation of outdoor scale-up cultivation and extraction, still exist. Several microalgal species can be considered as candidate producers for Fx to meet global market demands. The species, trophic mode, and bioreactor types which contribute to high biomass and enhanced productivities are still being developed and improved for commercial production. To achieve increased economic benefits, breeding, culturing, harvesting, and co-extraction should be cautiously considered. Fx as natural product may represent novel and functional ingredient for the treatment and/or prophylaxis of diseases. However, the application of Fx from microalgae in humans remains at an early stage of development and it is necessary to continue with the studies, to reach the successive clinical phases, more in vivo studies and, subsequently, in humans.

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Author contributions All authors declare that they have equally contributed to the manuscript in the following three parts: (1) the conception and design of the study, (2) drafting the article and revising it critically for important intellectual content and (3) final approval of the version to be submitted.

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Data availability All data supporting the findings of this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that might affect this work. All the authors confirmed authorship of the manuscript and agreed to submit it for peer review.

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