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This book is dedicated to the memory of Eduardo Rodrigues Lopes.

Foreword

The colours of terrestrial plants are very familiar. The predominant green of photosynthetic tissues, especially leaves, is due to chlorophylls and masks the yellow carotenoids that are also present, though these are revealed in autumn leaves, along with red anthocyanins, as the green chlorophyll is degraded prior to leaf fall. The bright colours of flowers and fruit provide striking contrast and attract insects and other vectors for pollination and seed dispersal. In the oceans of the world, terrestrial plants are replaced by algae, which may be macroalgae (seaweeds) or microalgae (phytoplankton). These do not have flowers or fruit, so major classes of plant pigments—anthocyanins and other flavonoids, betalains and quinones—are not produced in the aquatic environment. Algae, however, do use pigments for their natural photosynthesis.

Photosynthesis in algae of all classes is similar in principle to that in land plants, taking place in chloroplasts and requiring pigments to harvest and use the available light energy. The intensity of the available light, however, decreases as water depth increases, and not all wavelengths of light penetrate water, especially seawater, to the same extent. Red light, which would be absorbed by chlorophylls, is mostly absorbed in the first few metres of water depth, and it is largely blue and green light that penetrates to greater depths. So the role of accessory light-harvesting pigments, carotenoids and phycobiliproteins, is much more important, to make the most efficient use of the available light for photosynthesis. Also, algae that may become exposed to bright sunlight during part of the day must be able to withstand a high level of light energy. Adaptation to low or high light intensity is, therefore, an important feature of algal life patterns.

Algae, including microalgae, use three kinds of pigments in photosynthesis, generally located in pigment–protein complexes. Chlorophyll is essential for photosynthesis. Chlorophyll *a* occurs universally in algae of all classes. Chlorophyll *b* is largely or entirely restricted to green algae (Chlorophyta), whereas chlorophylls *c* and *d* occur in many classes. Many green algae have carotenoid compositions rather similar to those of green leaves, but other classes of algae contain many different carotenoids. These have occupied the attention of carotenoid chemists for many years, and carotenoid compositions have been used in chemosystematic

classification of algae. It is estimated that tens of millions of tonnes of fucoxanthin and peridinin are produced naturally in the world's oceans every year.

Phycobiliproteins are more specialised and restricted to the Cyanophyceae (blue-green algae, now classified as Cyanobacteria) and Rhodophyceae (red algae) in which they are localised in specialised aggregated structures, phycobilisomes and Cryptophyceae. There are two main types of phycobiliproteins, the blue phycocyanin and the red phycoerythrin, but both are usually present, though in differing proportions. Some 'blue-green algae' have a high proportion of phycoerythrin and are red and some 'red algae' have a high proportion of phycocyanin and are blue-green. The phycobilin prosthetic groups of these pigments are linear tetrapyrroles that are covalently linked to protein *via* cysteine residues.

Commercial activity is mainly focused on microalgae, which can be grown in monoculture. This is likely to expand as more commercial applications of these pigments are devised. Currently, two microalgae are used extensively for the commercial production of carotenoids. The green algae *Dunaliella* (*D. salina* or *D. bardawil*) under stress conditions can accumulate a high concentration of β-carotene, for use as a food colourant and health product. *Dunaliella* has the advantage that it tolerates high salt concentrations and can be grown cheaply, effectively as a monoculture, in large open ponds. Another green algae, *Haematococcus pluvialis*, is used to produce astaxanthin (as esters together with other carotenoids) for use in aquaculture feeds and for cosmetic and health purposes. This, though, is a freshwater species and is more expensive to produce because it must be grown in photobioreactors under sterile conditions to avoid contamination. Phycocyanin is under intensive investigation for possible use as a blue food colourant—safe and stable blue colourants are otherwise elusive—and phycoerythrin is under consideration as a red food colourant. The intense fluorescence of these phycobilins opens possibilities for their application in clinical diagnostics, e.g. in immunoassays.

This book is timely. There are many opportunities to develop new applications for pigments of microalgae and new ways of improving the production of the algae and their pigments. These aspects are covered extensively in this book, and exciting prospects are reported. The state of the natural environment, characterised by global warming due to increasing atmospheric concentration of carbon dioxide, is, however, a major concern for the future of our planet. Natural microalgae are a major contributor to fixing CO_2 from the atmosphere and generating O_2, but commercial production of microalgae may require the input of energy, and the addition of nutrients, which may lead to eutrophication. The impact of all environmental factors and the overall environmental balance may be different for different species, products and culturing conditions, and must, therefore, always be considered.

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Chapter 1
Chlorophylls in Microalgae: Occurrence, Distribution, and Biosynthesis

Jaqueline Carmo da Silva and Ana Teresa Lombardi

Abstract Chlorophylls (Chl) are the most abundant natural pigment supporting oxygenic photosynthesis in microalgae and Cyanobacteria, whereby they derive energy for metabolism and reproduction. In microalgae (eukaryotes) Chls are located in the chloroplast, but in Cyanobacteria (prokaryotes) in the photosynthetic lamellae. Chlorophylls are constituted by a large aromatic tetrapyrrole macrocycle (light absorption and redox chemistry), a central Mg ion (maximizes excited state lifetime), and a hydrocarbon tail (anchoring in thylakoids). Endosymbiosis Theory explains the photosynthetic eukaryotes plastids origin, postulating that Cyanobacteria ancestral was engulfed by eukaryotic host cell and gradually transformed into organelles that were further spread to other eukaryotes by additional rounds of endosymbiosis. Evolution distributed the Chls *a*, *b*, *c*, *d,* and *f* among microalgae and Cyanobacteria, with Chl *a* universally distributed; Chl *b* in Euglenophyta, Chlorophyta, and Charophyta; Chl *c* in Bacillariophyceae, Chrysophyceae, Xanthophyceae, Raphidophyceae, Phaeophyceae, Haptophyta, Cryptophyta, Dinophyta; Chl *d* in Rhodophyta; Chl *f* in Cyanobacteria. The pathways of Chl *a* biosynthesis were based in experiments with leaves and *Chlorella vulgaris*, dating back to the forties. Latter, it was documented the two genetically and biochemically different strategies for chlorophyll *a* biosynthesis, one being light dependent and one light independent co-exist. Chl *f* is the most recently discovered Chl, helping Cyanobacteria thrive in environments dominated by far-red light; far-red light photoacclimation, whereby 8% Chl *a* is replaced by Chl *f*, permits cyanobacteria expand light absorption range for oxygenic photosynthesis up to 800 nm allowing them to access 33% more photons than organisms that do not have Chl *f.*

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1.1 Introduction

The term *chlorophyll* was first proposed by Pelletier and Caventou (1818) to designate the green substance (Greek *chloros*) of leaves (Greek *phyllos*) for the pigments that could be extracted from leaves using organic solvent. Chlorophylls are the most abundant natural pigment that give microalgae their green color and are essential for the photosynthetic process, whereby microalgae derive their energy for metabolism and reproduction. The photosynthetic process provides direct and indirect energy source for all forms of life inhabiting our planet, and chlorophylls support such important process, therefore chlorophylls are not only the most abundant, but also the most important pigment. Chlorophyll (Chl) takes part in both light-harvesting process and energy conversion (Li and Chen 2015). In algae and plants (eukaryotes organisms) chlorophyll *a* is present in plastids, the chloroplast. In Cyanobacteria, Chl *a* is present in the photosynthetic lamellae distributed in the cell cytoplasm. The pigment contains four substituted pyrrole rings in addition to a fifth ring that is not a pyrrole but a porphyrin derivative. Chlorophyll a has a long hydrophobic phytyl side chain that anchors and orients the pigment molecule in the thylakoid membrane that is located inside the chloroplast (Lehninger 1987).

Nature has almost exclusively and universally selected chlorophylls (Chl) for the primary reactions of photosynthesis. The pigment has two main absorption bands, one near 660–665 nm (red light) and the other near 430 nm (blue light). In vivo chlorophylls occur as bound chromophores of pigment-protein complexes in the light-harvesting antennae and photosynthetic reaction centers of photosystems I (PSI) and II (PSII) in algae. In green algae and plants, Chl *a* is the most important pigment in the antenna. The antenna pigments contribute to increase the absorption cross-section of the reaction centers that are assisted by the intense absorptions of most Chls (Scheer 2006). Even though the individual absorption peaks of the chlorophylls are narrow, interaction with proteins of in vivo Chls can amplify the absorption bands and further spread them by combination with other pigments, including other Chls.

In the reaction centers, Chls are indispensable as primary electron donors and acceptors, transporting the electron within a few picoseconds across half the thylakoid membrane. They also serve to transport triplet excitation energy to the protective carotenoids (Angerhofer et al. 1998). In most photosynthetic organisms, Chls also provide the majority of light-harvesting pigments, which are only supplemented by linear tetrapyrroles and carotenoids (Sheer 2006). The large aromatic tetrapyrrole macrocycle is the basis for both the absorption and the redox chemistry of Chls, and the central metal (Mg) has a decisive influence on the excited state kinetics of tetrapyrroles, therefore, on their function in photosynthesis. Mg as the central metal maximizes excited state lifetime as well as the interactions of Chls with their proteins; in vivo chlorophylls are conjugated with protein (Markwell et al. 1979).

The structure of chlorophyll a (Chl *a*) was established in the forties according to information in Fischer and Strell (1947) that report research by the groups of Hans Fischer and James Conant (Fischer and Orth 1940) as important contributions. Chlorophylls can be defined as cyclic tetrapyrroles carrying a characteristic isocyclic five-membered ring that are functional in light harvesting or in charge separation in photosynthesis (Sheer 2006). Due to high absorption and long-lived excited states, chlorophylls are powerful photosensitizers. The safe transduction of this excited state into chemical energy is the basis of photosynthesis. Fischer and co-workers were the first to delineate the structure of the porphyrin ring, both deductively and by synthesis (Fisher and Orth 1937), as well as the fine structure of the degradation products which Professor Willstätter had prepared. He has been considered the father of the modern era on the study of chlorophyll chemistry with most of his research being summarized in "Untersuchungen über Chlorophyll" (Willstätter and Stoll 1913). Stokes (1854, 1864a, 1864b) suggested that chlorophyll consisted of two components and that the green fluorescent residue that remained after extraction of carotenoids would still be a mixture of two green substances that fluoresce in the red (Aronoff 1966). It dates of 1906–1908 by Tswett (1906, 1907, 1908) that the physical proof of the existence of two chlorophylls in green leaves had been demonstrated. This was possible with the advent of adsorption chromatography. In the very beginning of chlorophyll research, publications by Hoppe-Seyler (1879, 1880, 1881), Nencki and co-workers (Nencki and Zaleski 1901, Nencki and Marchlewski 1901), and Borodin (1882) had an important role in understanding the chlorophylls. Monteverde (1893) isolated the crystalline chlorophylls and determined their spectroscopic properties. It was Nencki in 1896 that predicted the similar chemical properties of chlorophyll (Mg) and heme (Fe), what would denote a common origin of plant and animal life. Moreover, Nencki (1896) proposed that comparison of similar compounds of flora and fauna would provide insights into the chemical and organismal evolution in nature.

Microalgae are a general name for a diverse polyphyletic group of microorganisms that convert sunlight into chemical energy through photosynthesis. A complex set of pigments that in addition to chlorophylls (Chl), can include carotenes, xanthophylls, and in some algal groups the biliproteins phycocyanin and phycoerythrin, contribute to optimization of light absorption during photosynthesis. The chlorophylls (Chl) and their absorption maximum (solved in methanol) that have been described in photosynthetic organisms are Chl *a* (the most abundant, 665 nm), Chl *b* (652 nm), Chl *c* (630 nm), Chl *d* (696 nm), and more recently Chl *f* (707 nm) (Chen et al. 2010, Chen et al. 2012, Chen 2014, Ferreira and Sant'Anna 2017). Chlorophyll *f* has been identified just in Cyanobacteria (Chen et al. 2012).

In the present chapter we concentrate on *chlorophylls*, focusing on their origin, distribution, and occurrence, including a short overview of its biosynthesis.

1.2 Occurrence and Distribution of Chlorophylls

Algae have diverse mechanisms of photosynthesis, particularly when it comes to light-harvesting pigments and assemblage in comparison to terrestrial plants. This can be attributed to the different plastid types and evolutionary history, despite the fact that endosymbiosis from Cyanobacteria and/or their ancestors are likely to have supplied the algae with the plastids (Douglas et al. 2003).

Oxygen evolving photosynthetic organisms contains Chl *a* that take part in the photochemical reactions of photosystem I (PSI), and photosystem II (PSII). Chlorophyll *a* is the primary photosynthetic pigment in these organisms and its concentration in microalgae is related to the mineral nutrition in culture media and exposure light. In healthy cells, its content has been reported to constitute about 5% of the dry biomass weight in *Chlorella* sp. cells (Marks 1966). Chlorophylls are located in intracellular organelles called chloroplasts, where they are attached to proteins forming the chloroplast lamellae. These are membranes that enclose flattened "sacs" called thylakoids (Dujardin et al. 1975), where light is absorbed during the photosynthetic process.

The evolutionary origin of photosynthetic eukaryotes is currently explained by the Endosymbiosis Theory, which involved capturing subsequent endosymbiosis of prokaryotic cells, a group characterized by the absence of nucleus, Golgi complex, endoplasmic reticulum, mitochondria, and plastids (De Duve 2007). According to this theory, the organelles of eukaryotic cells such as mitochondria and plastids, among them the chloroplasts, are supposed to have originated independently, from free-living microbes (van den Hoek et al. 1995). This is supported, among other facts, by the double-stranded DNA circular molecule similar to that of prokaryotic cells. According to the Endosymbiosis Theory, the original plastid was derived from Cyanobacteria ancestral that was incorporated into a eukaryotic host cell by primary endosymbiosis; then they were gradually transformed into organelles. Once established, primary plastids spread from that lineage to other eukaryotes by additional rounds of endosymbiosis between two eukaryotes occurred (Keeling 2004).

The primary endosymbiosis gave rise to three major clades: the green algae (Chlorophyta), the red algae (Rhodophyta), and the Glaucocystophyta. As photoautotrophic organisms, all have Chl *a*; the green algae share the presence of Chl *b* as an accessory pigment, while the red algae are characterized the presence of Chl *c* and its derivatives as accessory photosynthetic pigments (Falkowski et al. 2004). Subsequently, secondary endosymbiotic events resulted in most algal lineages we know today. This happened by the acquisition of plastids from the primary symbiont lineage in association with different eukaryotic host cells (van den Hoek et al. 1995). The secondary endosymbiotic events that involved the green algae gave rise to Euglenophytes, Chlorarachnophytes, and "green" dinoflagellates. The red primary symbiont was engulfed in a variety of eukaryotic host cells to give rise to Cryptophytes, Haptophytes (including coccolithophorids), Heterokonts (including diatoms), and peridinin-containing dinoflagellates (Delwiche 1999).

Evolution process distributed the Chl *a*, *b*, *c*, *d,* and *f* among the various groups of microalgae, and their presence or absence is of taxonomic importance (Ritchie 2006). Table 1.1 shows the taxonomic distribution of chlorophylls (Chl) in microalgae.

Chlorophyll *b* is present in Chlorophyta (green algae) and Euglenophyta; chlorophyll *c* is present in Bacillariophyta, Phaeophyta, Pyrrophyta, and Cryptophyta; chlorophyll *d* is present only in Rhodophyta (van den Hoek et al. 1995). This pigment is present in the prochlorophytes, green algae, and green plants (Scheer 1991). Chlorophyll *c* is widely distributed and abundant in the Chromophyte algae and diatoms, and usually consists of a mixture of two components c_1 and c_2 (van den Hoek et al. 1995). The two pigments differ only in two hydrogen atoms, Chl c_1 (Mg tetradehydro-pheoporphyrin) that has an ethyl group, and Chl c_2 (Mg hexadehydro-pheoporphyrin) that has a vinyl group at position C4 in ring II of the chlorophyll macrocycle (Strain et al. 1971, Wright and Jeffrey 2006). Chl *c* including its two components (Chl c_1 and Chl c_2) differ from Chls *a* and *b* in several important aspects: they are porphyrin rather than chlorin derivatives with ring IV unsaturated; they contain an acrylic rather than propionic side chain at C7; they occur as free acids (not esterified to phytol) (Larkum et al. 1994). Chlorophyll *c* has been considered an accessory Chl, but now it is better known as light-harvesting chlorophylls, i.e., they augment the light-harvesting properties of oxygenic photosynthetic organisms such as Cyanobacteria, algae, and plants by passing on light excitation to chlorophyll a (Larkum et al. 1994; Larkum and Kühl 2005). Another variety of Chl *c* is the Chl c_3, with high polarity compared to Chls c_1 and c_2. Chl c_3 has relatively low absorption in 630 nm in vivo compared to 580 nm. This pigment (Chl c_3) is present in Chrysophyceae, Haptophyta, and diatoms, but in the second it is found together with unusual fucoxanthin pigments (Stauber and Jeffrey 1988, Fawley 1989, Zapata et al. 2006). The absorption spectra of chlorophyll *c1* and *c2* in organic solvents are of the magnesium-porphyrin type, exhibiting a three-banded spectrum with very low absorption in the red region, at shorter wavelengths (628 nm) than that of chlorophyll *b* (645 nm) and chlorophyll *a* (660 nm) (Jeffrey 2013).

Chlorophyll *d* is a pigment that has been reported to occur in a number of species of the Rhodophyta (red algae), a group that contains Chl *a* but, at most, only traces of Chl *b* (Manning and Strain 1943). The occurrence of Chl *d* in red algae, together with the probable absence of Chl *b* and *c*, is further evidence for the remoteness of any phylogenic connection between this large group of algae and most of the other groups of photosynthetic organisms. Chl *d* differs markedly from all other chlorophylls due to absorption spectra in the near-infrared; The Chl *d* in vivo absorption peak occurs between 708 and 720 nm (Miyashita et al. 1996, Nieuwenburg et al. 2003, Larkum and Kühl 2005).

The most recently described chlorophyll, the Chl *f*, has been reported in 2010 (Chen et al. 2010). As chemical structure of this pigment (Chl *f*), the [2-formyl]-chlorophyll *a*, was isolated from stromatolites of the specie *Halomicronema hongdechloris*, Western Australia. The morphological features of stromatolites provide a unique environment for specific but diverse cyanobacterial communities (Chen et al. 2010). *Halomicronema hongdechloris* is the thinnest filamentous cyanobacterium known to date, which contains not only Chl *a*, but also Chl

Table 1.1 The distribution of chlorophylls (Chl) in algal groups (+ means pigment presence; empty means pigment absent). *present just in the genus *Vaucheria* [87]

	Cyanobacteria	Prochlorophyta	Glaucophyta	Rhodophyta	Chrysophyceae	Xanthophyceae	Eustigmatophyceae	Raphidophyceae
Chl a	+	+	+	+	+	+	+	+
Chl b								
Chl c1					+	+*		+
Chl c2					+			+
Chl c3					+			
Chl d				+				
Chl f	+							

	Dictyochonophyceae	Phaeophyceae	Bacillarophyta	Haptophyta	Cryptophyta	Dinophyta	Euglenophyta	Chlorarachniophyta	Chlorophyta	Charophyta
Chl a	+	+	+	+	+	+	+	+	+	+
Chl b							+		+	+
Chl c1		+	+	+						
Chl c2		+	+	+	+	+				
Chl c3			+	+						
Chl d										
Chl f										

f (Allakhverdiev et al. 2016). Chl *f* has been shown to have absorption peak at about 706 nm and a maximum fluorescence emission at 722 nm at room temperature in methanol, making it the most red-shifted chlorophyll discovered to date (Chen et al. 2010). The photophysical and photochemical functions of Chl *f* are still poorly known (Li et al. 2012). The photophysical and photochemical properties and function of Chl *f* are being now investigated (Li et al. 2012, Gan et al. 2014, Ho 2018, Kurashov et al. 2019).

1.3 Biosynthesis of Chlorophylls: A Short Overview

The pathways of chlorophyll *a* biosynthesis were based in experiments with leaves (Smith 1948), a mutant of *Chlorella* that accumulated protoprophyrin IX but not chlorophyll (Granick 1948a), and additional observations with heme (Fe protoporphyrin IX) (Shemin and Wittenberg 1951). In details, the history and the pigment biosynthesis can be found in Marks (1966) and Bogorad (1967). Hendry and Jones (1980) present a detailed study comparing heme and chlorophylls, with the biosynthetic pathways of tetrapyrroles and their natural occurrence. A more recent text on the biochemistry and regulation of chlorophyll biosynthesis can be found in Cahoon and Timko (2003).

The first indication of relation between the structure of the green pigment of plants (chlorophyll) and heme, the prosthetic group of the red blood pigment hemoglobin, dates back to 1880, as reported in Marks (1966). In spite of the differences in these two molecules (chlorophylls and heme), the arrangement of their side chains is identical. Their structural similarity has generated the idea that the biosynthesis of heme and chlorophylls could have similar pathways. This was further confirmed by Granick and co-workers (Granick 1948a, 1948b) in experiments with a mutant of the microalgae *Chlorella vulgaris* Beyerinck. This mutant produced no chlorophyll but was able to accumulate either protoporphyrin IX or Mg protoporphyrin IX. From these studies, it was shown that heme synthesized in animal's red blood cells followed the same biosynthetic pathway and with the same intermediate compounds as the synthesis of protoporphyrin IX in the chloroplast of green plants (Granick 1954). Thus, it became apparent that protoporphyrin IX was the last common molecule in the biosynthetic pathways for heme and chlorophyll production. The insertion of Fe yields the iron protoporphyrin (heme) molecule, while Mg insertion yields Mg protoporphyrin, a precursor of protochlorophyll that will lead to chlorophyll molecule (Marks 1966). It was around 1940 (Fischer and Orth 1943) that the structure of heme was elucidated and in 1960, the total synthesis of chlorophyll was finally published (Woodward et al. 1960). A general and synthetic sequence of steps in chlorophyll *a* biosynthesis is presented in Fig. 1.1, as modified from Bogorad (1967); the chemical structure of heme, protoporphyrins IX, and chlorophyll a are shown in Fig. 1.2.

Chlorophyll *a* biosynthesis in algae is a complex process that comprises several enzymatic reactions and stereo-specific reductions that will produce the chlorophyllide a from protochlorophyllide *a*. The first is converted into chlorophyll *a*, being the

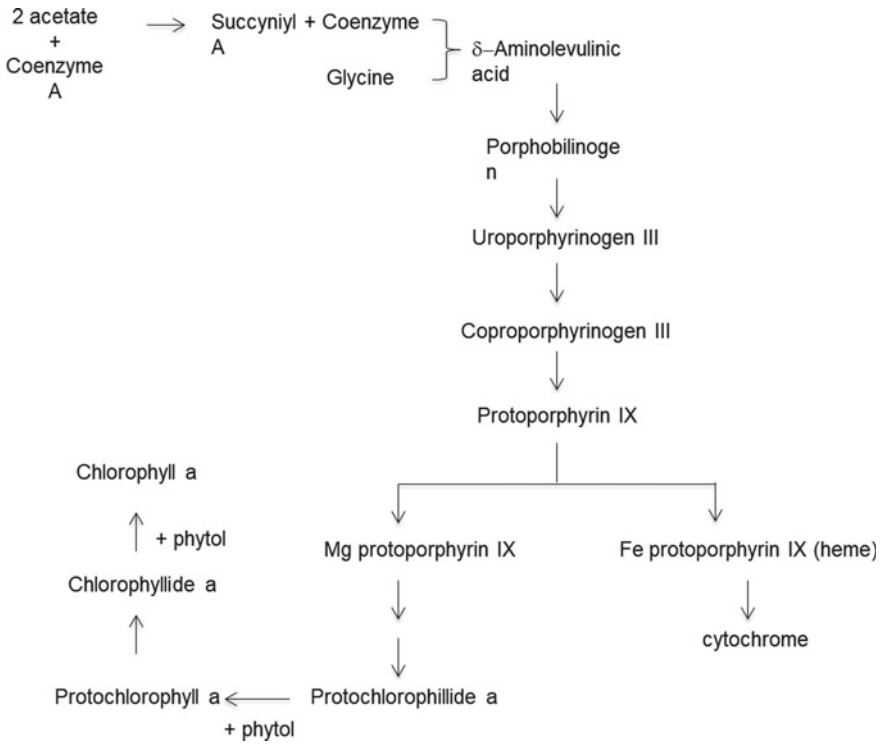

Fig. 1.1 Scheme for the biosynthesis of chlorophyll *a* (modified from Bogorad 1967)

enzymatic reduction of protochlorophyllide *a*, a key regulatory step in this process (Armstrong 1998). The pathway from protoporphyrin IX to chlorophyll *a* is presented in von Wettstein et al. (1995) and in Cahoon and Timko (2003). Figure 1.3 shows as presented in von Wettstein et al. (1995).

The existence (and co-existence) of two genetically and biochemically different strategies for the biosynthesis of chlorophyll *a* that depends on the light requirements of the reactions has been documented (Galova et al. 2008). When the enzymatic reduction of protochlorophyllide *a* to chlorophyllide *a* has light as cofactor, so occurring upon illumination, chlorophyll *a* biosynthesis is said to be light dependent. But, if it occurs in the absence of light, it is said to be light independent and can occur in the dark. Most microalgae and many other photosynthetic organisms can produce their chlorophyll from both pathways, under light and dark conditions (Bogorad 1976, von Wettstein et al. 1995, Porra 1997). As presented in Marks (1966) protochlorophyllide a can be reduced to chlorophyllide *a* by an enzymatic reaction (Smith 1960), so occurring in the dark (as with *Chlorella* sp), and then the chlorophyllide *a* is phytylated to yield chlorophyll. This reaction occurs rapidly at environmental temperatures, but not at low temperatures, near 0 °C, confirming it is an enzymatic driven reaction (Loeffler 1955, Wolff and Price 1957). Alternatively, the

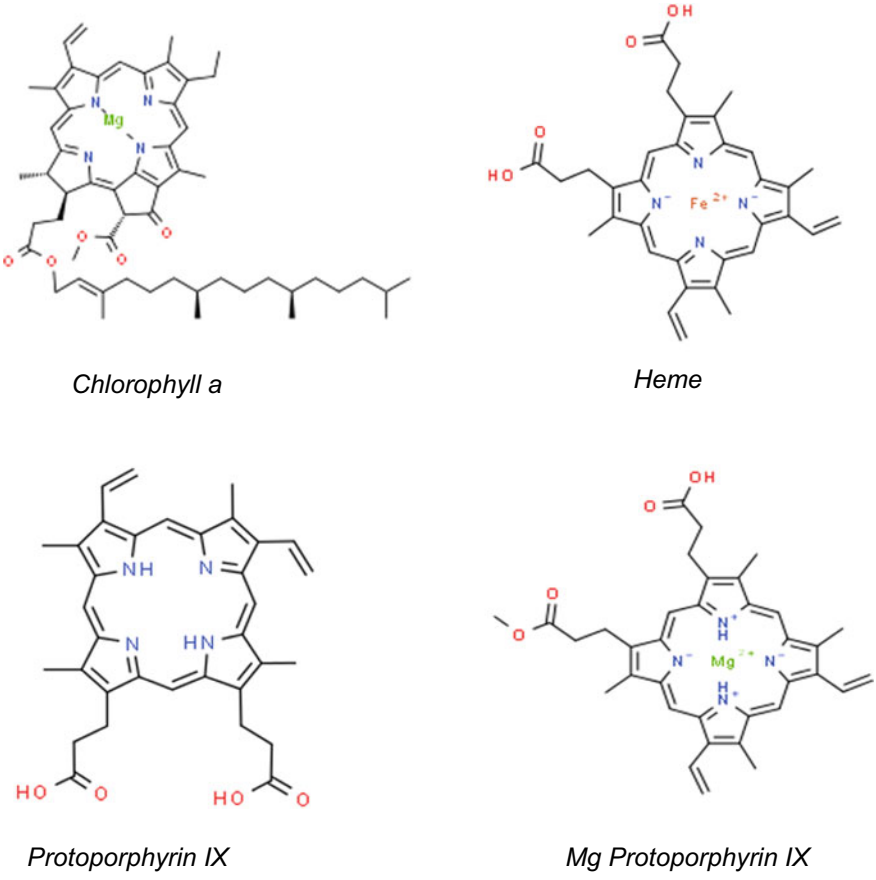

Fig. 1.2 Molecular structure of chlorophyll *a*, heme, protoporphyrin, and Mg protoporphyrin

other pathway is the phytylation of protochlorophyllide to yield protochlorophyll *a*, which is subsequently reduced photochemically to yield chlorophyll *a*.

The light dependent and independent pathways have been further confirmed. The algae chloroplasts ancestor came with two distinct nonhomologous enzymes for the reduction of protochlorophyllide to chlorophyllide: light dependent (LPOR) and the light independent protochlorophyllide oxidoreductase (DPOR) that can be active in the dark (Schulz and Senger 1993, Armstrong 1998, Reinbothe et al. 2010). Both are genetically encoded, and while LPOR is encoded by the nuclear gene *por* (Hunsperger et al. 2015), DPOR is encoded by the genes *chlL*, *chlN*, and *chlB* in the chloroplast genome (Suzuki and Bauer 1992, Li et al. 1993) in all photosynthetic eukaryotes and is active just when its substrate, the protochlorophyllide molecule, absorbs light (Shui et al. 2009, Hunsperger et al. 2015). According to Bogorad (1976) in the microalgae *Chlorella*, chlorophyll *a* can normally be produced via protochlorophyllide *a*. According to Kirk and Tilney-Bassett (1978) and Armstrong (1998) the

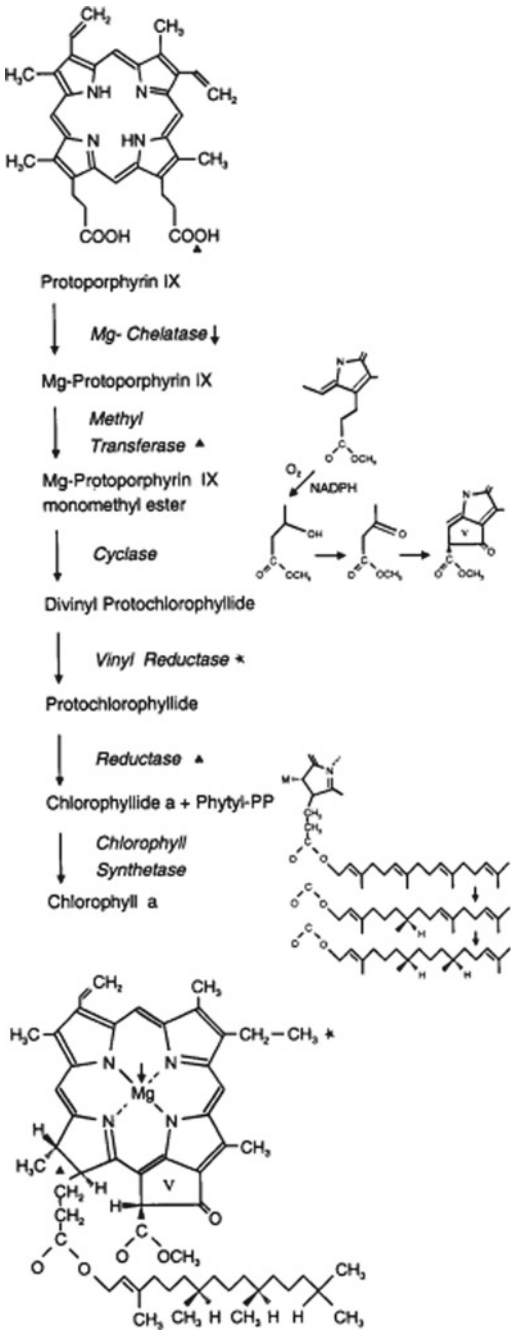

Fig. 1.3 Pathway from protoporphyrin IX to chlorophyll *a* with specification of enzymes (von Wettstein et al. 1995)

protochlorophyllide reduction in the absence of light of most algae correlates with the presence of the *chlB*, *chlL,* and *chlN* genes. It is accepted that these genes encode all of the subunits of DPOR in oxygenic-evolving photosynthetic prokaryotes and eukaryotes (Galova et al. 2008).

1.3.1 Chlorophyll b

In green algae, both Chl *a* and Chl *b* have their functions in the photosynthetic process, in the light reactions. While Chl *a* is present in the core of antenna and reaction centers, and is important for the light-harvesting and electron transfer (Larkum 2016), Chl *b* is associated with PSII antenna (Pocock et al. 2007, Szyszka et al. 2007), mostly present in the peripheral antenna complexes (Green and Durnford 1996, La Roche et al. 1996), and besides light harvesting it acts in the stabilization of the peripheral antenna complexes. The Chl *a*:Chl *b* is usually 3:1 in green algae, but Chl *b* can increase in shade adapted green algae, decreasing the ratio to 1.8–2.2 (Falkowski and Owens 1980). Chlorophyll *b* is the second most abundant among the chlorophyll pigment in nature and is found in the light-harvesting apparatus, with important function as accessory pigment (Cahoon and Timko 2003).

Figure 1.4 shows the structure of Chl *a* and Chl *b*. Chlorophyll *b* that has a molecular weight of 907 g/mol differs from Chl *a* (M.W. 839 g/mol) in the third position of its chlorin ring, which is a methyl group in Chl *a* and an aldehyde (formyl) group in Chl *b*. According to Rüdiger (2002) Chl *a* and Chl *b* follow the same biosynthetic pathway and the difference is only a step, the transformation of the 7-methyl group to the 7-formyl group. Experiments performed with ^{14}C on Chl *a* and Chl *b* in *Chlorella pyrenoidosa* and *Scenedesmus obliquus* by Blass et al. (1959) showed that Chl *a* may be a precursor of Chl *b*. It has also been shown that Chl *a* appears

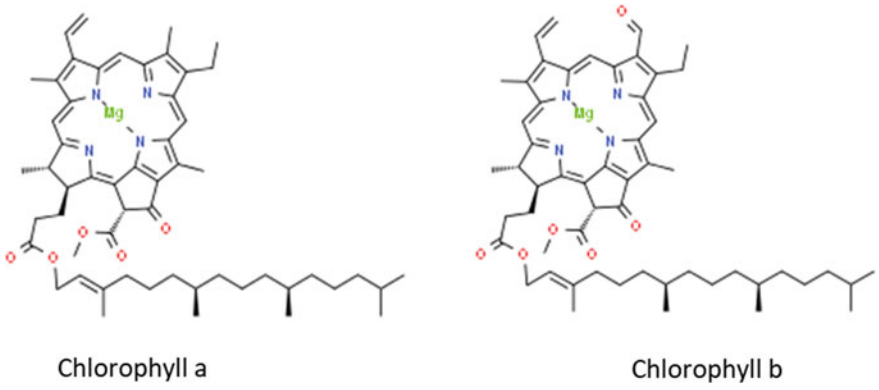

Fig. 1.4 Schematic representation of Chlorophyll *a* and Chlorophyll *b*. In Chl *a* the side group in the third carbon is a methyl group (-CH3) while in Chl *b* it is an aldehyde group (-CHO)

before Chl *b* when chlorophyll synthesis begins after irradiation of angiosperm plants (Bogorad 1976). In green algae and plants, the assembly of stable light-harvesting complexes (LHC) requires the presence of Chl *b*. Eggink et al. (2004) showed that the transformation of the 7-methyl in Chl *a* group to the 7-formyl group in Chl *b* is a reaction that uses molecular oxygen and is catalyzed by the enzyme chlorophyllide *a* oxygenase (CAO). Based on the location of the CAO the authors suggest that the envelope membranes of chloroplast are the initial site of Chl *b* synthesis. Some years later, Nakagawara et al. (2007) showed that a chloroplast protease (Clp) is involved in regulating chlorophyll *b* biosynthesis through the destabilization of CAO protein in response to the accumulation of chlorophyll *b*.

1.3.2 Chlorophylls c, d, *and* f

Chlorophyll *c* is present in three forms, Chl *c1* (no phytol tail), Chl *c2*, and Chl *c3* (no phytol tail) (Cahoon and Timko 2003). They are found in chromophyte algae, which belong to the Chromista kingdom that includes the alveolates, and that have secondary plastids (Larkum 2016). Figure 1.5 shows the structure of chlorophyll *c* and *d*.

According to they all have secondary plastids. Although Chl *c* has been described in the Chromista and alveolates, no real function for this pigment has been agreed (Fookes and Jeffrey 1989); most algae that have Chl *c*, possess both Chl *c1* and Chl *c2*, but the Dinophyceae and Cryptophyceae have only Chl *c2*; in some cases a third Chl *c, Chl c3* can be found (Prymnesiophytes). It has been suggested that Chl *c* biosynthesis occurs as a branch from Chl *a*, more precisely from protochlorophyllide or its related 3,8-divinyl protochlorophyllide (Beale 1999), thus closely related to

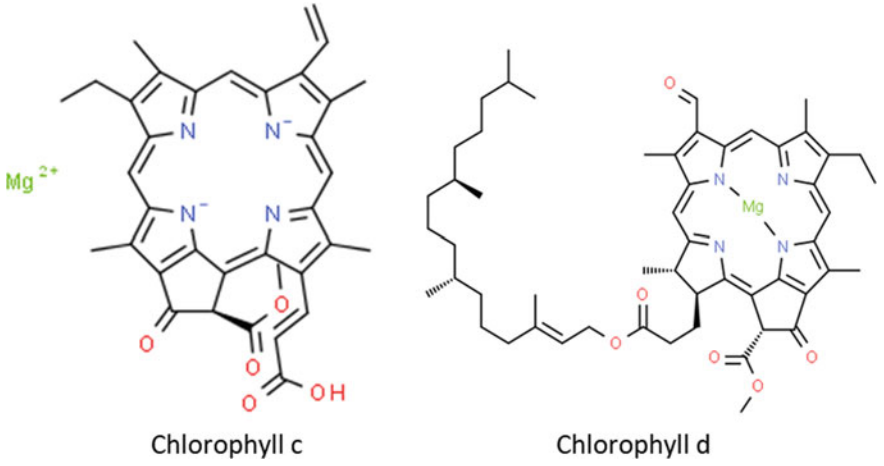

Fig. 1.5 Schematic representation of Chlorophyll *c* and *d*

protochlorophyllide, not to other chlorophylls. Figure 1.5 shows the structure of chlorophyll *c*. Additional studies on the biosynthesis of Chl *c* and their role in the Chromophyte algae are clearly needed (Chen 2014).

Chlorophyll *d* was first identified in Rhodophyta in 1943 (Manning and Strain 1943) as a minor, green, and Mg containing pigment. At first, and because of its structural similarity to an oxidation derivative of Chl *a*, Chl *d* was speculated to be an artifact due to algae aging or even generated during pigment extraction process. However, in the late nineties its discovery in the oxygenic photosynthetic prokaryote organism *Acaryochloris marina*, a Cyanobacteria, in which Chl *d* is an important primary photosynthetic pigment, confirmed the natural origin of Chl *d* (Cahoon and Timko 2003, Miyashita et al. 1996, 1997, Partensky and Garczarek 2003).

Schliep et al. (2013) proposed that in *A. marina* Chl *d* may replace Chl *a* in a photochemical role in PSI and PSII, but Chl *d* would be important in the near-infrared light (700–750 nm). Chen (2014) reported that in *A. marina*, Chl *d* accomplishes 95% of the photosynthetic pigments, while Chl *a* just 5%. In other oxygenic photosynthesis conditions, Chl *a* is likely to dominate. As pointed in Larkum (2016), the near-infrared light is the region where bacteriochlorophylls are active for anoxygenic photosynthesis.

Chlorophyll *f* is the most recently discovered chlorophyll. It absorbs in the far-red light, particularly in 700–800 nm region of the light spectra. This pigment was first described in cyanobacteria that colonize niches that are rich in the far-red light and near-infrared light, such as caves (cavernous cyanobacteria), soils, plant shaded areas (Behrendt et al. 2015, Ho et al. 2017, Shen et al. 2019). They thrive in such environment through an acclimation process known as far-red light photoacclimation (FaRLiP), whereby ~ 8% of Chl *a* molecules in the photosystems are replaced by Chl *f*, and a small amount of Chl d is produced (~1%) (Kurashov et al. 2019). The presence of chl *f* permits the cyanobacteria to expand the light absorbing range for oxygenic photosynthesis down to the near-infrared light and up to 800 nm (Ho et al. 2016). This in turn allows them to access 33% more photons than organisms that do not have Chl *f* and that are able to absorb just visible light (Chen and Blankenship 2011). Gan et al. (2014), Ho et al. (2017), and Herrera-Salgado et al. (2018) showed that PSII, PSI, and phycobilisomes are involved in the FaRLiP process. It has been shown that while Chl *f* is associated with PSII and PSI, Chl *d* is exclusively associated with PSII (Ho 2018, Nurnberg et al. 2018). It is known that chlorophyll *d* is synthesized from Chl *a*, but the synthesis of chlorophyll *c* and *f* from chlorophyll *a* needs to be clarified (Chen 2014, Ho et al. 2016).

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Chapter 2
Carotenoids in Phototrophic Microalgae: Distributions and Biosynthesis

Shinichi Takaichi

Abstract Phototrophic organisms necessarily synthesize not only carotenoids but also chlorophylls for photosynthesis. Phototrophic microalgae are usually single-cell organisms; however, as the pigment compositions of both single-cell and macrophytic types are almost identical, both are included in this chapter. Further, many kinds of carotenoids have been identified and, recently, taxonomic studies of algae have been developed. In this review, the relationship between the distributions of carotenoids and the phylogeny of oxygenic phototrophs, including cyanobacteria, red algae, brown algae, and green algae, in sea and fresh water is summarized. These phototrophs contain division- or class-specific carotenoids, such as fucoxanthin, peridinin, diadinoxanthin, and siphonaxanthin. Carotenogenic pathways are discussed in terms of the chemical structures of carotenoids and known characteristics of the enzymes involved with carotenogenesis in other organisms, as the algal genes and enzymes associated with carotenogenesis have not yet been identified. Additionally, some procedures for the general identification of carotenoids are outlined.

Keywords Algal phylogeny · Biosynthesis of carotenoids · Chlorophyll · Distribution of carotenoids

2.1 Introduction

Phototrophic algae are found in many divisions of the Plant Kingdom. Their sizes range from the single cells of picophytoplankton to macrophytic seaweeds. Attempts have long been made to cultivate single-cell algae for a long time, with limited success. However, with the recent development of culture techniques, some single-cell species can now be cultured so that their characteristics, including pigment composition, can be studied. With the development of taxonomic technology that includes the use of DNA base sequences of 16S or 18S rRNA and some genes, the understanding of algal phylogenetics underwent recent advantages (Takaichi 2011).

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More than 800 structurally defined carotenoids have been defined in organisms including land plants, algae, bacteria (including cyanobacteria and photosynthetic bacteria), archaea, fungus, and animals (Britton et al. 2004). Apart from animals, these organisms can synthesize many kinds of carotenoids using diverse carotenogenesis pathways. These carotenoids and their pathways can be used as chemotaxonomic markers (Rowan 1989; Bjørnland and Liaaen-Jensen 1989; Liaaen-Jensen 1990; Mackey et al. 1996; Jeffrey and Vesk 1997; Takaichi 2011). In addition, characteristics of the enzymes and genes involved in carotenogenesis have been investigated. Some of the genes show high base sequence similarity from bacteria to land plants, but others have low similarity. Although some homologous genes have been proposed (Frommolt et al. 2008; Bertrand 2010), carotenogenesis enzymes and genes, especially algae-specific ones, have not yet been identified. Takaichi (2011) summarized "Carotenoids in Algae" around 10 years ago, and this review offers a revised and updated version of the work.

In this review, the term algae refer to oxygenic phototrophs found in both sea and fresh water habitats. The group includes the cyanobacteria but excluding land plants. The distribution of carotenoids, carotenogenesis enzymes, and pathways are summarized. Furthermore, some procedures for the general identification of carotenoids are outlined as, recently, the identification of carotenoids as reported in some reports has been inadequate.

2.2 Distribution of Carotenoids

Many different carotenoids are found in algal species. The structures of some of the important carotenoids in phototrophic microalgae are illustrated in Fig. 2.1. Among them, approximately 30 types exhibit possible photosynthetic functions, and others may be intermediates of carotenogenesis or accumulated carotenoids. Some carotenoids are found only in certain algal divisions or classes; therefore, these carotenoids and also chlorophylls can be used as chemotaxonomic markers. Their distribution in algae is summarized in Table 2.1 (Rowan 1989; Bjørnland and Liaaen-Jensen 1989; Liaaen-Jensen 1990; Mackey et al. 1996; Jeffrey and Vesk 1997; Takaichi 2011).

Carotenoids contain some unique functional groups. The allene group (C=C=C) is a unique structure in natural compounds and is found mainly in carotenoids (Dembitsky and Maoka 2007) including fucoxanthin in brown algae and diatoms, 19′-acyloxyfucoxanthin in Haptophyta and Dinophyta, peridinin in dinoflagellates, and 9′-*cis* neoxanthin in green algae and land plants. The acetylene group (C≡C) is also a unique structure, and acetylenic carotenoids are found only in algae as part of the composition of alloxanthin, crocoxanthin, and monadoxanthin in Cryptophyta, and diadinoxanthin and diatoxanthin in Heterokontophyta, Haptophyta, Dinophyta, and Euglenophyta. The acetylated carotenoids, (C–O–CO–CH_3) such as fucoxanthin, peridinin, and dinoxanthin, are also mainly found in algae including Heterokontophyta, Haptophyta, and Dinophyta. These carotenoids are specific to certain

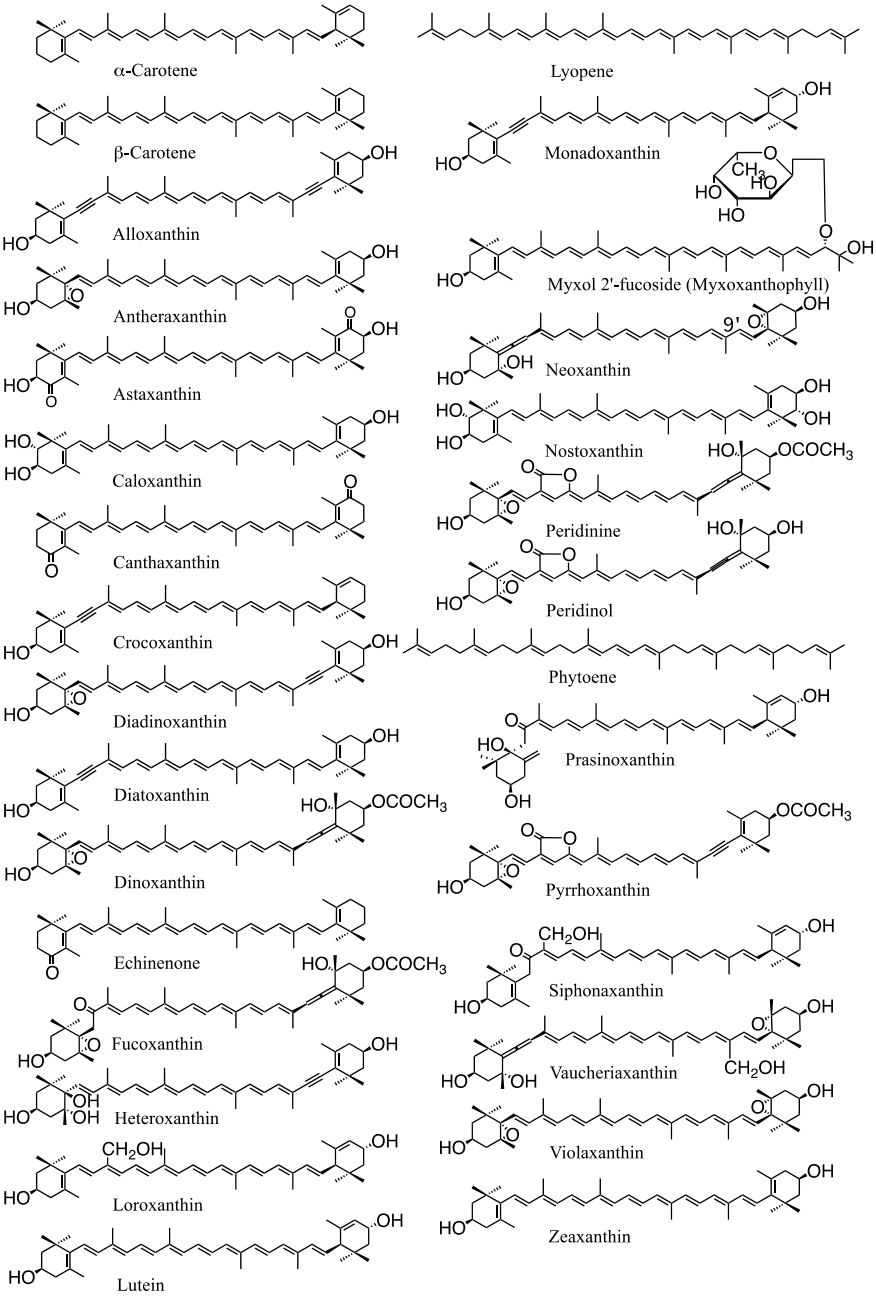

Fig. 2.1 Structures of some carotenoids

Table 2.1 Distribution of carotenoids and chlorophylls in oxygenic phototrophs

Division	Carotene		Xanthophyll												Chlorophyll		
Class	β	α	Ze	Vi	Ne	Da	Dd	Fx	Va	Lu	Lo	Sx	Others		*a*	*b*	*c*
Cyanophyta	H	L	H										No, L; Ec, H; My, H; Sy, L		H	L	
Glaucophyta	H		H												H		
Rhodophyta																	
ZEA-type	H		H												H		
ANT-type	H		H										An, H		H		
LUT-type	H	L	L							H					H		
Cryptophyta		H	L										Al, H; Cr, L; Mo, L		H		H
Heterokontophyta																	
Chrysophyceae	H		L			L	L	H	L						H		H
Raphidophyceae	H		H	L		L	L	L							H		H
Bacillariophyceae	H		L			L	L	H							H		H
Phaeophyceae	H		H	H		L	L	H							H		H
Xanthophyceae	H		L			H	H						Va-FA, L		H		H
Eustigmatophyceae	H			H					L						H		
Haptophyta	H		L			L	H	H					Fx-FA, L		H		H
Dinophyta	L		L			L	H						Pe, H		H		H
Euglenophyta	H		L		L	L	H				L	L			H	H	
Chlorarachniophyta	H		L	L	L					L	L		Lo-FA, L		H	H	
Chlorophyta																	
Prasinophyceae	H	L	L	H	H					L	L	H	Pr, L; Lo-FA, L; Sx-FA, H		H	H	
Chlorophyceae	H	H	L	H	H					H	L	L	Sx-FA, L		H	H	
Ulvophyceae	H	L	L	H	H					L	L	L	Sx-FA, H		H	H	
Trebouxiophyceae	H		L	H	H					H					H	H	
Charophyceae	H		L	H	H					H					H	H	
Land Plants	H	L	L	H	H					H					H	H	

H, Major carotenoid in most species of the class; L, Low content in most species or major carotenoid in some species.
α,α-carotene; β,β-carotene; Al, alloxanthin; An, antheraxanthin; Cr, crocoxanthin; Da, diatoxanthin; Dd, diadinoxanthin; Ec, echinenone; -FA, fatty acid ester; Fx, fucoxanthin; Lo, loroxanthin; Lu, lutein; Mo, monadoxanthin; My, myxol glycosides and oscillol glycosides; Ne, neoxanthin; No, nostoxanthin; Pe, peridinine; Pr, prasinoxanthin; Sx, siphonaxanthin; Sy, synechoxanthin; Va, vaucheriaxanthin; Vi, violaxanthin; Ze, zeaxanthin.
Some cyanobacteria contain chlorophyll *d*or*f*.

H: Major carotenoid in most species of the class; L: Low content in most species or major carotenoid in some species
α: α-carotene; *β*: *β*-carotene; Al: alloxanthin; An: antheraxanthin; Cr: crocoxanthin; Da: diatoxanthin; Dd: diadinoxanthin; Ec: echinenone; -FA: fatty acid ester; Fx: fucoxanthin; Lo: loroxanthin; Lu: lutein; Mo: monadoxanthin; My: myxol glycosides and oscillol glycosides; Ne: neoxanthin; No: nostoxanthin; Pe: peridinine; Pr: prasinoxanthin; Sx: siphonaxanthin; Sy: synechoxanthin; Va: vaucheriaxanthin; Vi: violaxanthin; Ze: zeaxanthin
Some cyanobacteria contain chlorophyll *d* or *f*

algal divisions and classes, and are summarized in Table 2.1 based on our results (Takaichi and Mimuro 1998; Yoshii et al. 2005; Takaichi and Mochimaru 2007; Takaichi 2011) and some references (Rowan 1989; Bjørnland and Liaaen-Jensen 1989; Liaaen-Jensen 1990; Mackey et al. 1996; Jeffrey and Vesk 1997).

Many cyanobacteria contain β-carotene, zeaxanthin, echinenone, and myxol pentosides (myxoxanthophyll), while certain species lack some of these, and others contain additional carotenoids, such as nostoxanthin, canthaxanthin, and oscillol dipentoside (Table 2.1, Fig. 2.1) (Takaichi and Mochimaru 2007). In addition, the carotenoid compositions of cyanobacteria are different from those of chloroplasts in algae; consequently, during symbiosis of cyanobacteria to eukaryotic cells, carotenoids may have been considerably restructured (Takaichi and Mochimaru 2007). Note that, since the name of myxoxanthophyll cannot specify the glycoside moieties, we proposed the name of myxol glycosides to specify the glycosides, such as myxol 2′-α-L-fucoside, 4-ketomyxol 2′-rhamnoside, and oscillol dichinovoside (Takaichi et al. 2001; Takaichi and Mochimaru 2007; Takaichi 2011). Additionally, most species contain chlorophyll *a*, whereas some species contain additional chlorophylls *b*, *d,* or *f* (Table 2.1).

Carotenoid composition is very diverse in Rhodophyta (red algae). They can be divided into three groups based on their carotenoid composition and phylogenetics: the unicellular group contains only *β*-carotene and zeaxanthin (ZEA-type); whereas the macrophytic group contains additional antheraxanthin (ANT-type); and the Bangiophyceae and some others contain α-carotene and lutein along with ZEA-type carotenoids (LUT-type) (Table 2.1, Fig. 2.1) (Takaichi et al. 2016). The Glaucophyta contains only *β*-carotene and zeaxanthin, and the major carotenoid in the Cryptophyta is acetylenic alloxanthin. The latter group also contains *α*-carotene and its acetylenic derivatives, crocoxanthin and monadoxanthin, which are only found in this division (Takaichi et al. 2016).

The Heterokontophyta, Haptophyta, and Dinophyta contain β-carotene and its derivatives as well as chlorophyll *c* (Table 2.1, Fig. 2.1). These divisions, except for the Eustigmatophyceae, which lack chlorophylls *c*, contain the unique acetylenic carotenoids of diadinoxanthin and diatoxanthin. Fucoxanthin and its derivatives are found in only four classes of the Heterokontophyta (Chrysophyceae, Raphidophyceae, Bacillariophyceae, and Phaeophyceae) and in the Haptophyta and Dinophyta. Peridinin and its derivatives are found only in the Dinophyta. In fact, fucoxanthin and peridinin exhibit unique structures (Fig. 2.1) and are class-specific carotenoids (Table 2.1) (Takaichi 2011).

The Euglenophyta, Chlorarachniophyta, and Chlorophyta contain the same carotenoids including *β*-carotene, violaxanthin, 9′-*cis* neoxanthin (Takaichi and Mimuro 1998), and lutein, as well as chlorophyll *a* and *b*, which are found in land plants (Table 2.1, Fig. 2.1). Some classes contain additional carotenoids, such as loroxanthin, siphonaxanthin, and prasinoxanthin, which are derivatives of lutein, and are class specific (Takaichi 2011).

Note that identification of some carotenoids is lacking because they have not yet been fully investigated. Moreover, some algal names have been changed due to new developments in taxonomic technology and phylogenetic classification.

2.3 Carotenogenesis Pathways, Enzymes, and Genes

Carotenogenesis pathways and enzymes among oxygenic phototrophs have been mainly investigated in cyanobacteria (Takaichi and Mochimaru 2007) and land plants (Britton 1998). Especially in land plants, carotenogenesis pathways and characteristics of enzymes are studied in detail (Fig. 2.2). However, algae contain common pathways to land plants and also additional algae-specific pathways, which have been proposed solely based on the chemical structures of carotenoids (Fig. 2.2) (Takaichi 2011). Some common carotenogenic genes in algae are suggested from homology with other known genes (Frommolt et al. 2008; Bertrand 2010), but most of the genes and enzymes involved in the algae-specific pathways remain unknown (Fig. 2.2). In cyanobacteria, since carotenoid compositions are different from those in land plants and algae, the pathways and enzymes are also different from those in Fig. 2.2, and are shown instead in Fig. 2.3. In addition, the carotenogenic enzymes and genes, whose

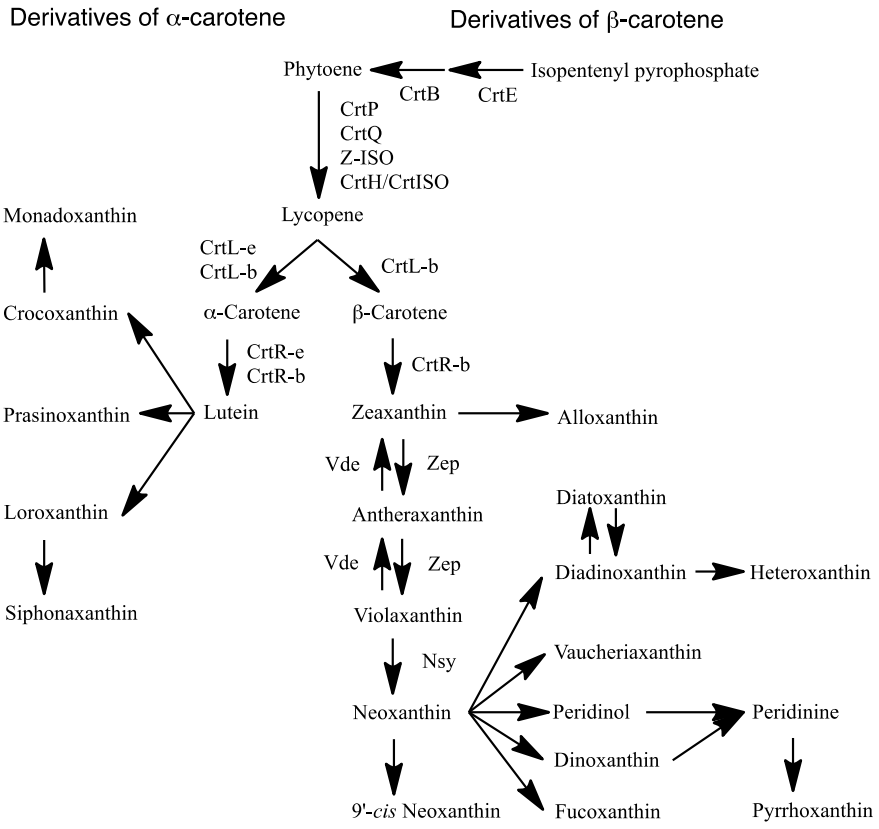

Fig. 2.2 Carotenogenesis pathways and enzymes, whose functions are confirmed, in oxygenic phototrophs

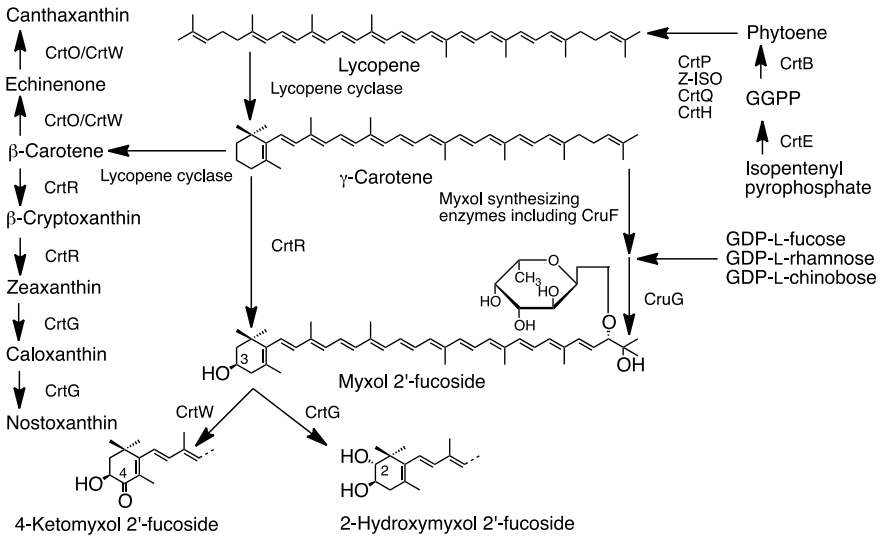

Fig. 2.3 Carotenogenesis pathways and enzymes, whose functions are confirmed, in cyanobacteria

functions have been confirmed in algae, including cyanobacteria, are summarized in Table 2.2. Unfortunately, these enzymes are mostly from cyanobacteria and green algae.

2.3.1 Lycopene Synthesis

2.3.1.1 Isopentenyl Pyrophosphate to Phytoene Synthesis

Two independent pathways of isopentenyl pyrophosphate (IPP) synthesis exist: the classical mevalonate (MVA) pathway and the alternative, non-mevalonate, 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway, which was identified in the 1990s (Lichtenthaler 1999; Eisenreich et al. 2004). In the MVA pathway, acetyl-Coenzyme A is converted to IPP through mevalonate, and the enzymes and genes have been well studied. The pathway is found in the plant cytoplasm, animals, and some bacteria (Lichtenthaler 1999; Miziorko 2011). In DOXP pathway, pyruvate and glycelaldehyde are converted to IPP. The DOXP pathway is found in cyanobacteria, the plastids of algae and land plants, and some bacteria (Lichtenthaler 1999). Carotenoids are synthesized in plastids. Exceptionally, among oxygenic phototrophs, the Euglenophyceae demonstrate only the MVA pathway, and the Chlorophyceae demonstrate only the DOXP pathway (Lichtenthaler 1999; Takaichi 2011).

All carotenoids in oxygenic phototrophs consist of eight IPP units. Farnesyl pyrophosphate (C_{15}) is synthesized from three IPPs, after which one IPP is added to farnesyl pyrophosphate by geranylgeranyl pyrophosphate synthase (CrtE, GGPS)

Table 2.2 Carotenogenesis genes and enzymes, whose functions are confirmed, in phototrophic microalgae

Gene	Enzyme	Species	References
crtE, ggps	Geranylgeranyl pyrophosphate synthase	*Thermosynechococcus elongates* BP-1 *Porphyra umbilicalis* UTEX LB-2591 *Euglena gracilis*	Ohto et al. (1999) Yang et al. (2016) Kato et al. (2016)
crtB, pys, psy	Phytoene synthase	*Gloeobacter violaceus* PCC 7421 *Synechococcus elongatus* PCC 7942 *Synechocystis* sp. PCC 6803 *Phaeodactylum tricornutum* 646 *Chlamydomonas reinhardtii* *Haematococcus pluvialis* NIES-144 *Euglena gracilis*	Steiger et al. (2005) Chamovitz et al. (1992) Martínez-Férez et al. (1994) Dambek et al. (2012) McCarthy et al. (2004) Steinbrenner and Linden (2001) Kato et al. (2016)
crtI	Phytoene desaturase (bacterial type)	*Gloeobacter violaceus* PCC 7421	Steiger et al. (2005) Tsuchiya et al. (2005)
crtP, pds	Phytoene desaturase (plant type)	*Arthrospira platensis* NIES-39 *Synechococcus elongatus* PCC 7942 *Synechocystis* sp. PCC 6803 *Phaeodactylum tricornutum* 646 *Chlamydomonas reinhardtii* *Chlorella zofingiensis* ATCC 30412 *Euglena gracilis*	Sugiyama et al. (2020) Chamovitz et al. (1992) Martínez-Férez and Vioque (1992) Dambek et al. (2012) Vila et al. (2008) Huang et al. (2008) Liu et al. (2010) Kato et al. (2019)
crtQ, zds	ζ-Carotene desaturase	*Anabaena* sp. PCC 7120 *Synechocystis* sp. PCC 6803 *Phaeodactylum tricornutum* 646 *Euglena gracilis*	Linden et al. (1993) Breitenbach et al. (1998) Dambek et al. (2012) Kato et al. (2019)
Z-ISO	ζ-Carotene isomerase	*Arthrospira platensis* NIES-39 *Euglena gracilis*	Sugiyama et al. (2020)
crtH, crtISO	ζ-Carotene isomerase	*Synechocystis* sp. PCC 6803	Masamoto et al. (2001) Breitenbach et al. (2001)

(continued)

Table 2.2 (continued)

Gene	Enzyme	Species	References
crtL, crtL-b, lcy-b	Lycopene β-cyclase	*Synechococcus elongatus* PCC 7942 *Prochlorococcus marinus* MED4 *Cyanidioschyzon merolae* NIES-1332 *Phaeodactylum tricornutum* 646 *Dunaliella salina* CCAP 19/30 *Haematococcus pluvialis* NIES-144	Cunningham et al. (1994) Stickforth et al. (2003) Cunningham et al. (2007) Dambek et al. (2012) Ramos et al. (2008) Steinbrenner and Linden (2003)
cruA	Lycopene β-cyclase	*Arthrospira platensis* NIES-39 *Synechococcus* sp. PCC 7002 *Synechocystis* sp. PCC 6803	Sugiyama et al. (2017) Maresca et al. (2007) Xiong et al. (2017)
cruP	Lycopene β-cyclase	*Synechococcus* sp. PCC 7002	Maresca et al. (2007)
crtL-e, lcy-e	Lycopene ε-cyclase	*Prochlorococcus marinus* MED4 *Chlromochloris zofingiensis* SAG 211-14	Stickforth et al. (2003) Cordero et al. (2012)
crtR	β-Carotene hydroxylase	*Anabaena* sp. PCC 7120 *Anabaena variabilis* ATCC 29413 *Synechocystis* sp. PCC 6803 *Haematococcus pluvialis* NIES-144	Mochimaru et al. (2008) Makino et al. (2008) Masamoto et al. (1998) Lagarde and Vermaas (1999) Lagarde et al. (2000) Linden (1999)
CYP97, chy1	β-Carotene hydroxylase (P450-type)	*Porphyra umbilicalis*	Yang et al. (2014)
crtG	β-Carotene 2-hydroxylase	*Thermosynechococcus elongates* BP-1	Iwai et al. (2008)
zep, npq	Zeaxanthin epoxidase	*Madagascaria erythrocladioides* *Phaeodactylum tricornutum* *Chlamydomonas reinhardtii* CC-125	Dautermann and Lohr (2017) Eilers et al. (2016) Baroli et al. (2003)
vde	Violaxanthin de-epoxidase	*Mantonilla squamata*	Goss (2003)

(continued)

Table 2.2 (continued)

Gene	Enzyme	Species	References
crtO	β-Carotene ketolase	*Anabaena* sp. PCC 7120 *Gloeobacter violaceus* PCC 7421 *Synechocystis* sp. PCC 6803	Mochimaru et al. (2005) Steiger et al. (2005) Makino et al. (2008) Lagarde et al. (2000) Fernández-González et al. (1997)
crtW, bkt	β-Carotene ketolase	*Anabaena* sp. PCC 7120 *Gloeobacter violaceus* PCC 7421 *Nostoc punctiforme* PCC 73102 *Chlorella zofingiensis* ATCC 30412 *Haematococcus pluvialis* NIES-144 *Haematococcus pluvialis* strain 34/7	Makino et al. (2008), Mochimaru et al. (2005) Steiger et al. (2005) Tsuchiya et al. (2005) Steiger and Sandmann (2004) Huang et al. (2006a) Kajiwara et al. (1995) Huang et al. (2006b) Lotan and Hirschberg (1995)

to yield geranylgeranyl pyrophosphate (C_{20}). In a condensation of the two C_{20} compounds, the first carotene, phytoene (C_{40}), is formed by phytoene synthase (CrtB, Pys, Psy) using ATP (Sandmann 1994, Armstrong 1997). This pathway has been confirmed by cloning genes from two species of *Rhodobacter* (purple bacteria) and two species of *Pantoea* (Misawa et al. 1990; Sandmann 1994; Armstrong 1997). Among oxygenic phototrophs, the functions of CrtE and CrtB have also been confirmed (Table 2.2). The *crtE* and *crtB* genes exhibit high DNA sequence similarity from bacteria to land plants, respectively (Takaichi 2011).

2.3.1.2 Phytoene to Lycopene Synthesis

Four desaturation steps are needed in the conversion of phytoene to lycopene. Oxygenic phototrophs require four enzymes: phytoene desaturase (CrtP, Pds), ζ-carotene desaturase (CrtQ, Zds), ζ-carotene isomerase (Z-ISO), and *cis*-carotene isomerase (CrtH, CrtISO) (Fig. 2.2). CrtP catalyzes the first two desaturation steps from phytoene to ζ-carotene via phytofluene, and CrtQ catalyzes two additional desaturation steps from ζ-carotene to lycopene via neurosporene. During desaturation by CrtP, 9,15,9′-*tri-cis* ζ-carotene is produced, and this is converted to 9,9′-*di-cis* ζ-carotene, as in *Arabidopsis* by Z-ISO. Light is also effective for this reaction (Sugiyama et al. 2020). During desaturation by CrtQ, neurosporene and lycopene are isomerized to poly-*cis* forms, and then CrtH isomerizes to all-*trans* forms. Light is also effective for their photoisomerization to all-*trans* forms (Masamoto et al. 2001). The functions of these enzymes have mainly been confirmed in cyanobacteria, green algae and land plants (Table 2.2), CrtP and Z-ISO from *Arthrospira platensis* and

Euglena gracilis (Sugiyama et al. 2020) and CrtH from *Synechocystis* sp. PCC 6803 (Masamoto et al. 2001; Breitenbach et al. 2001). The CrtP of *Synechococcus elongatus* PCC 7942 is stimulated by NAD(P) and oxygen as a possible final electron acceptor (Schneider et al. 1997). CrtQ only from *Anabaena* sp. PCC 7120 showed sequence homology with bacterial phytoene desaturase (CrtI) and CrtH (Linden et al. 1993), while other CrtQs show sequence homology with CrtP and plant CrtQ.

In contrast, the bacterial type uses only one enzyme, phytoene desaturase (CrtI), to convert from phytoene to lycopene. The primitive cyanobacterium of *Gloeobacter violaceus* PCC 7421 uses this type of CrtI, and homologous genes of *crtP*, *crtQ* and *crtH* are not found in the genome (Steiger et al. 2005; Tsuchiya et al. 2005); therefore, *G. violaceus* is the only oxygenic phototroph that has been shown to use this type (Table 2.2). These observations suggest the following evolutionary scheme for this step in the reaction: the desaturation of phytoene was initially carried out by CrtI in ancestral cyanobacteria; *crtP* and related desaturase genes were acquired, and ultimately, there was replacement of *crtI* by *crtP* occurred (Tsuchiya et al. 2005). Among anoxygenic phototrophs, purple bacteria, green filamentous bacteria, heliobacteria, and newly identified *Gemmatimonas* use CrtI, whereas green sulfur bacteria and newly identified *Chloracidobacterium* use CrtP, CrtQ and CrtH without Z-ISO (Takaichi 2009, 2011; Sugiyama et al. 2020).

2.3.2 β-Carotene and α-Carotene Synthesis by Lycopene Cyclases

All carotenoids in oxygenic phototrophs are dicyclic carotenoids, β-carotene, α-carotene, and their derivatives, and are derived from lycopene (Figs. 2.1 and 2.2). Exceptionally, myxol glycosides and oscillol diglycosides in cyanobacteria are monocyclic and acyclic carotenoids, respectively.

Lycopene is cyclized into either β-carotene via γ-carotene or α-carotene via δ-carotene. Three distinct families of lycopene cyclases have been identified in carotenogenic organisms (Krubasik and Sandmann 2000; Takaichi and Mochimaru 2007; Maresca et al. 2007). One large family contains CrtY, found in some bacteria, except cyanobacteria, and CrtL (CrtL-b, Lcy-b), found in some cyanobacteria and land plants. Lycopene ε-cyclases (CrtL-e, Lcy-e) from land plants are also included. Their amino acid sequences exhibit five conserved regions (Krubasik and Sandmann 2000; Sandmann 2002; Ramos et al. 2008), and have an NAD(P)/FAD-binding motif (Harker and Hirschberg 1998). Some CrtLs have been functionally confirmed (Table 2.2). Note that Krubasik and Sandmann (2000) and Takaichi (2011) indicate these are part of the same family, whereas Maresca et al. (2007) divide this family into two CrtY and CrtL families.

Two cyanobacteria also contain CrtL-type enzymes (Table 2.2). *Synechococcus elongatus* PCC 7942 contains a functional CrtL (Cunningham et al. 1994). *Prochlorococcus marinus* MED4 contains two lycopene cyclases, which exhibit sequence

homology to CrtL. CrtL-b exhibits lycopene β-cyclase activity, while CrtL-e is a bifunctional enzyme having both lycopene ε-cyclase and lycopene β-cyclase activities (Stickforth et al. 2003). The combination of these two cyclases allows the production of β-carotene, α-carotene, and ε-carotene. These enzymes may have originated from the duplication of a single gene. The characteristics of this CrtL-e are somewhat different from those in land plants (Cunningham and Gantt 2001). In addition, the β-end groups of both β-carotene and α-carotene (left half) might be hydroxylated by CrtR to zeaxanthin through β-cryptoxanthin and 3-hydroxy-α-carotene, respectively, in *P. marinus*. *Acaryochloris marina* MBIC 11017 produces (6*S*)-α-carotene, which displays opposite chirality at C-6 to the usual (6*R*)-α-carotene, and contains only one *crtL*-like gene from genome sequence (Takaichi et al. 2012).

The second family of lycopene cyclases contain a heterodimer (CrtYc and CrtYd) from bacteria, a monomer (CrtYc-Yd) from bacteria and archaea, and fused and bifunctional CrtYBs from fungi (Hemmi et al. 2003; Iniesta et al. 2008), but these are not found in phototrophs.

At first, a new family of functional lycopene cyclase in the form of CruA was identified in *Chlorobaculum tepidum* (green sulfur bacterium) (Maresca et al. 2005). Homologous genes, *cruA* and *cruP*, have been found in the genome of *Synechococcus* sp. PCC 7002, and their main products are γ-carotene in *E. coli*, which produces lycopene (Maresca et al. 2007). On the other hand, Bradbury et al. (2012) have reported that *cruP* of *Synechococcus* sp. PCC 7002 has not lycopene cyclase activity. Recently, using lycopene producing *E. coli*, Sugiyama et al. (2017) reported that CruA from *Arthrospira platensis* NIES-39 exhibits lycopene cyclase activity in *E. coli*. Furthermore, Xiong et al. (2017) determined that CruA from *Synechocystis* sp. PCC 6803 has lycopene cyclase activity requiring bound chlorophyll *a* in *cruA* deletion mutant of *Synechococcus* sp. PCC 7002.

Homologous genes of *cruA* and *cruP* are widely distributed in the genome of some cyanobacteria; however, information about the activities of these *cruA*- and *cruP*-like genes are limited, as described above. Phylogenetic analysis of the functional CruA-, CruP- and CrtL-type lycopene cyclases, and their homologs in cyanobacteria shows that they form three individual clusters (Sugiyama and Takaichi 2020). Therefore, further studies of distributions of functional lycopene cyclases (CrtL- and CruA-like, or others) in cyanobacteria are required.

The distribution of α-carotene, CrtL-e, is limited in some algae classes (Table 2.1). In some species of land plants, the characteristics of CrtL-e have been investigated (Cunningham and Gantt 2001), and are shown to have sequence homology with *crtL-b*. Lycopene is first converted to δ-carotene by CrtL-e, and then to α-carotene by CrtL-b. γ-Carotene, produced by CrtL-b, is not a suitable substrate for CrtL-e (Takaichi 2011).

2.3.3 β-Carotene Derivatives and Their Synthesis

2.3.3.1 Cyanobacteria

Some cyanobacteria produce zeaxanthin, and some produce both zeaxanthin and nostoxanthin (Fig. 2.3). First, the C-3 and C-3′ hydroxyl groups of zeaxanthin are introduced to β-carotene by β-carotene hydroxylase (CrtR, CrtR-b) via β-cryptoxanthin. Then, the C-2 and C-2′ hydroxyl groups of nostoxanthin are introduced by 2,2′-β-hydroxylase (CrtG) via caloxanthin (Table 2.2) (Masamoto et al. 1998, Mochimaru et al. 2008; Makino et al. 2008; Iwai et al. 2008). The same enzymes, CrtR and CrtG, can also introduce hydroxyl groups to deoxymyxol and myxol to produce myxol and 2-hydroxymyxol, respectively (Lagarde and Vermaas 1999; Takaichi and Mochimaru 2007; Iwai et al. 2008); consequently, the same enzymes are used in two pathways, and they have no sequence homology (Takaichi and Mochimaru 2007).

Cyanobacteria contain ketocarotenoids, namely, echinenone, canthaxanthin, and 4-ketomyxol, and two distinct β-carotene ketolases, CrtO and CrtW, are known (Table 2.2) (Takaichi and Mochimaru 2007). CrtO catalyzes β-carotene to echinenone, and the final product is canthaxanthin (Fernández-González et al. 1997; Lagarde et al. 2000; Steiger et al. 2005; Mochimaru et al. 2005; Makino et al. 2008). CrtW can introduce a keto group into β-carotene, zeaxanthin, and myxol to produce canthaxanthin, astaxanthin, and 4-ketomyxol, respectively (Fig. 2.3) (Steiger and Sandmann 2004; Mochimaru et al. 2005; Steiger et al. 2005; Tsuchiya et al. 2005; Makino et al. 2008); therefore, these ketolases are used in two pathways, β-carotene, and myxol, depending on the species (Takaichi and Mochimaru 2007).

The pathway and the enzymes required to produce the right half of myxol 2′-pentoside compound are, however, not clear (Fig. 2.3) (Takaichi and Mochimaru 2007); although, two enzymes, carotene 1′,2′-hydratase (CruF) and glycosyltransferase (CruG), have been functionally confirmed in *Synechococcus* sp. PPC 7002 (Graham and Bryant 2009).

2.3.3.2 Land Plants

Most of the carotenogenic pathways in land plants are known, and their enzymes have been functionally confirmed (Fig. 2.2). Hydroxyl groups are introduced by β-carotene hydroxylase (CrtR, CrtR-b, BCH) to β-carotene to produce zeaxanthin. Epoxy groups are introduced into zeaxanthin by zeaxanthin epoxidase (Zep, NPQ) to produce violaxanthin via antheraxanthin. Under high light conditions, violaxanthin is converted into zeaxanthin by violaxanthin de-epoxidase (Vde) to disperse excess energy from excited chlorophylls. One end group of violaxanthin is converted to an allene group of neoxanthin by neoxanthin synthase (Nsy). Because all neoxanthin in chloroplasts is in the 9′-*cis* form, an unknown 9′-isomerase that converts all-*trans* neoxanthin to 9′-*cis* neoxanthin is likely to be present (Takaichi and Mimuro 1998).

2.3.3.3 Algae

Little is known about the carotenogenic pathways among algae, but some theories have been proposed based on the chemical structures of carotenoids (Fig. 2.2). Functionally confirmed enzymes have been reported mainly in Chlorophyceae including *Chlorella*, *Chlamydomonas*, *Dunaliella,* and *Haematococcus* for CrtB, CrtP, CrtL-b, CrtR-b, Zep, Vde, and CrtW (Table 2.2) (Takaichi 2011).

In the cell-free preparation of *Amphidinium carterae* (Dinophyta), ^{14}C-labellled zeaxanthin was incorporated into allenic neoxanthin, and then into acetylenic diadinoxanthin and C_{37} peridinin (Fig. 2.2). In addition, the three carbon atoms C-13′,14′,20′ of peridinin were eliminated from neoxanthin (C-13,14,20) (Swift and Milborrow 1981; Swift et al. 1982). In terms of organic chemistry, the C-7,8 double bond of zeaxanthin can be oxidized to the triple bond (acetylenic group) of alloxanthin (Britton 1998). Consequently, alloxanthin in Cryptophyta may be synthesized from zeaxanthin, since no epoxy carotenoids exist (Table 2.1). Diadinoxanthin and diatoxanthin may be synthesized via neoxanthin, as they are asymmetric structures (Figs. 2.1 and 2.2).

Allenic carotenoids are very limited in algae. From their chemical structures, all-*trans* neoxanthin might be converted to fucoxanthin, dinoxanthin, peridinin, vaucheriaxanthin, and diadinoxanthin, but the pathways and enzymes remain unknown (Figs. 2.1 and 2.2).

Under stressful conditions, such as high light, UV irradiation, or nutrition stress, some of the Chlorophyceae including *Haematococcus*, *Chlorella,* and *Scenedesmus*, accumulate ketocarotenoids, canthaxanthin, and astaxanthin, which are synthesized by combining CrtR-b and *β*-carotene ketolase (CrtW, BKT) (Table 2.2) (Kajiwara et al. 1995; Lotan and Hirschberg 1995; Huang 2006a, b; Lemoine and Schoefs 2010). Note that although *β*-carotene ketolase of *Haematococcus* and *Chlorella* were named CrtO at first (Huang 2006a, b), they are the CrtW-type, not the CrtO-type, according to the amino acid sequences (Table 2.2).

2.3.4 α-Carotene Derivatives and Their Synthesis

In *Arabidopsis thaliana*, *β*-carotene is hydroxylated mainly by the non-heme di-iron enzymes, BCH1 and BCH2 (CrtR-b), to produce zeaxanthin, while α-carotene is mainly hydroxylated by the cytochrome P450 enzymes, CYP97A3 for the *β*-end group and CYP97C1 for the *β*- and *ε*-end groups, to produce lutein (Kim et al. 2009). In *Porphyra umbilicalis*, *β*-carotene hydroxylase of CYP97 has been functionally confirmed (Yang et al. 2014) (Table 2.2).

Lutein and its derivatives are found only in the Rhodophyta (LUT-type), Cryptophyta, Euglenophyta, Chlorarachniophyta, and Chlorophyta (Table 2.1), but no details have yet been confirmed about the hydroxylation of α-carotene. From the

chemical structures of siphonaxanthin (Yoshii et al. 2005), loroxanthin, prasinoxanthin, and monadoxanthin, it could be considered that they are derived from lutein, but the pathways and enzymes remain unknown (Figs. 2.1 and 2.2) (Takaichi 2011).

2.4 Identification Protocol of Carotenoids

Dr Liaaen-Jensen (1995), authorities on carotenoid chemistry, recommended a procedure to identify carotenoids; "For identification, the three minimum criteria, *i.e.* co-chromatography with an authentic sample, UV/Vis spectrum, and mass spectrum of a quality that allows identification of the molecular ion, must be fulfilled." She also recommends eight criteria for structure elucidation.

Other criteria have previously been suggested (Takaichi 2014), and here I summarize some revisions to the general identification procedures.

(i) Extraction and purification

These processes should be carried out under dark, dim-light, or red-light conditions. Carotenoids are easily isomerized by light; however, low temperature can prevent isomerization and oxidation.

In some reports, crude extracts of pigments from phototrophs were analyzed by C_{18} HPLC, and some *cis* forms of carotenoids were indicated. This might be due to photoisomerization during preparation. Usually, carotenoids in phototrophs are all-*trans* forms. Exceptionally, neoxanthin in chloroplasts is in the 9′-*cis* form (Takaichi and Mimuro 1998).

(ii) Identification of chromophore (conjugated C=C and C=O double bond system)

The wavelength of absorption maximum shows the number of conjugated C=C double bonds (Takaichi and Shimada 1992; Takaichi 2000; Britton et al. 2004). The value of %III/II, which is the ratio of the peak heights of the longest and the middle wavelength absorption bands from the trough between the two peaks, shows the number of β-end groups, and that of $\%D_B/D_{II}$ shows the height of *cis* peak (Fig. 2.4). A broad absorption spectrum indicates the presence of conjugated carbonyl group (C=O). Note that the absorption spectrum depends on the solvent used.

In some reports, the absorption spectra of carotenoids did not fit their names, since the authors depended only on LC-MS data and ignored absorption spectra. Nostoxanthin and β-carotene have the same chromophores, and their absorption spectra are the same (Takaichi et al. 1990).

(iii) Estimation of polar group(s) from C_{18} HPLC retention time

Xanthophylls contain oxygenic groups, such as hydroxyl, keto, and epoxy groups. The type and number of these polar group(s) may be estimated from the C_{18} HPLC retention times compared with those of authentic samples.

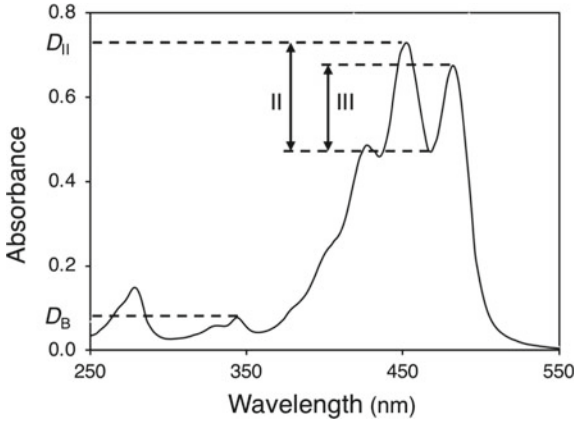

Fig. 2.4 Expression of %III/II and $\%D_B/D_{II}$ used in expressing the shape and fine structure in the absorption spectrum of carotenoid

In some of the published literature, lycopene (carotene with no oxygenic group) was shown to elute earlier than zeaxanthin (xanthophyll with two hydroxyl groups) due to ignore the polarity. Nostoxanthin (four hydroxyl groups), caloxanthin (three), zeaxanthin (two), β-cryptoxanthin (one), and β-carotene (zero) are eluted in this order (Iwai et al. 2008). Carotenoid fatty acid esters and carotenoid glycoside fatty acid esters are eluted later, due to hydrophobicity of fatty acids (Yoshii et al. 2005).

(iv) Molecular mass from mass spectrum

Mass spectra are used to determine the relative molecular masses of carotenoids. Since carotenoids consist of C, H, and O atoms, the molecular masses are essentially even numbers. At present, LC-MS is usually used, and from high-resolution mass spectra, the molecular formula can easily be obtained. Fragmentation patterns are strongly depending on ionization conditions. The Mass Bank from The Mass Spectrometry Society of Japan (MSSJ) is a useful database (http://www.massbank.jp/Index).

In some reports, authors identified carotenoids by only comparing their data with the mass spectra provided by the supplier, the reliability of whom is not always certain. Hence, comparison with additional absorption spectra data and the HPLC retention times is essential. Fragmentation patterns should be analyzed under the same conditions using the same authentic Écarotenoids.

(v) Structure elucidation from ^{1}H-NMR and ^{13}C-NMR spectra

NMR is the most powerful technique for structure elucidation (Englert 1995). Since the conjugated double bonds of the olefinic region of carotenoids are long, the NMR spectra of both end-groups are independent of each other. In the case of asymmetrical carotenoid, the total NMR spectrum is the addition of that derived from left and right halves (Englert 1995). 2D-NMR analysis is very useful for structure elucidation.

NMR inevitably detects impurities including lipids, if present. In some reports, these have been the cause of misidentification. With ^{1}H-NMR in $CDCl_3$, chemical

shifts at 0.88, 1.25, and 1.56 ppm are due to the terminal methyl groups of fatty acids, olefinic methylene of fatty acids, and water, respectively. Nostoxanthin, caloxanthin, zeaxanthin, β-cryptoxanthin, and β-carotene show corresponded identical ^{1}H-NMR spectra of the β-end group, 3-hydroxy β-end group, and 2,3-dihydro β-end group (Takaichi et al. 1990). An example of NMR data of neurosporene is presented in Takaichi and Maoka (2015).

(vi) Chirality elucidation from circular dichroism (CD) spectra

Some carotenoids such as zeaxanthin and α-carotene have chiral carbon(s). Absolute configurations of carotenoids are determined with CD spectra (Buchecker and Noack 1995).

Natural α-carotene and its derivatives, such as lutein and siphonaxanthin, have a (6′*R*) conformation at the ε-end group. Exceptionally, α-carotene from cyanobacteria of *Acaryochloris* exhibits opposite chirality, (6′*S*)-α-carotene. Both CD spectra are mirror images (Takaichi et al. 2012).

(vii) "Carotenoids Handbook"

This book (Britton et al. 2004) contains more than 700 types of carotenoids with their spectroscopic data, sources of organisms, and references, and is useful for checking the identity of any newly isolated carotenoids.

(viii) Compare with phylogenetic systematics

In general, natural compounds and their metabolic pathways are strongly related to phylogenetic systematics. These are very useful controls for analysis of carotenoids and useful tools for thinking about metabolic pathways. In purple bacteria, the variation of carotenoids and carotenogenic pathways can be explained as the deletion and/or addition of one or two enzymes (Takaichi 2009). In cyanobacteria (Fig. 2.3), it can be explained as the presence or absence of carotenogenic enzymes and characteristics of the enzymes (Takaichi and Mochimaru 2007). Genus or family-specific carotenoids have been reported including synechoxanthin (Graham et al. 2008) and myxol glycoside (Takaichi and Mochimaru 2007) in cyanobacteria, and siphonaxanthin esters in the Chlorophyta (Yoshii et al. 2005). Exceptionally in prokaryote, the horizontally transfer of a gene cluster to other bacteria has been found (Petersen et al. 2012).

In some reports, a bacterium contained diverse carotenoids with unrelated synthetic pathways. However, it is not likely that the bacterium contains so many carotenogenic enzymes. Therefore, this result is likely to be due to misidentification of carotenoids.

(ix) Carotenogenic genes from genome DNA sequences

Recently, determining genomic DNA sequences has become easier, especially among prokaryotes. After a homologous search for carotenogenic genes, in some cases,

the products of carotenoids can be predicted. The presence or absence of genes or carotenogenic pathways can also be predicted. This is a new approach.

Note that not all homologous genes of base sequence demonstrate the same functions; CrtI, CrtH, CrtO, and CrtD exhibit relatively homologous base sequences but different functions. Some cyanobacterial homologous genes of lycopene cyclases have not yet had their functions confirmed (see Sect. 3.2). However, finding unknown enzymes using this method is impossible.

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Chapter 3
Phycobiliproteins in Microalgae: Occurrence, Distribution, and Biosynthesis

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Abstract Photosynthetic performance of microalgae is predominantly dependent on the intracellular pigments composition and its functionality. Phycobiliproteins (PBPs) are the major accessory multi-colored pigment-protein assembly in thylakoid membrane that play an eminent role in harvesting solar spectrum for the carbon fixation. The wide spectral compositions of PBPs are due to assembly of mainly three subunits such as phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC). The subunits of PBPs are widely distributed in various microalgae inhabiting diverse habitats. They are metabolically produced by systematic catalytic regulation of lyases enzymes in bilin biosynthesis. The multi-functional properties of PBPs play an important role in various fields of life sciences. Comprehensive knowledge of occurrence, distribution, and biosynthesis of various PBPs in microalgae is still lacking. This chapter will mainly focus on the PBPs composition of microalgae, its distribution, biosynthetic mechanisms, recent approaches in efficient production technology, and commercial role of PBPs.

Keywords Biosynthesis · Commercialization · Metabolic pathway · Microalgae · Phycobiliproteins

3.1 Introduction

Phycobiliproteins (PBPs) are brilliantly colored, autofluorescent, and water-soluble accessory light-harvesting pigments that play a key role in photosynthesis by transferring solar energy to the reaction center in cyanobacteria, red algae, glaucophytes, and cryptomonads. PBPs constitute about 20–30% dry weight of the cell whose concentration may rise up to 40–50% in low light intensity (Muramatsu and Hihara

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2012). PBPs are made up of different colored subunits such as phycoerythrin, phycocyanin and allophycocyanin, and colorless linker polypeptides. Each PBPs subunit has a characteristic heterodimer linear tetrapyrrole chromophore with four rings (A, B, C, and D) which covalently attached to thiol linked cysteine residue at C-3 position in ring A and ring D by C-18 position (Kannaujiya and Sinha 2017a). According to chromophore absorbance and structure, PBPs are broadly divided into three classes such as phycocyanin (PC; λ_{max} = 610–620 nm), phycoerythrin (PE; λ_{max} = 560–570 nm), and allophycocyanin (APC; λ_{max} = 650–660 nm) (Sidler 1994, Kannaujiya and Sinha 2016a) (Fig. 3.1).

PBPs are made up of two basic heterodimer α and β subunits which aggregate and functionalize into monomeric, trimeric $(\alpha\beta)_3$ (APC), or $(\alpha\beta)_6$ hexameric conformation (PE, PC). Pathway of energy transfer from solar spectrum is initiated from PE to PC to APC and ultimately to reaction center for proper carbon fixation during photosynthesis. The molecular structure of PC and APC are embedded on the periphery and core region of photosynthetic membrane (Singh and Montgomery 2013). PE is attached at the distal end of PBPs and plays a crucial role in light adaptation (Kannaujiya and Sinha 2016a, b). Most PBPs subunits are made up of hetero-monomer united by two subunits such as α and β with molecular weight ranging from 12–20 kDa and 15–22 kDa, respectively (Galland-Irmouli et al. 2000).

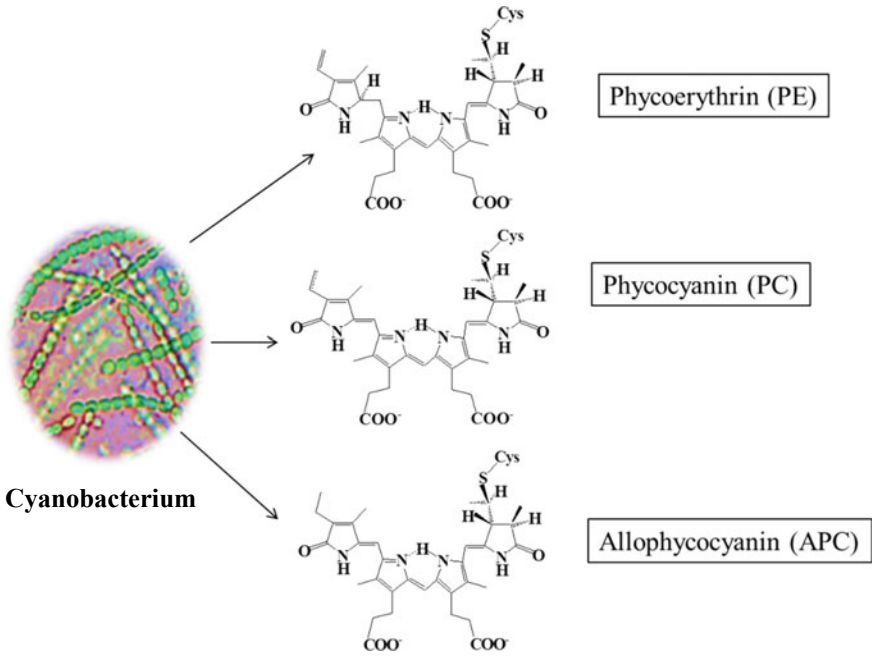

Fig. 3.1 Chemical structure of phycobiliproteins isolated from cyanobacteria (Adapted and modified from Kannaujiya et al. 2018)

PBPs have great potential in food, biopolymers, colorants, pharmaceuticals, cosmetics, molecular biology, and biomedical sciences (Mishra et al. 2012). PBPs have auto-fluorescent nature, so it can be used for the development of fluorescence compounds for various biomedical, pharmaceutics, and experimental molecular biology (Eriksen 2008). Worldwide, several patents have been reported on the broad application of PBPs (Sekar and Chandramohan 2008). Many companies have initiated the development of value-added products from PBPs (Sekar and Chandramohan 2008). This chapter highlights the distribution of PBPs among cyanobacteria, biosynthetic machinery, production and commercial utilization of value-added PBPs in various fields of sciences.

3.2 Ecological Distribution of Microalgae

Cyanobacteria are the most ancient obligate photosynthetic prokaryotic organisms (Wood et al. 2008). They originated on early Earth surface about 2.8–3.5 billion years ago and presently have cosmopolitan distribution ranging from hot to cold water habitats (Fischer 2008). They are important primary producers and carbon fixers in both aquatic as well as terrestrial ecosystems and contribute immensely in net primary productivity (Häder et al. 2007). Cyanobacteria are well known for their role in global CO_2 and nitrogen fixation (Singh et al. 2010) and oxygen production (Parmar et al. 2011). Atmospheric nitrogen-fixing property of cyanobacteria makes them ecologically valuable as they increase the biomass and productivity of crop plants (Vaishampayan et al. 2001). As cyanobacteria reside in a variety of adverse habitats, they are also used for the reclamation of desert soil. Cyanobacteria are also a better substitute for commercially available N-fertilizer, whose production needs a huge amount of fossil fuels (Grizeau et al. 2016). Solar spectrum mainly consists of visible radiation (PAR; 400–700 nm) and ultraviolet (UV; 100–400 nm) radiation. Moreover, ultraviolet radiation consists of ultraviolet-B radiation (UV-B, 280–315 nm) which is deleterious to living organisms and causes several inhibitory effects on biological organisms (Sinha et al. 1995, Häder et al. 2011, Paul et al. 2012). Cyanobacteria are directly exposed to deleterious UV-B radiation due to their photoautotrophic nature and hence the most important photosynthetic and nitrogen-fixing abilities of cyanobacteria may be impaired (Cassier-Chauvat and Chauvat 2015). However, nucleic acids and proteins are more susceptible to damage by the high intensity of UV-B irradiation (Rastogi et al. 2010). UV-B irradiation also adversely affects the key physiological and biochemical life processes, such as morphology, growth, development, heterocyst frequency, pigmentation, motility and orientation, N_2 metabolism, phycobiliproteins composition, lipid peroxidation, $^{14}CO_2$ uptake, membrane permeability, PS-II activity, nutrient uptake, and various other metabolic processes of cyanobacteria (Sinha et al. 2002, Rastogi et al. 2012, Singh et al. 2013, Kannaujiya et al. 2017d).

3.3 Occurrence and Distribution of Phycobiliproteins

PBPs are the major antennae complex of rods subunits stacked biliproteins with linear tetrapyrrole chromophore which is covalently bound to cysteine residue (Adir 2005, Su et al. 2010, Watanabe and Ikeuchi 2013, Kannaujiya and Sinha 2015). More than ten different PBPs have been detected in cyanobacteria and red algae (Stadnichuk, and Tropin 2017). There are large numbers of cyanobacteria having a different composition of PBPs (Table 3.1).

3.4 Chromophores

PBPs are chemically similar to bile pigments, thus it is called phycobilins. Pigments with the chemical structure of bilins are also found in bacteria of different habitats, mushrooms, higher plants, and a range of invertebrates. Along with PBPs, bilins are enriched with cofactors of different proteins such as leghemoglobin in the root nodules of beans and phytochromes of plants. Hemoglobin derived pigments are also found in spiders, myriapods, and other arthropod tissues, the fins and muscles of fishes, and coral skeleton (Rüdiger and Scheer 1983). Phycobilins are assigned to linear tetrapyrroles, which contain four pyrrole rings A, B, C, and D connected by single carbon bridges. The pyrrole rings A and D, contains one oxygen atom whereas middle pyrrole rings, B and C, are associated with propionic acid residues (Glazer 1989). The phycoerythrobilin and phycourobilin incorporated in phycoerythrins can have a second covalent bond with the protein in the D ring.

3.5 Biosynthesis

The core biosynthetic pathway of PBPs has followed the similar route as tetrapyrroles biosynthesis. The core mechanism of tetrapyrroles biosynthesis has been originated from evolution of photosynthetic bacteria. There are more than 19 enzymes involved in the core pathway of conversion to initial precursor protoporphyrin IX from glutamate (Fujita et al. 2015). Certain enzymes including uroporphyrinogen synthase, hydroxymethylbilane, hydroxymethylbilane synthase, porphobilinogen, and porphobilinogen synthase are extremely conserved from prokaryotes to eukaryotes (Dailey et al. 2017). Recently, several pathways were found in bacteria related to heme biosynthesis in higher plants. A set of enzymes used for catalytic conversion from coproporphyrin intermediates in Gram-positive bacteria, (Dailey et al. 2015, Lobo et al. 2015, Dailey and Gerdes 2015) whereas protoporphyrin intermediate in Gram-negative bacteria were found for biosynthesis of heme (Dailey et al. 2015). However, certain sulfate-reducing bacteria and archaea have utilized another intermediate such as siroheme for molecular conversion to protoheme (Kuhner et al. 2014). There is

Table 3.1 Diversity of phycobiliproteins composition found in various microalgae

Cyanobacteria	Phycobiliproteins	References
Phormidium rubidium A09DM	Phycoerythrin (PE) Phycocyanin (PC) Allophycocyanin(APC)	Rastogi et al. (2015)
Synechocystis sp. PCC 6803	Phycocyanin (PC) Allophycocyanin (APC)	Sidler (1994)
Fremyella diplosiphon	Phycoerythrin (PE) Phycocyanin (PC) Allophycocyanin(APC)	Tandeau de Marsac(1983)
Nostoc sp. strain HKAR-11	Phycoerythrin (PE) Phycocyanin (PC)	Kannaujiya and Sinha (2015)
Nostoc sp. strain HKAR-2	Phycoerythrin (PE) Phycocyanin (PC)	Kannaujiya and Sinha (2015)
Spirulina platensis	C-Phycocyanin (C-PC) Allophycocyanin (APC)	Boussiba and Richmond (1979)
Limnothrix sp. strain 37–2-1	C-Phycocyanin (C-PC)	Gantar et al. (2012)
Phormidium sp. A27DM	Phycoerythrin (PE)	Parmar et al. (2011)
Lyngbya sp. A09DM	Phycoerythrin (PE)	Parmar et al. (2011)
Halomicronema sp. A32DM	Phycoerythrin (PE)	Parmar et al. (2011)
Spirulina (Arthrospira) fusiformis	C-Phycocyanin (C-PC)	Minkova et al. (2003)
Nostoc sp. PCC 9202	Phycocyanin (PC) Phycoerythrin (PE)	Reis et al. (1998)
Oscillatoria sp. N9DM	Phycoerythrin (PE) Phycocyanin (PC) Phycoerythrocyanin(PEC)	Singh et al. (2012)
Oscillatoria cf. *corallinae*	Phycoerythrin (CU-PE)	Hoffmann et al. (1990)
Synechococcus CCMP 833	Allophycocyanin C-phycocyanin Phycoerythrin	Viskari and Colyer (2003)
Phormidium fragile	C-Phycocyanin (C-PC)	Soni et al. (2008)
Oscillatoria quadripunctulata	C-Phycocyanin (C-PC)	Soni et al. (2006)
Geitlerinema sp. A28DM	Allophycocyanin	Parmar et al. (2010)
Rhodella violacea	B- Phycoerythrin (B-PE)	Koller and Wehrmeyer (1975)
Porphyridium cruenfum	R- Phycocyanin (R-PC) Allophycocyanin B- Phycoerythrin (B-PE)	Tchernov et al. (1993)
Gracilaria chilensis	R- Phycocyanin (R-PE)	Vásquez-Suárez et al. (2018)
Anabaena sp. PCC 7120	Allophycocyanin Phycocyanin Phycoerethrocyanin	Ducret et al. (1996)

C: Cyanophyta, R: Rhodophyta, U: Phycourobilin

an utmost need of an aerobic environment for catalytic regulation of systematic enzymatic process. Apart from several enzymes, there are few enzymes essentially required for catalysis of biosynthesis process while other enzymes are regulated in anaerobic condition. Thus, anaerobic environment would exhibit reduction in biosynthesis of bilins including chlorophylls by mark inhibition in gene expression (Fujita et al. 2015).

3.5.1 Glutamyl-tRNA to Porphyrin Pathway

Heme (iron-containing porphyrins) is widely distributed among eukaryotic photosynthetic bacteria and certain archaeal species. In a bacterial system, heme compositions are diverse with different functionality and specific catalytic binding proteins under diverse environmentally regulated respiratory cytochromes (Mayfield et al. 2011). However, some non-respiratory-chain cytochromes (P450) are utilizing protoheme in the form of cofactor to metabolize broad range of substrates by using monooxygenase enzyme (Moody and Loveridge 2014). In photosynthetic bacteria, protoheme synthesized endogenously is used as substrate for heme oxygenase for synthesis of the linear tetrapyrrole biliverdin for synthesis of phycobilins (Frankenberg-Dinkel and Terry 2009). Many bacteria are utilizing heme for release of iron after heme degradation (Runyen-Janecky 2013). The routes of heme sysnthesis start with precursor of 5-aminolevulinate (ALA). The mechanism of synthesis of ALA is widely distributed in archaea, plants, and most of the bacteria (Jahn et al. 1992). In enzymatic reaction, glutamyl-tRNA is synthesized by glutamyl-tRNA synthetase and converted into labile intermediates glutamate-1-semialdehyde which is further converted into ALA by glutamate-1-semialdehyde-2, 1-aminomutase enzyme (GsaM) (Randau et al. 2004). In the next pathway, ALA is converted into monopyrrole porphobilinogen by catalytic action of porphobilinogen synthase (PbgS). In the series of transformation, pyrrole building block elements PBG formed macrocyclic uroporphyrinogen III intermediates such as hydroxymethylbilane synthase (HmbS) and uroporphyrinogen synthase (UroS). In most bacteria, these enzymes are encoded by genes *hemC* and *hemD* (Jordan 1991). The biosynthesis of protoheme is regulated by siroheme-dependent pathway in archaea, sulfate-reducing, and denitrifying bacteria. Systematically, protoheme is a result for conversion of uroporphyrinogen III and coproporphyrinogen III which is catalyzed by uroporphyrinogen III decarboxylase (Jordan 1990). The coproporphyrin-dependent pathway is generally found in most of Gram-positive bacteria. Coproporphyrin or protoporphyrin is not found in archaea and they utilize coproheme intermediate for the synthesis (Kuhner et al. 2014). Now, coproporphyrinogen III is converted into protoporphyrinogen IX, by decarboxylation mechanism (del Batlle et al. 1965) (Fig. 3.2). This reaction is catalyzed by the action of coproporphyrinogen decarboxylase in the presence of molecular oxygen whereas another enzyme coproporphyrinogen dehydrogenase is active in anaerobic conditions (Layer et al. 2010). Now protoporphyrinogen IX is oxidized to protopor-

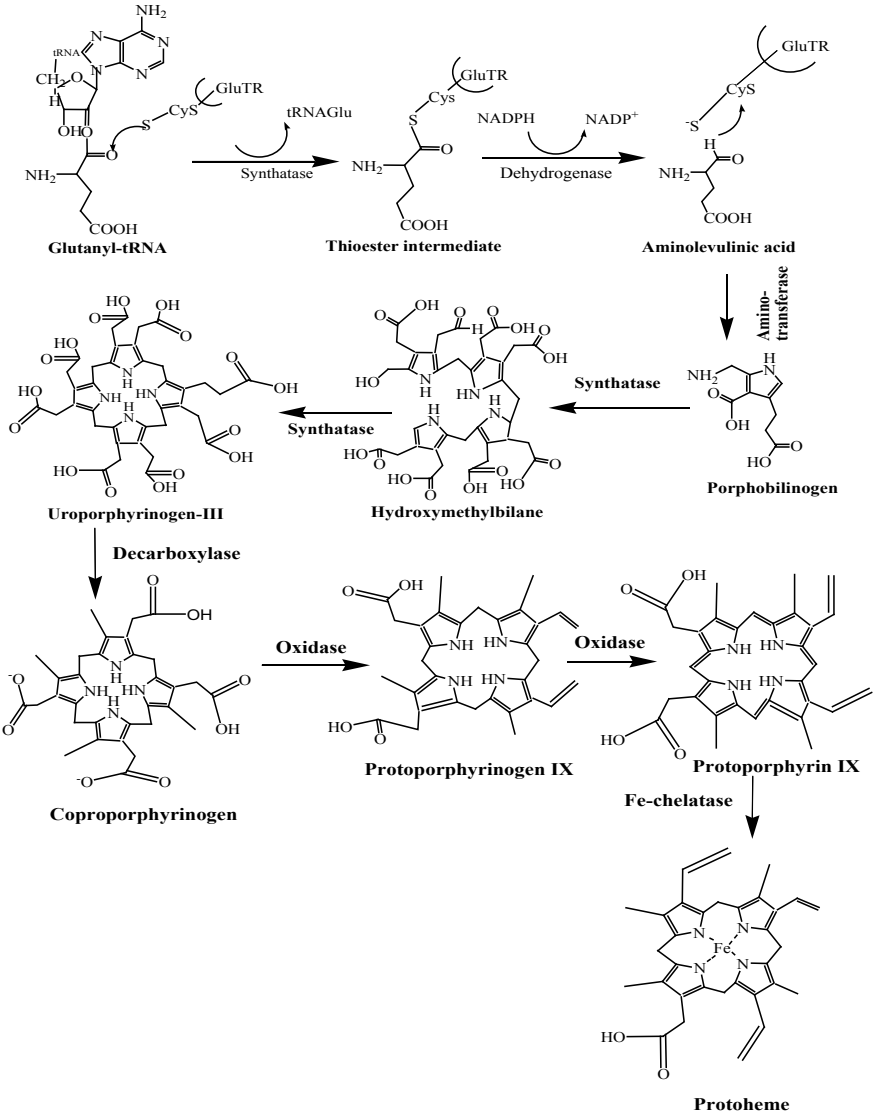

Fig. 3.2 Biosynthetic pathway from Glutanyl-tRNA to protoheme (Adapted and modified from Kannaujiya et al. 2017c, Dailey et al. 2017, Pagels et al. 2019)

phyrin IX by oxygen-dependent enzymes. The incorporation of an Mg atom leads to the formation of protoporphyrin whereas binding of Fe to protoporphyrin IX leads to the formation of protoheme.

3.5.2 Heme to Biliverdin IXα

The biosynthesis from heme to biliverdin IXα (BV) is catalyzed by heme oxygenase (HOs) (Dammeyer and Frankenberg-Dinkel 2008). Heme oxygenase enzyme is catalytically triggered by molecular oxygen and reducing electrons (Kannaujiya et al. 2017c, d). Sometimes, biliverdin IXα is also converted into bilirubin IXα by catalysis of biliverdin reductase recorded in most of the fresh and marine cyanobacteria (Kannaujiya et al. 2017c). However, prominent functionality of enzymes requires reducing sugar such as NAD (Sugishima et al. 2005) and reduced methine bridge located between B and C of biliverdin (Overkamp et al. 2014).

3.5.3 Biliverdin IXα to Phycobilins

Phycobilins are initially formed by the cleavage of carbon bridges in protoheme while the reconstruction of pyrrole rings (A or D) occurs by the action of bilin reductases enzymes (Stadnichuk and Tropin 2017). In the next step of conversion, FDBR family gene (ferredoxin-dependent bilin reduction) (Frankenberg et al. 2001) produces certain enzymes such as 15, 16-dihydrobiliverdin: ferredoxin oxidoreductase (*pebA*), phycoerythrobilin: ferredoxin oxidoreductase (*pebB*), phycocyanobilin: ferredoxin oxidoreductase (*pcyA*) and phycoerythrobilin synthase, (*pebS*) and heme oxygenase (*HO*) play a distinctive role in the conversion of BV to phycyanobilin (PcyA) and phycoerythrobilin (Peb A, B) (Beale and Cornejo 1991, Dammeyer et al. 2008, Scheer et al. 2015). Heme oxygenase is crucially dependent on oxygen concentration for optimum activity. In the cyanobacterium *Synechocystis* 6803, two isoform genes *ho1* and *ho2* have been recognized which encodes heme oxygenase with 50% similarity between amino acids (Sugishima et al. 2005, Aoki et al. 2011, Fujita et al. 2015). The HO2 also shows potential catalytic activity with oxygen as compared to HO1. However, HO is also found in anaerobic bacterium *Clostridium perfringens* and *Clostridium tetani* (Brüggemann et al. 2004). In addition, the catalytic products of heme oxygenase, biliverdin IXα, and its derivative bilirubin play as a potent oxidative inhibitor (Wegiel et al. 2014). The crystallography analysis of α helix exhibits the presence of highly conserved residues including His and Asp which provide native polar contacts in the BV IXα conversion to PC (Unno et al. 2015) (Fig. 3.3). PebS is a catalytic protein which is composed of 233 different amino acids that regulate alternate pathway for the conversion from BV to PEB including intermediate products 15, 16-DHBV (Martiny et al. 2006). However, other intermediate components like PVB are produced by molecular isomerization of cystein-84 residue by the activity of lyase enzymes PecE/F in *Mastigocladus laminosus* (Böhm et al. 2007). The activities of bilin lyase enzymes ensure the proper functionality of Cyst integrated chromophore in bilin proteins (Arciero et al. 1988). The isomerization is a key feature of lyase enzyme which converts PVB into PCB and PUB into PEB by isomerization of native chromophores (Blot et al. 2009). Methylation of asparagine amino acid is commonly

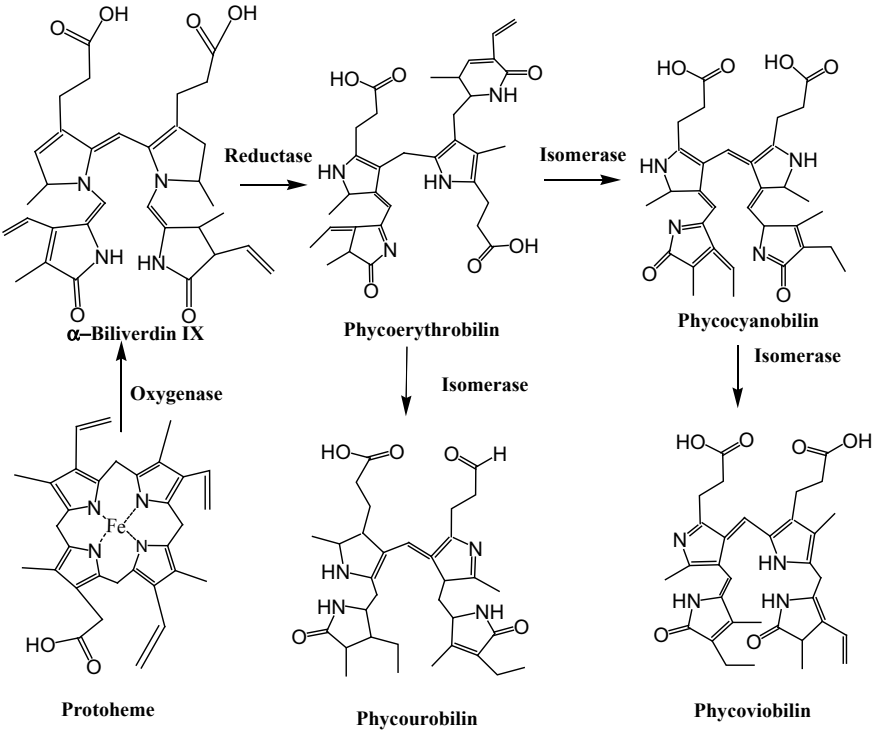

Fig. 3.3 Biosynthesis of phycobiliproteins from protoheme (Adapted and modified from Kannaujiya et al. 2017c, d, Pagels et al. 2019)

found in cyanobacteria, red algae, and cryptomonads and involve in the modification of the β-subunits of bilin proteins (Apt et al. 2001, Kannaujiya et al. 2017c, d).

3.5.4 Bilin Lyases and Its Significance

PBPs lyases are functionally similar to chaperones-like proteins which transiently attached the functional chromophore with bilin proteins and also exhibited isomerization in chromophores (Overkamp et al. 2014, Zhao et al. 2017, Gasper et al. 2017).

In most of cyanobacterial species, four types of bilin lyase have been recognized including E/F-type, S/U-type, T-type, and Y/Z-type (Biswas et al. 2010). The first bilin lyase is designated as heterodimeric CpcE/CpcF expressed by orthologs genes of *cpcE* and *cpcF* present on downstream of *cpcBA* which play important role in chromophore attachment of α-subunits of PC (Zhou et al. 1992, Fairchild and Glazer 1994).

In ceratin cyanobacterium such as *Synechocystis* sp. PCC 6803, CpcE/F proteins have HEAT repeat motifs in nucleotide sequence which facilitate interaction for stable conformation of proteins (Morimoto et al. 2003). Another type of CpeF is required for ligation and functionalization of chromophore from Cys-48/Cys-59 residue in CpeB of PE subunits in the presence of chaperone-like proteins CpcZ (Kronfel et al. 2019). However, S/U-type bilin lyase is expressed as CpeS, CpeU, CpcS, CpcU, and CpcV proteins (Kannaujiya et al. 2017d). Various types of CpcS exist in cyanobacteria such as *Synechococcus* sp. PCC7002 (CpcS-I), marine *Synechococcus* sp. (CpcS-II), *Anabaena* sp. PCC 7120 (CpcS-III) which catalyze the chromophore attachment reaction of β-PC, β-APC, and PEB chromophores (Shen et al. 2008) (Fig. 3.4).

Sometimes, null mutants (*cpcS-I* and *cpcU*) in *Synechococcus* sp. PCC 7002 have also affected the productivity of PC (Kannaujiya et al. 2017d, Shen et al. 2008). S/U lyase exhibits broad substrate association with Cys residue in PBPs subunits as compared to E/F-type (Scheer and Zhao 2008). More specifically, they catalyze chromophores' attachment reaction at Cys-81 residue in allophycocyanins and Cys-84 in β-subunits in PC, PE, and PEC (Zhao et al. 2017). CpcS-III is structurally dimeric in nature and found in *T. elongatus* BP1 (Kuzin et al. 2007). Most of lyase

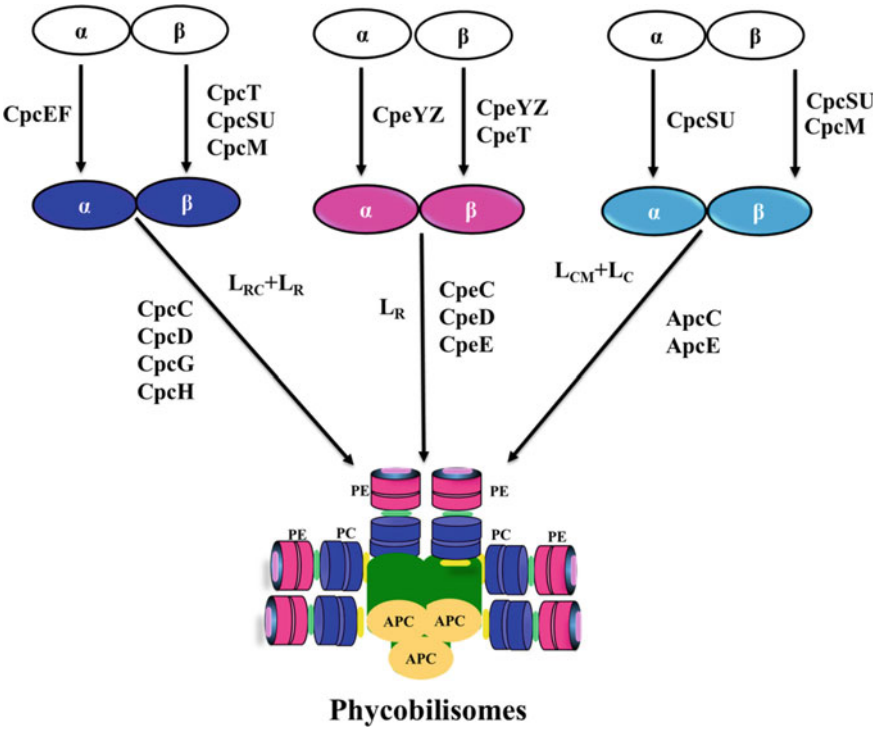

Fig. 3.4 Model of bilin lyase-induced post-transcriptional modification and functionalization of biosynthesis of phycocyanin, phycoerythrin, and allophycocyanin to form a complete structure of phycobilisomes. (Adapted and modified from Kannaujiya et al. 2017d)

are found in homodimers, heterodimers, and tetramers and remain in bind state with fatty acids, vitamins, and carotenoids ligands (Grzyb et al. 2006). Another type of bilin lyase is named as Cpc T which is expressed by *cpcT* gene and *cpeCDESTR* gene cluster in *Fremyella diplosiphon* (Cobley et al. 2002). Certain cryptophytic *Guillardia theta* also encodes Cpc T lyase enzymes (Bolte et al. 2008). In addition, certain cyanophage such as *Prochlorococcus* P-HM1 also contains Φ*cpeT* which play an important role in the functional integration of open chain chromophores to Cys-155 of β-subunits (Gasper et al. 2017). Recently, the crystal structure of CpcT (T-type lyase) has been demonstrated in the cyanobacterium *Nostoc* (*Anabaena*) sp. PCC 7120 (Zhou et al. 2014). The Y/Z-type lyases are playing an important role in the functionality of PE-I and PE-II. Lyases are heterodimer and catalyze chromophore binding in α- and β-subunits of PE (Scheer and Zhao 2008). Recently, an isomerase enzyme (Mpe Z) has been reported in the cyanobacterium *Synechococcus* sp. which catalyzed chromophore interaction in α-subunit of PEII and also involves in the conversion of PEB to PUB (Shukla et al. 2012) (Table 3.2).

3.6 Stress Adaptability and Commercial Productivity

In the current scenario, production of value-added compounds from microalgae is of great interest for industries. Among value-added compounds, most of the industry focuses on production of PBPs in large scale. However, the rate of production is severally affected in various abiotic stress conditions. Thus there is an utmost need to acclimatize and optimize cyanobacteria for large scale production of PBPs from most of the cyanobacteria. In this section, we have elaborated the effects of stress on cyanobacteria related to production and adaptation of methodology for commercial production of PBPs.

3.6.1 Light

Nitrogen-fixing cyanobacteria have an obligatory mechanism of photosynthesis in the presence of solar spectrum. In PBPs subunits, PC absorbs in the yellow–red regions, whereas PE absorbs in the green range of solar spectrum. The environmental adaptability of cyanobacteria under various light regimes is not uniformly constant and it varies among the cyanobacteria. It has been reported that ultraviolet radiation exhibits several effects on the structure and productivity of PBPs. UV radiation has high intensity of energy that is able to produce different types of reactive oxygen species (ROS) inside the cell which indirectly cause several damages in biomolecules such as proteins, lipids, and nucleic acids (Gao et al. 2007, Singh et al. 2014, Kannaujiya et al. 2014). Tyagi et al. (1992) PE-rich strain of *Nostoc* sp. was found to be more tolerant under UV-B irradiation as compared to PC-rich cyanobacteria. UV radiations also affect the composition of linker polypeptides

Table 3.2 Types of bilin lyase and site of action in chromophores (adapted and modified from Kannaujiya et al. 2017c, d)

Lyase enzymes	Function	Site of catalysis	Cyanobacterium	References
CpcE/CpcF	PCB lyase	PC to Cys-84-αPC PC to Cys-84-αPC	*Synechococcus* sp. PCC 7002 *Synechocystis* sp. PCC 6803 *Nostoc* sp. PCC 7120	Fairchild et al. (1992) Tooley et al. (2001) Zhao et al. (2017)
PecE/PecF	PCB lyase isomerase	PEC to Cys-84-αPEC	*Mastigocladus laminosus*	Storf et al. (2001) Zhao et al. (2005)
CpcT/ CpeT	PCB lyase	PC to Cys-153-βPC PCB to Cys 155 of PecB PEB at Cys-155 of beta-PEI	*Synechococcus* sp. PCC 7002 *Anabaena* sp. PCC 7120/ *Nostoc* sp. PCC 7120 Prochlorococcus marinus MED4	Shen et al. (2006) Zhao et al. (2007a, b) Gasper et al. (2017)
CpeS-I	PCB lyase	PC to Cys-84PC PE to Cys-84PE, PEC PEB:Cys-80 beta-PE lyase	*Nostoc* sp. PCC 7120/ *Anabaena* sp. PCC 7120 Fremyella diplosiphon strain UTEX481	Zhao et al. (2007a, b) Biswas et al. (2011)
CpeY/Z	PEB lyase	PEB to PE α or β	*Fremyella diplosiphon*	(Kahn et al. 1997)
CpcS-III	PCB lyase	PC to Cys84-βPC PEC to βPEC APC to αAPC, βAPC	*Anabaena* sp. PCC 7120/ *Nostoc* sp. PCC 7120	Zhao et al. (2006) Zhao et al. (2007a, b)
CpcS-I/CpcU	PCB lyase	PC to Cys82-βPC; APC to Cys81-αAPC, βAPC	*Synechococcus* sp. PCC 7002	Saunée et al. (2008) Shen et al. (2008)

of the PBPs (Kannaujiya and Sinha 2015). Long-term exposure of UV-B inhibits the oxygen evolution and also disturbs PBPs composition in *Synechococcus* sp. by partial uncoupling of energy transfer (Kulandaivelu et al. 1989). Sah et al. (1998) reported that low dose of UVB irradiation altered the linker polypeptide (75 kDa) in *Synechococcus* sp. 7942. Similarly, Rajagopal et al. (1998) reported that anchor polypeptide was altered in PBPs of *Spirulina platensis* after UV-B irradiation. The fluorescent nature of PC/PE is dependent on cysteine-linked integration of linear tetrapyrrole chromophores in α and β monomers. The high intensity of PAR and

UV radiation reduces the fluorescent nature of PBPs (Breinig et al. 2003, Kannaujiya and Sinha 2017a, b). The intensity of light also affects PBP composition in cyanobacteria. Apart from UV radiation, light intensity of visible spectrum also affects the growth of cyanobacteria. It has been noticed that low and medium light intensity are being widely used for the efficient productivity of PBPs (Pagels et al. 2019). The divergence of growth at different light intensities shows that 25 μmol photons/m2/s was the best-suited intensity for optimum growth of *Spirulina* sp. (Tomasseli et al. 1995, 1997), *Synechococcus* NKBG 042,902 (Takano et al. 1995), and *Synechocystis* sp. (Hong and Lee 2008). However, optimum growth was recorded after 50% reduction in light intensity (12.5 μmol photons/m2/s) in cyanobacteria *Nostoc muscorum* (Ranjitha and Kaushik 2005) and *Nostoc* UAM 206 (Poza-Carrion et al. 2001). Interestingly, it has been suggested that bilin proteins for PBPs are more stimulated in low light intensities due to minimum energy consumption in the maintenance of the photosystem (Grossman et al. 1993). Eukaryotic red algae required higher irradiance for growth and development such as 40 μmol photons/m^2/s intensity optimum for *Gracilaria tenuistipitata* (Carnicas et al. 1999) and 65 μmol photons/m^2/s for *Audouinella*, *Batrachospermum*, and *Compsopogon* (Zucchi and Neechi 2001). Certain cyanobacterium such as *Arthronema africanum* required high light intensity of up to 150 μmol photons/m^2/s for optimum production of PBPs (Chaneva et al. 2007). The optimum intensity of color of light may enhance the productivity of PBPs in certain cyanobacteria. It has been reported that red light may affect the growth of cyanobacteria and stimulate production of PC in *Anacystis nidulans* (Lonneborg et al. 1985), *Synechococcus* (Takano et al. 1995), *Calothrix* 7601 (Liotenberg et al. 1996), *Nostoc* UAM206 (Poza-Carrion et al. 2001), and *Nostoc muscorum* (Ranjitha and Kaushik 2005). The wavelength of the blue spectrum has shown stimulatory effects for PE synthesis in red algae such as *Rhodella reticulata* (Mihova et al. 1996), *Porphyra leucosticta* (Tsekos et al. 2002), *Chondrus crispus* (Franklin et al. 2002), and *Halymenia floresii* (Godinez-Ortega et al. 2008). The dynamic fluctuation of light/dark photoperiod exhibits light periods-induced change in accessory light-harvesting capacity for photosynthesis (Kono and Terashima 2014). The fluctuation in UV-B radiation suggests induction of photoprotective mechanism to protect PBPs from damage (Chukhutsina et al. 2015). In industry, a open and closed system has been used for the large scale production of PBPs. The open system is categorized into four such as tanks, circular ponds, raceway, and shallow pond. However, cyanobacterial growth has been inhibited by the variable environmental condition. To obtain high productivity rate, a paddle-wheel attached for proper aeration in raceway ponds and stress adaptable organisms are being used (Kannaujiya et al. 2017d). However, close bioreactors have an advantage over the open bioreactor in reducing the chance of contamination, water evaporation, and efficient utilization of nutrients for higher productivity. There are four types of close photobioreactors such as annular, airlift, plate, and tube system for large scale production of biomass or value-added compounds. However, costs of production of biomass are higher in comparison to open photobioreactor system (Tredici et al. 2015). From economical point of view, open photobioreactors/pond system are more feasible as compared to others.

3.6.2 Temperature Stress

Temperature is the most fundamental physiological factor that affects physiological, biochemical, and metabolic processes of an organism. In aquatic system, high temperature reduces availability of free oxygen which may be a primary effect on the photosynthesis machinery. Temperature exhibit several impacts on productivity of PBPs in different cyanobacterial species (Pagels et al. 2019). Hemlata and Fatma (2009) have found an optimum temperature of about 30 °C for the synthesis of PBPs in the cyanobacterium *Anabaena* NCCU-9.

However, alteration in optimum temperature (30 °C) may reduce the growth significantly with optimum yield of PBPs. Moreover, other scientists have reported the optimum temperature to be 36 and 37 °C for *Synechococcus* (Sakamoto and Bryant 1998) and *Arthronema africanum* (Chaneva et al. 2007), respectively. Upon exposure to a higher temperature, heat shock proteins (HspA) are produced by the cyanobacterium *Synechococcus* sp. strain PCC 7942 which interact with PC of PBPs and suppress the inactivation of functional properties by heat-enabled denaturation (Nakamoto and Honma 2006). They also thrive well at high temperature (Richa and Sinha 2015). In *Synechococcus* sp. PCC7 942 HspA has been suggested to protect phycobiliproteins from degradation under heat stress as well as oxidative stress (Nakamoto et al. 2000, Nakamoto and Honma 2006). It has been found that *Spirulina platensis* is able to grow efficiently in large fluctuation of temperature except below 20 °C and greater than 40 °C (Kumar et al. 2018).

3.6.3 Salt Stress

The maintenance of salt concentration is crucial for proper cell functioning, ion regulation, membrane potential, osmotic balance, and metabolic activity (Pandhal et al. 2008, 2009). The consequent increase of salt concentration adversely affect non-tolerant organism by inhibition of electron transport system. The composition and function of PBPs in cyanobacteria changed in response to stress conditions (Grossman et al. 1993). Salt stress mainly decreases PC concentration, and thereby energy transfer between PBPs and PSII may be interrupted (Lu et al. 1999, Lu and Vonshak 2002). It was found that the lowest concentration of salt (10 mM) resulted in an increase in PBP content in cyanobacterium *Anabaena* sp. NCCU-9 as compared to untreated sample, while further increase in salt concentration resulted in gradual decline in growth (Hemlata and Fatma 2009). To increase salt concentration, the ionic movement of sodium ions might be increased, and induced detachment of PBPs leads to inhibition of energy transfer reaction between PBPs and PSII (Rafiqul et al. 2003). Moreover, *Spirulina fusiformis* exhibit optimum growth in the absence of salt (Rafiqul et al. 2003).

3.6.4 Carbon and Nitrogen Stress

The carbon and nitrogen ratio is crucial for the growth and development of cyanobacteria and that plays a distinguished role in the production of value-added compounds or PBPs. Generally, carbon sources such as fructose, glucose, glycerol, and sucrose are used for the large scale production of cyanobacteria (Kovač et al. 2017). It has been reported that addition of glucose with optimum light condition play a significant role in increasing the concentration of PC in cyanobacteria Moreover, high concentration of glucose and glycerol exhibit increase in the production of PBPs in *Anabaena* C2 and *Spirulina* S2 strains (Kovač et al. 2017). Khattar et al. (2015) have also found sucrose as another good carbon source for the growth of *Anabaena fertilissima* than glucose and fructose. Cyanobacteria require range of nitrogen components such as NO_{3-}, NH_4 or carbamide, and $NaNO_3$ for their growth and development. The cyanobacterium *Fischerella* sp. produced more amounts of PBPs under nitrate or ammonium medium as compared to nitrogen-free growth media (Soltani et al. 2007). Hemlata and Fatma (2009) have reported about the toxicity of ammonium in growth medium, which reduces growth that leads to cell death in *Spirulina platensis*, *Nostoc* sp., *Calothrix* sp. PCC7601, and *Nodularia spumigena* (Belkin and Boussiba 1991, Liotenberg et al. 1996).

3.6.5 Metal Stress

Heavy metals play a leading role to induce toxicity of undesirable pollutants on an organism. The toxicity of heavy metals may further increase due to their non-biodegradable nature and ability to enter into the food chain. Heavy metals severely affect normal physiological processes by interference of essential enzyme activity and pigment-protein developmental process (Bertrand and Guary 2002). Micronutrient heavy metals include boron, manganese, zinc, molybdenum, copper, and cobalt required in very low quantities. Boron is a rare metal and keenly required by plants, but has no role in the growth of fungi as well as animals. Anderson and Jordan (1961) found that boron required for the dinitrogen fixation in *Azotobacter*. Boron is an essential micronutrient for rapid growth and development in nitrogen-deprived condition in certain cyanobacteria like *Nostoc muscorum*, *Calothrix parietina*, and *Anabaena cylindrica* (Gerloff 1968). The boron-induced growth of cyanobacteria may signify rapid synthesis of PBPs. Manganese and zinc are essential elements for the growth of cyanobacteria particularly in different enzymatic reactions involved in photosynthetic processes (Casarett and Doull 1980). Metal-induced changes in the arrangement and structure of the light-harvesting complex in cyanobacteria have been well documented (Sersen and Kralova 2001). Murthy and Mohanty (1991) have found that energy transfer from PC to chlorophyll *a* (Chl *a*) is rapidly inhibited by heavy metals. This eventually could study the effects of cadmium on three different

cyanobacterial genera. Cyanobacteria evolved as organisms capable of extreme adaptation, at several types of conditions. These adaptations are often related to the production of defense machinery, producing many natural products and high-valued compounds.

3.7 Commercial Significance of PBPs

In the past few years, PBPs are being used as emerging protein sources for the development of natural dyes, pharmaceuticals, autofluorescent chemical, neutraceuticals, and many therapeutic and non-therapeutic bioproducts. Currently, a green approach for generation of value-added therapeutic and daily use products have been widely accepted by common people (Sekar and Chandramohan 2008). In current scenario, PC and PE are frequently used as coloring agents for the development of colored food products without using health-hazardous synthetic color (Prasanna et al. 2007). Commercially, they are used in manufacturing edible food products, cold beverages, milk products, and autofluorescent sweet candies (Eriksen 2008, Kannaujiya et al. 2017d). Now, certain companies of different countries focus on the development of PE/PC based natural cosmetic products such as soft cream, lotion, beauty soaps, colored eyeliner, and lipsticks. Recently, PBPs application has been elaborated with novel potential in the field of cosmetics industry and biomedical research for the treatment of many diseases (Richa et al. 2011, Kannaujiya et al. 2017c, d). PBPs are also used as potent anti-oxidative agents due to reactive oxygen level scavenging property (Wu et al. 2016). It has been observed that PC and PCB exhibit effective scavenging property of peroxynitrite which accelerate the DNA damage (Bhat and Madyastha 2001). In addition, PC is further characterized by Oxygen Radical Absorbance Capacity (ORAC) which indicates strong anti-oxidative potential (Benedetti et al. 2010). In addition, PBPs have also been used as food supplements for enhancing immune system (Levi et al. 2018). Apart from antioxidant behavior, PC shows immune-modulatory and anti-inflammatory agents (Datla 2011). Certain biological experiment suggests that it has neuro-protective property (Cervantes-Llanos et al. 2018) and combination of PC- α-synuclein inhibit fibrils formation in brains which cause reduction in development of neurodegenerative disease such as Parkinson's and Alzheimer (Liu et al. 2019, Kannaujiya et al. 2017c, d). They are also hepatic-protective due to reduction in enzymes activity such as liver transaminases and alkaline phosphatase which is accelerated by oxidative stress (Gdara et al. 2018). PC is specifically toxic to cancer cells and inhibits the tumor growth (Jiang et al. 2018, Ravi et al. 2015). Recently, PC has been used as a potent photosensitizer as drug delivery for photodynamic therapy (Wan et al. 2017). It has been shown that PC prevents the UV-induced apoptosis by regulation of proteins kiase (Kim et al. 2018). Moreover, Chakdar and Pabbi (2015) recognized that PC has a great ability for reduction of animal cholesterol, low-density lipoproteins, and harmful level of triglycerides.

3.8 Conclusion

The autofluorescent, brilliantly colored PBPs have indispensable property in every sector of life science for more than 50 year. Several properties of PBPs are still to be discovered for useful applications. The diversity of cyanobacteria and availability of differentially colored PBPs have to be searched out for the welfare of humans. The biosynthetic mechanisms of PBPs in various groups of cyanobacteria are still to be discovered. PBPs play a vital role in the development of various biotechnological, foods and pharmaceutical products including natural dyes, target specific apoptotic agent, anti-cancer, anti-inflammatory, anti-bacterial, anti-viral, anti-oxidative medicines, value-added products and colored fluorescence dye for human welfare. Under the current scenario, many products are under developing stage including photodynamic therapy, biofuel solar cell, diagnostic, and many medicines for the treatment of various diseases. The bright future and wide commercial uses of PBPs may open large economic growth for industry as well as availability of novel products for human welfare.

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Chapter 4
Carotenoid Synthesis and Accumulation in Microalgae Under Environmental Stress

Shota Kato and Tomoko Shinomura

Abstract In oxygenic phototrophs including cyanobacteria, algae, and land plants, carotenoids serve the light-harvesting function together with chlorophylls and photoprotective functions. High-intensity irradiation can cause excess excitation pressure on photo system II (PSII) of chloroplasts and can generate reactive oxygen species resulting in the photodamage to PSII. In general, to prevent the photodamage to photosynthetic apparatus, carotenoids dissipate excitation energy of singlet-state chlorophylls as heat (xanthophyll-dependent non-photochemical quenching) and also quench triplet-state chlorophylls in antenna complexes of PSII. Carotenoids also quench singlet oxygen in the reaction center of PSII. Light is one of the most striking environmental cues that alter the expression of carotenoid biosynthetic genes and induce the carotenogenesis in microalgae. Light-induced accumulation of carotenoids is reported in certain algae such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella zofingiensis*, and *Euglena gracilis*. Besides light stress, various abiotic stresses, such as heat, low temperature, drought, salinity, and oxidative stress, can enhance the extent of photoinhibition in photosynthetic organisms and can also induce the accumulation of carotenoids in microalgae. Here, we summarize studies on the regulation of biosynthesis and accumulation of carotenoids in eukaryotic microalgae in response to environmental stimuli especially focusing on the light and temperature. In addition, here we show our recent findings on the carotenoid biosynthesis in *Euglena gracilis* in response to increasing light intensity and light-induced stress enhanced by decreasing temperature.

Keywords Photoprotection · Light stress · Cold stress

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4.1 Introduction

Carotenoids, isoprenoid compounds with 40 carbon backbones, are the most widespread pigments in nature. They are found in various organisms including bacteria, archaea, fungi, algae, plants, and animals. So far, more than 750 naturally occurring carotenoids have been isolated and structurally identified from photosynthetic and non-photosynthetic organisms (Britton et al. 2004). Carotenoids are indispensable components of photosynthetic machinery in photoautotrophs, forming chlorophyll- and carotenoid-binding protein complexes in thylakoid membranes of cyanobacteria, eukaryotic algae, and land plants. They serve crucial roles in light harvesting to drive photosynthesis and protecting photosystem II (PSII) from photodamage by thermal dissipation and scavenging of reactive oxygen species (Müller et al. 2001; Triantaphylidès and Havaux 2009; Telfer 2014).

Photosynthetic organisms are exposed to various abiotic stresses such as high irradiance that can induce photodamage to PSII and heat, low-temperature, drought, salinity, and oxidative stress that can enhance the extent of photoinhibition by inhibiting the repair of photodamaged PSII (Nishiyama et al. 2008; Takahashi and Murata 2008). Those environmental stimuli can alter the biosynthesis of photosynthetic pigments. Here, we summarize studies on the regulation of biosynthesis and accumulation of carotenoids in eukaryotic microalgae in response to light and temperature. In addition, we would like to introduce our recent findings on the physiology of carotenoid biosynthesis of *Euglena gracilis* in response to those environmental stimuli.

4.2 Carotenoids in Photosynthetic Machinery

Photosystem I (PSI) and PSII core complexes contain carotenoids. X-ray structural studies identified 22 and 12 carotenoid molecules in the monomeric PSI at 2.5 Å resolution and PSII at 2.9 Å resolution, respectively, of *Thermosynechococcus elongatus* (Jordan et al. 2001; Guskov et al. 2009). Umena et al. (2011) assigned 11 β-carotenes in the monomeric PSII of *Thermosynechococcus vulcanus* at 1.9 Å resolution. PSII reaction center of *T. vulcanus* contains two molecules of β-carotene in the vicinity of reaction center chlorophylls (Kamiya and Shen 2003). β-carotenes in the PSII reaction center are considered to function primarily in scavenging of singlet oxygen produced via triplet-state P_{680} (Triantaphylidès and Havaux 2009; Telfer 2014). The core light-harvesting complex (LHC) of PSII also contains β-carotene and peripheral LHC of PSII contains several xanthophylls.

Major xanthophylls in the peripheral LHC vary depending on algal divisions/classes: e.g., lutein, violaxanthin, and 9′-*cis* neoxanthin in land plants; alloxanthin in Cryptophyta; fucoxanthin in Chrysophyceae, Bacillariophyceae, Phaeophyceae, and Haptophyta; peridinin in Dinophyta; and diadinoxanthin and diatoxanthin in Haptophyta, Heterokontophyta, Dinophyta, and Euglenophyta (Takaichi

2011). X-ray structural analyses indicated that the monomer of peripheral LHC of PSII (LHC-II) of land plants has four carotenoid-binding sites for two luteins, one neoxanthin, and one violaxanthin or zeaxanthin (Liu et al. 2004; Standfuss et al. 2005). Carotenoids in peripheral LHCII can transfer their excitation energy to their nearest/neighbor chlorophylls.

Bacillariophyceae and diatoms possess fucoxanthin-chlorophyll *a*/*c* binding proteins (FCP). FCP associates with core complex of PSI and PSII, and functions as the peripheral antenna in the light harvesting and in energy transfer (Nagao et al. 2007; Ikeda et al. 2008; Nagao et al. 2014). Dinoflagellate employs peridinin-chlorophyll-protein (PCP), a unique water-soluble protein located in the thylakoid lumen, for the light harvesting, in addition to the LHC in the thylakoid membrane (Polívka et al. 2007). The PCP complex of a dinoflagellate *Amphidinium carterae* consists of trimeric subunits with two pseudo-identical domains containing densely packed peridinin and chlorophyll *a* in a stoichiometric ratio of 4:1 (Hofmann et al. 1996).

4.3 Photoprotection by Carotenoids

In the photochemical reaction of photosynthesis, excess light energy can generate various reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) (Edreva 2005, Krieger-Liszkay 2005; Asada 2006; Telfer 2014). ROS can cause the cleavage of D1 protein in PSII and can inhibit the repair of photodamaged PSII resulting in the photoinhibition of photosynthesis (Mishra and Ghanotakis 1994; Miyao et al. 1995; Okada et al. 1996; Nishiyama et al. 2004).

Xanthophylls in peripheral LHCII (mainly lutein in the L1 and L2 sites of LHC-II) can quench triplet-state chlorophylls in close van der Waals contact to prevent the formation of singlet oxygen (Standfuss et al. 2005; Triantaphylidès and Havaux 2009). In addition, violaxanthin and zeaxanthin in a pocket at the monomer interface of LHC-II participate in the regulation of inducible energy-dependent non-photochemical quenching (qE) via deepoxidation/epoxidation of xanthophylls called a xanthophyll cycle (violaxanthin cycle) (Müller et al. 2001; Liu et al. 2004; Standfuss et al. 2005). Several algae such as Haptophyta, Dinoflagellate, and diatoms employ another type of xanthophyll cycle called diadinoxanthin cycle, which consists of a conversion of diadinoxanthin to diatoxanthin, for the non-photochemical energy dissipation (Goss and Jakob 2010).

In the PCP complex of dinoflagellate, peridinin can quench triplet-state chlorophyll *a* (Bautista et al. 1999; Alexandre et al. 2007; Schulte et al. 2009). Cyanobacteria utilize the orange carotenoid protein (OCP), a water-soluble photosensory protein, for the photoprotection. OCP contains 3′-hydroxyechinenone as the photoactive chromophore and triggers light-induced non-photochemical quenching by interacting with light-harvesting phycobilisomes under blue-green light (Kerfeld et al. 2003; Wilson et al. 2006, 2008; Harris et al. 2016). OCP can also function as a singlet oxygen quencher in cyanobacteria (Sedoud et al. 2014).

4.4 Carotenogenesis of Algae in Response to Environmental Stimuli

4.4.1 Light-Induced Carotenoid Accumulation

Light is one of the most striking environmental cues that can induce and alter the carotenogenesis in algae as well as land plants. To avoid excess photochemical reaction in photosystem of chloroplast, photosynthetic organisms tightly control the biosynthesis of photosynthetic pigments in response to light environment.

When grown under circadian light/dark cycle, algal cells show periodic changes in the cell division rate, photosynthetic activity, expression of photosynthetic apparatus genes, and contents of chlorophylls and carotenoids (Goto and Johnson 1995; Monnier et al. 2010; Sorek et al. 2013; Miyagishima et al. 2014). Fábregas et al. (2002) observed that, when a marine alga *Nannochloropsis gaditana* was grown under a 12 h/12 h light/dark cycle, the cellular content of chlorophyll *a* and carotenoids increased during the light phase and reached the peak at the end of the light period. Sorek et al. (2013) reported that a coral symbiotic dinoflagellate *Symbiodinium* sp. exhibited diel fluctuations of concentrations of peridinin, diatoxanthin, and diadinoxanthin in free-living cells under a light/dark cycle (12 L/12 D).

High-intensity light can induce the biosynthesis and accumulation of carotenoids in certain algae such as *Chlorella zofingiensis*, *Dunaliella* spp., *Haematococcus pluvialis*, and *Euglena gracilis*. Li et al. (2009) reported that illumination at 150 μmol photon m^{-2} s^{-1} caused 5.3-, 2.2-, and 2.8-fold increases in the content of zeaxanthin, canthaxanthin, and astaxanthin, respectively, in *C. zofingiensis* compared with those in the cells grown under 30 μmol photon m^{-2} s^{-1}. When grown under high-intensity light at 460 and 920 μmol photon m^{-2} s^{-1}, *C. zofingiensis* cells showed a 3-fold higher astaxanthin content than that in cells grown at 90 μmol photon m^{-2} s^{-1} (0.04 pg $cell^{-1}$) (Campo et al. 2004). In *Dunaliella salina*, a shift of light intensity from 200 to 1400 μmol photon m^{-2} s^{-1} increased the intracellular concentration of β-carotene 7.6-fold accompanied by a cell volume increase (Lamers et al. 2010). Lamers et al. (2010) reported that the production of β-carotene in *D. salina* was immediately induced within a day in response to a stepwise increase in light intensity in a range from 150 to 650 μmol photon m^{-2} s^{-1}.

H. pluvialis accumulates ketocarotenoid astaxanthin in the cytoplasm of motile vegetative cells and cyst cells under intense light conditions. Lv et al. (2016) reported that 80 μmol photon m^{-2} s^{-1} illumination caused 8.5- and 14.6-fold increases in the content of total carotenoids and astaxanthin, respectively, on dry weight basis. Steinbrenner and Linden (2001) showed that continuous illumination at 125 μmol photon m^{-2} s^{-1} for 72 h caused astaxanthin accumulation of approximately 6 mg g^{-1} dry weight. Similarly, Wang et al. (2003) reported that illumination at 350 μmol photon m^{-2} s^{-1} induced the accumulation of astaxanthin-rich lipid globules in the cytoplasm of *H. pluvialis* and increased the cellular carotenoid content from 13 to 98 pg $cell^{-1}$, yielding red cell cultures. When *H. pluvialis* cells were grown under illumination at a range of 10–250 μmol photon m^{-2} s^{-1}, higher light intensity induced

higher astaxanthin accumulation in the vegetative cells of this microalga, although the total content of chlorophyll and carotenoids (except for astaxanthin) was the highest in the cells grown under illumination at 10 μmol photon m^{-2} s^{-1} (Steinbrenner and Linden 2003). Wang et al. (2003) suggested that those carotenoid-accumulated red cells were more tolerant to high-intensity irradiation (3000 μmol photon m^{-2} s^{-1}) than green cells, comparing the turnover of D1 protein and O_2 evolution rate between the green and red cell cultures.

We previously reported high-intensity light-induced accumulation of carotenoids in *E. gracilis* (Kato et al. 2017) (Fig. 4.1a). *E. gracilis* contains mainly β-carotene, neoxanthin, diadinoxanthin, and diatoxanthin (Kato et al. 2017). We revealed that continuous illumination at 920 μmol photon m^{-2} s^{-1} caused a 25% increase in the cellular content of total major carotenoids, compared with the cells grown under 55 μmol photon m^{-2} s^{-1}, whereas the cellular chlorophylls content was decreased by 58% (Kato et al. 2017). Transmission electron microscopy suggested that the high-light-induced accumulation of carotenoids in *E. gracilis* might be partly relevant to the accumulation of lipid globules in the cytoplasm and plastoglobules in the interthylakoid space of chloroplasts (Kato et al. 2017).

Król et al. (1997) reported a 5-fold increase in the xanthophyll pool size (violaxanthin + antheraxanthin + zeaxanthin) of *D. salina* under illumination at 2500 μmol photon m^{-2} s^{-1} on chlorophyll *a* basis relative to the cells grown under 150 μmol photon m^{-2} s^{-1}. We showed that, under the continuous illumination at 920 μmol photon m^{-2} s^{-1}, the pool size of diadinoxanthin cycle pigments (diadinoxanthin + diatoxanthin) in *E. gracilis* cells showed a 1.2-fold increase relative to the cells grown under 55 μmol photon m^{-2} s^{-1} (Kato et al. 2017). Under high-intensity light at 160–180 μmol photon m^{-2} s^{-1}, a diatom *Cyclotella meneghiniana* showed 3- to 4-fold increases in the content of diadinoxanthin cycle pigments on chlorophyll *a*

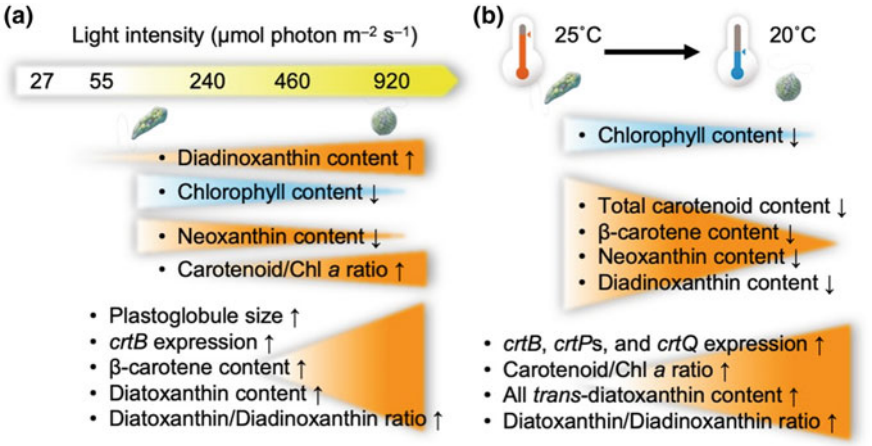

Fig. 4.1 Biosynthesis of photosynthetic pigments in *Euglena gracilis* in response to **a** increasing light intensity (Kato et al. 2016, 2017) and **b** decreasing temperature (Kato et al. 2019)

basis in the cell and thylakoid fraction, respectively, compared with the cells grown under illumination at 10–15 μmol photon m^{-2} s^{-1} (Lepetit et al. 2010). In a diatom *Phaeodactylum tricornutum*, although diatoxanthin formation was not the only factor to control NPQ, kinetics of NPQ was shown to be closely correlated with diatoxanthin concentration in the alga (Lavaud et al. 2002; Ruban et al. 2004). In *E. gracilis* cells, we found that the molar ratio of cellular diatoxanthin content to diadinoxanthin content (Dtx/Ddx ratio) increased from 0.05 (55 μmol photon m^{-2} s^{-1}) to 0.09 after the irradiation of intense light (920 μmol photon m^{-2} s^{-1}), suggesting that diatoxanthin might participate in photoprotection of *E. gracilis* during the long-term acclimation to intense light condition (Kato et al. 2017). On the other hand, in *C. meneghiniana*, the majority of diadinoxanthin cycle pigments localized in the peripheral antenna FCP (in particular FCPa) complexes and that the largest part of high-light-induced newly synthesized diadinoxanthin cycle pigments might not be protein bound and did not participate in the NPQ of *C. meneghiniana* (Lepetit et al. 2010). Furthermore, Lepetit et al. (2010) suggested that those light-induced additional pigments were dissolved in a monogalactosyldiacylglycerol lipid shield around FCP complexes, serving an antioxidant function in the thylakoid membrane.

4.4.2 Light-Induced Expression of Carotenoid Biosynthetic Gene

Light-induced expression of carotenoid biosynthetic genes was reported in several microalgae. Steinbrenner and Linden (2001) reported increases in the expression of phytoene synthase gene (*psy*) and carotenoid hydroxylase gene (*chy*) of *H. pluvialis* cells after continuous illumination at 125 μmol photon m^{-2} s^{-1} for 12 h. Steinbrenner and Linden (2003) also observed a strong induction of the expression of *psy*, phytoene desaturase gene (*pds*), lycopene β cyclase gene (*lyc*), and *chy* of *H. pluvialis* grown under illumination at 50 μmol photon m^{-2} s^{-1} for 24 h, compared with those in the cells illuminated at 10 μmol photon m^{-2} s^{-1}. Irradiation at 120 μmol photon m^{-2} s^{-1} induced the expression of *pds* in *C. zofingiensis* (Huang et al. 2008). In *P. tricornutum*, the expression of 1-deoxy-D-xylulose 5-phosphate synthase gene (*dxs*) and *psy* was elevated during several hours after the dark-light transition (Eilers et al. 2016). In *E. gracilis*, our previous study revealed that continuous illumination at 920 μmol photon m^{-2} s^{-1} induced a 1.3-fold increase in the expression of phytoene synthase gene (*crtB*) relative to the cells illuminated at 55 μmol photon m^{-2} s^{-1}, indicating that the carotenoid synthetic pathway of this alga responded to the intense light at the transcriptional level (Kato et al. 2016) (Fig. 4.1a).

Bohne and Linden (2002) reported that, in the dark-light transition, blue light induced the gene expression of *psy* and *pds* of *Chlamydomonas reinhardtii* to a comparable level to those in cells irradiated with white light, whereas red light had no obvious effect on the expression level of those genes. On the contrary, Steinbrenner and Linden (2003) reported that blue and red light similarly induced the expression

of *psy*, *pds*, *lyc*, and *chy* genes, while blue light was more effective in the induction of astaxanthin accumulation in *H. pluvialis* cells than red light. Furthermore, Steinbrenner and Linden (2003) indicated that the redox state of the plastoquinone pool related to the light induction of carotenoid synthetic gene expression and astaxanthin accumulation in *H. pluvialis*. In contrast, Bohne and Linden (2002) indicated that the redox state of plastoquinone pool of the photosynthetic electron transport was no relevant to the light-induced expression of *psy* and *pds* of *C. reinhardtii*.

Sun et al. (2010) reported that *C. reinhardtii* showed a clear diurnal expression pattern of genes in methylerythritol phosphate (MEP) pathway for the biosynthesis of isoprenoids and in the carotenoid metabolism, when the cells were grown under a 12 h/12 h light/dark condition. In addition, Sun et al. (2010) revealed that *C. reinhardtii* displayed higher transcript level of *psy*, *pds*, and lycopene β-cyclase gene (*lcyb*) in the light period than those in the dark period and discussed that the gene expression of *dxs* and geranylgeranyl pyrophosphate synthase gene (*ggps*) of isoprenoid biosynthetic pathway and of *lcyb* was co-regulated by the internal circadian clock and external light signals.

4.4.3 Temperature-Stimulated Carotenoid Accumulation

Temperature is also one of the major environmental stimuli which can alter the photosynthetic pigment production as well as light. Campo et al. (2004) reported that, in a temperature range from 20 to 28 °C, higher temperature induced higher cellular content of lutein in *C. zofingiensis* cells. At elevated temperatures over 30 °C, *H. pluvialis* showed 15- to 20-fold increases in the cellular carotenoid content compared with the algal cells cultured at 20 °C (Tjahjono et al. 1994). In *D. salina*, Gómez and González (2005) reported that the β-carotene content was higher at 26 °C than at 15 °C and that temperature considered to be more effective than irradiance in changing the carotenoid composition.

Decreasing temperature can also stimulate the carotenoid production in microalgae. In *Dunaliella bardawil*, Ben-Amotz (1996) reported that decreasing temperature from 30 to 10 °C caused a 2-fold increase in the cellular β-carotene content without any significant change of the cellular chlorophyll content resulting in an increase of the β-carotene/chlorophyll ratio from 4.4 at 30 °C to 8.5 at 10 °C. Orset and Young (1999) revealed that, when *D. salina* cells were transferred from 34 to 17 °C, the cells showed a 7.5-fold increase in α-carotene level. Furthermore, decreasing growth temperatures can alter isomeric composition of carotenoids such as β-carotene in *D. bardawil* (Ben-Amotz 1996) and *D. salina* (Orset and Young 1999) and diatoxanthin in *E. gracilis* (Kato et al. 2019).

Król et al. (1997) showed that cultivation at 13 °C induced a 5-fold increase in the pool size of xanthophyll cycle pigments (violaxanthin + antheraxanthin + zeaxanthin) in *D. salina* cells on chlorophyll *a* basis compared with cells grown at 30 °C under 150 μmol photon m^{-2} s^{-1}. Król et al. (1997) also revealed that the low temperature-induced accumulation of carotenoids in *D. salina* cells was the

comparable level of carotenoids in the cells grown at 30 °C under high-intensity illumination at 2500 μmol photon m^{-2} s^{-1}, suggesting that the carotenoid level of this alga was controlled depending on excitation pressure on PSII in response to temperature and irradiance changes.

In *E. gracilis*, our previous study revealed that the gene expression of *crtB*, phytoene desaturase genes (*crtP1* and *crtP2*), and ζ-carotene desaturase gene (*crtQ*) was increased by 1.3- to 1.8-fold by cultivation at 20 °C under 240 μmol photon m^{-2} s^{-1} relative to the cells grown at 25 °C, whereas the cellular content of total major carotenoids, β-carotene, neoxanthin, and diadinoxanthin was decreased by more than half compared with the cells grown at 25 °C (Kato et al. 2019) (Fig. 4.1b). On the other hand, *E. gracilis* cells grown at 20 °C contained a comparable level of diatoxanthin to that in cells grown at 25°C and showed an increase in Dtx/Ddx ratio and total carotenoid/chlorophyll *a* ratio (Kato et al. 2019). Our findings suggested that cold stress enhanced light-induced stress to this alga and diatoxanthin might participate in reducing the excitation pressure on PSII under cold and intense light condition (Kato et al. 2019).

4.5 Perspectives

Molecular mechanisms of the regulation of carotenogenesis in microalgae have still been elusive. In land plants, regulatory mechanisms of carotenoid biosynthesis have gradually come to light: for example, direct regulation of phytoene synthase at transcriptional level by phytochrome interacting factors (PIFs) and long hypocotyl 5 (HY5) (Toledo-Ortiz et al. 2014) and at post-translational level by ORANGE (OR) and Clp protease (Welsch et al. 2018) and redox-dependent ligand switching of 15-*cis*-ζ-carotene isomerase (Z-ISO) (Beltrán et al. 2015). To respond environmental stimuli at different time scale, microalgae would also have developed multiple regulatory systems at transcriptional and post-transcriptional levels to control the carotenoid biosynthesis. Elucidation of those mechanisms will lead to a deeper understanding of physiological functions of carotenoids in microalgae under various environmental conditions and would contribute to an efficient production of valuable carotenoids in the food and pharmaceutical industries.

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Chapter 5
Carotenoid Overproduction in Microalgae: Biochemical and Genetic Engineering

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Abstract Carotenoids are one the most frequently coloured molecules encountered in our environment. Beside their colouring effect, carotenoids are famous for their photoprotection and antioxidant properties. Because carotenoids conserve their properties in vitro, the interest for these natural molecules increased. To satisfy the growing demand for carotenoids, new sources are searched and microalgae emerged as organisms with a very high potential. Using the most recent publications, we established that the production of carotenoids by microalgae is a tailor-made process requiring the proper biological and environmental factors. The optimization of the biotechnological processes aiming at producing carotenoids from microalgae requires a deeper knowledge in the regulation of the carotenoid biosynthetic pathways, that might be used to engineer microalgal strains.

Keywords Bioactive molecules · Biotechnology · Microalgae · Molecular biotechnology · Stress

5.1 Introduction

Carotenoids are together with chlorophylls the most frequently coloured molecules found in our environment. Carotenoids are yellow to red coloured molecules famous for their in vivo light-harvesting ability, photoprotection, protein stabilization and antioxidant roles in the photosynthetic process. The latter activity is crucial because the aerobic metabolism, typical of eukaryotic cells, continuously generates reactive oxygen species (ROS) that can cause cellular damages. Large quantities of ROS

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may happen when cells experience a stress (e.g. Tan and Norhaizan 2019; Liu et al. 2017). Because carotenoids conserve their properties in vitro, the interest for these natural molecules has progressively shifted from the curiosity of some to the general interest at the end of World War II when it became clear that lifestyle will be directed toward the consumption of more proceeded food. Food processing is usually destructive for natural molecules that, therefore, need to be replaced by food supplements. Carotenoids enter in this category and, since, has also attracted the attention for their antioxidant and colouring properties (Ambati et al. 2014; Solymosi et al. 2015). With the exceptions of aphids (Moran and Jarvik 2010) and spotted spider mites (Altincicek et al. 2011), carotenoids cannot be synthesized by animal cells and therefore must be acquired through the alimentation, before being eventually modified after ingestion (Solymosi et al. 2015). Today, carotenoids are of considerable interest for numerous applications including food and feed, cosmetics and medicine (Garg et al. 2019; Le Goff et al. 2019, 2020; Gateau et al. 2017; Tan and Norhaizan 2019; Mimouni et al. 2012). For instance, carotenoids can help in fighting age-related macular degeneration, a disease leading causes of vision loss (Edelshtain et al. 2019). These being not the topic of this chapter, some examples of applications have been summarized in Table 5.1 (for reviews, see Novoveská et al. (2019), Cezare-Gomes et al. (2019)).

The new usages of carotenoids have increased the demand and contributed to an accelerated depletion of natural resources (Alexandratos and Bruinsma 2012). To avoid endangering natural species, new sources for carotenoids are intensively

Table 5.1 Some example of putative and real application fields using carotenoids

	β-carotene	Astaxanthin	Canthaxanthin	Fucoxanthin	Lutein
Food antioxidant	+ (Guedes et al. 2011)	+ (Ao and Kim 2019)	No longer (Esatbeyoglu and Rimbach 2017)	+ Neumann (Neumann et al. 2019; Sellimi et al. 2017)	–
Food colourant	+ (Giménez et al. 2015)	+ (Pogorzelska et al. 2018)	No longer (Esatbeyoglu and Rimbach 2017)	+ (Sellimi et al. 2017)	+ (Grčević et al. 2019)
Medicine (in use or putative)	+ (Le Goff et al. 2019)	+ (Gateau et al. 2017; Zuluaga Tamayo et al. 2019)	+ (Palozza and Krinsky 1992)	+ (Neumann et al. 2019; Garg et al. 2019)	+ (Shimazu et al. 2019)
Feed	+ (Vincent et al. 2017)	+ (Langi et al. 2018)	+ (Alonso-Alvarez et al. 2018; Esatbeyoglu and Rimbach 2017)	–	+ (Mehariya et al. 2019)
Food supplement	+ (Hemilä 2018)	+ (Tamjidi et al. 2018)	+ (Arab et al. 2019)	+ (Sellimi et al. 2017)	+ (Jaswir et al. 2011)
Cosmetics	+ (Freitas and Gaspar 2016)	(Anunciato and da Rocha Filho 2012)	− (Sujac (Sujak 2009)	–	+ (Anunciato and da Rocha Filho 2012)

searched (Fernández-Sevilla et al. 2010) and microalgae emerged as organisms with a very high potential. In addition to the primary carotenoids they synthesize for photosynthesis, microalgae are also able to produce secondary carotenoids under stress conditions (Table 5.2). Indeed, with the development of molecular engineering, the idea that microalgae constitute the next platform organisms for the production of high value molecules has emerged (Levitan et al. 2014).

The use of carotenoids is continuously increasing boosting the carotenoid market. It was estimated to be ~1.24 billion USD in 2016, and is projected to increase to ~1.53 billion USD by 2021, at a compound annual growth rate (CAGR) of 3.78% from 2016 to 2021 (http://www.bccresearch.com) (Liu et al. 2014, Ambati et al. 2019) (Fig. 5.1a). The markets for each carotenoid has no equivalent. As displayed in Fig. 5.1b, the most important are those related to astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione), lutein (β,ε-carotene-3,3'-diol) and β-carotene (β,β-carotene). The total production of natural carotenoids (with major carotenoids including fucoxanthin, lutein, violaxanthin and neoxanthin) has been estimated as 100 million tons/year (Delgado-Vargas et al. 2000). The amount of publications about all the aspects related to carotenoids is yearly increasing (Fig. 5.2) reaching more than 14,000 publications listed in the Web of Science. Therefore, the writing of this chapter was not possible without making chooses and we apologize to the colleagues whose data of interests are not cited in this contribution. We tried to focus the selection of data on the most recent ones in order to show that the production of carotenoids by microalgae is a tailor-made process that should take into account biological factors such as the taxon (origin and the genome composition) but also the factors related to the environment such as temperature, light quality and quantity, salinity etc. but also rely on general biological responses to stress.

5.2 The Best Alga for the Highest Carotenoid Production: Does the Taxon Matter?

When reviewing literature, it appears clearly that the carotenoid production capacity is (at least) strain dependent (Table 5.3). For instance, Xu and Harvey (2019) reported that the total carotenoids accumulated by several strains of *Dunaliella* grown under red light varied from 1 to 3 according to the strain considered. In another study, 15 strains of chlorophycean microalgae were screened for the capacity of lutein production (Del Campo et al. 2000). Because tens of thousands microalga taxa have been described (Guiry 2012), and new species are continuously described (e.g. Schoefs et al. (2020)), exploring the biodiversity looks to be a promising strategy for finding putative new taxon with either a unique carotenoid composition or productivity. This requires quick and precise tools not only for estimating the classification of the alga but also the amount of pigments. Carotenoid diversity and sometimes specific distribution in the different taxonomic branches (Table 5.4) offers the possibility to use the pigment composition for chemotaxonomic through a multivariate analysis (Paliwal

Table 5.2 Putative sources and alternative sources among microalga for the production of carotenoids and carotenoid precursors for industrial applications. The shaded lines indicate carotenoids that are produced under stress conditions. TC: Total carotenoids. Completed after Raposo et al. (2015) and Gateau et al. (2017)

Carotenoids	Traditional sources	Microalgal alternative source	Cellular concentration
α-carotene		Red alga	
α-carotene		*Dunaliella salina* (Christaki et al. 2013)	
Astaxanthin	Yeast: yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) (Rodríguez-Sáiz et al. 2010; Schmidt et al. 2011)	*Botryococcus braunii, Chlorella zofingiensis, Diacronema vlkianum, Euglena rubida, Haematococcus pluvialis, Neochloris wimmeri, Scotiellopsis oocystiformis* (Lemoine and Schoefs 2010; Kopecky et al. 2000; Christaki et al. 2013; Ranga Rao et al. 2010; Del Campo et al. 2004; Orosa et al. 2000; Zhang and Lee 1997; Durmaz et al. 2009)	*Haematococcus pluvialis*: up to 7% DW/40 mg g^{-1} DW (Sathasivam and Ki 2018; Lorenz and Cysewski 2000), 75% TC (Raposo et al. 2015) *Chlorella zofingiensis*: up to 3.7% DW (Liu et al. 2014)
β-carotene	Industrial production: Chemistry, fungus: *Blakeslea trispora* (Britton et al. 2009); dietary: carrots and other vegetables	*Arthrospira platensis, Botryococcus braunii, Chlorococcum sp., Dunaliella salina, Parietochloris sp., Synechocystis sp., Chlorella zofingiengis* (Del Campo et al. 2007; Solovchenko et al. 2008)	*Arthrospira sp.*: 80% TC (Raposo et al. 2015), *Chlorella zofingiengis*: 50% TC (Raposo et al. 2015), 0.9% DW (Sathasivam and Ki 2018) *Dunaliella salina*: up to 13% DW (Sathasivam et al. 2019), 50%
Canthaxanthin	Bacterium: *Gordonia jacobaea* (Veiga-Crespo et al. 2005)	*Anabaena sp.* (Shahidi and Brown 1998), *Coelastrella striolata var. multistriata* (Abe et al. 2007), *Chlorella vulgaris* (Raposo et al. 2015)	*Coelastrella striolata var. multistriata*: 4.75% DW (Abe et al. 2007), *Chlorella vulgaris*: 45% TC (Raposo et al. 2015)
Echinenone		*Botryococcus braunii* (Tonegawa et al. 1998)	*Botryococcus braunii*: 0.17% DW (Tonegawa et al. 1998)

(continued)

Table 5.2 (continued)

Carotenoids	Traditional sources	Microalgal alternative source	Cellular concentration
Fucoxanthin	–	*Cyclotella cf. cryptica, Cyclotella meneghiniana, Cylindrotheca closterium, Eustigmatos magnus, Eustigmatos polyphem, Eustigmatos vischeri, Isochrysis* aff. *galbana, Mallomonas* sp. SBV13, *Nitzschia cf. carinospeciosa, Odontella aurita, Paralia longispina, Phaodactylum tricornutum, Vischeria helvetica, Vischeria punctata, Vischeria stellata* (Kim et al. 2012a; Li et al. 2012a, b)	*Cyclotella cf. cryptica*: 0.7 mg g^{-1} (Petrushkina et al. 2017) *Cyclotella meneghiniana*: 2.3 mg g^{-1} (Petrushkina et al. 2017) *Cylindrotheca closterium*: 0.52% DW (Rijstenbil 2003) *Isochrysis* aff. *galbana*: 1.8% DW (Kim et al. 2012b) *Mallomonas* sp. SBV13: 26.6 mg g^{-1} (Petrushkina et al. 2017) *Nitzschia cf. carinospeciosa*: 5.5 mg g^{-1} (Petrushkina et al. 2017) *Odontella aurita*: up to 2.2% DW (Xia et al. 2013) *Paralia longispina*: 1.4 mg g^{-1} (Petrushkina et al. 2017) *Phaodactylum tricornutum*: 1.65% DW—10.2 mg g^{-1} (Sathasivam and Ki 2018; Petrushkina et al. 2017; Kim et al. 2012a; Ragni and D'Alcala 2007)

(continued)

Table 5.2 (continued)

Carotenoids	Traditional sources	Microalgal alternative source	Cellular concentration
Lutein	*Tageta erecta* (Marigold petals) (Fernández-Sevilla et al. 2010; Lin et al. 2015)	*Auxochlorella protothecoides, Botryococcus braunii, Chlorella minutissima, Chlorella protothecoides, Chlorella pyrenoidosa, Chlorella sorokiniana, Chlorella zofingiensis, Chlorococcum citriforme, Coelastrella* sp., *Galdieria sulphuraria, Muriellopsis* sp., *Neospongiococcus gelatonosum, Parachlorella kessleri, Scenedesmus almeriensis, Scenedesmus bijugus, Vischeria stellata* (Dineshkumar et al. 2015; Fernández-Sevilla et al. 2010; Del Campo et al. 2007; Ranga Rao et al. 2010; Cuaresma et al. 2011; Durmaz et al. 2009; Ghosh et al. 2015)	*Auxochlorella protothecoides*: 0.76 mg g^{-1} (Minhas et al. 2016), *Chlorella protothecoides*: 5.4 mg g^{-1} (Shi et al. 2002) *Chlorella pyrenoidosa*: 0.2–0.4% DW (Sathasivam and Ki 2018; Wu et al. 2007) *Chlorella sorokiniana*: 5.90 mg g^{-1} (Minhas et al. 2016) *Chlorella* sp.: 2.26 mg g^{-1} (Minhas et al. 2016), *Chlorella vulgaris* : 45% TC (Mendes et al. 1995) *Coelastrella* sp.: 6.49 mg g^{-1} (Minhas et al. 2016) *Galdieria sulphuraria*: 0.40 mg g^{-1} (Graziani et al. 2013) *Parachlorella kessleri*: 0.28 mg g^{-1} (Minhas et al. 2016) *Scenedesmus almeriensis*: 0.54% DW (Sánchez et al. 2008) *Scenedesmus bijugus*: 2.9 mg g^{-1} (Minhas et al. 2016) *Scenedesmus* sp: 1.8 mg g^{-1} (Minhas et al. 2016) *Vischeria stellata*: 1.5 mg g^{-1} (Minhas et al. 2016)
Phytoene	Tomato extract (von Oppen-Bezalel et al. 2015; Engelmann et al. 2011)	*Dunaliella bardawil* (Ben-Amotz et al. 1987, 1988)	*Dunaliella bardawil*: 80 mg g^{-1} (Ben-Amotz et al. 1987; Soudant et al. 2002), tomato: 2–9 μg g^{-1} DW (Laje et al. 2019), *Chlorococcum* UTEX B 3056: 33 μg mg^{-1} DW (Laje et al. 2019)
Siphonaxanthin	–	*Nephoselmis* (Yoshii et al. 2005)	

(continued)

Table 5.2 (continued)

Carotenoids	Traditional sources	Microalgal alternative source	Cellular concentration
Violaxanthin		*Chlorella ellipsodea* (Sathasivam and Ki 2018)	
Zeaxanthin	Chemistry, *Flavobacterium* sp. (Sajilata et al. 2008)	*Arthrospira* sp., *Botryococcus braunii, Chlamydomonas acidophila, Dunaliella salina, Microcystis aeruginosa, Neospongiococcum excentricum, Porphyridium cruentum* (Christaki et al. 2013; Ranga Rao et al. 2010; Sajilata et al. 2008; Cuaresma et al. 2011; Sathasivam and Ki 2018; Schubert et al. 2006)	*Porphiridium cruentum*: 94.5% TC (Schubert et al. 2006)

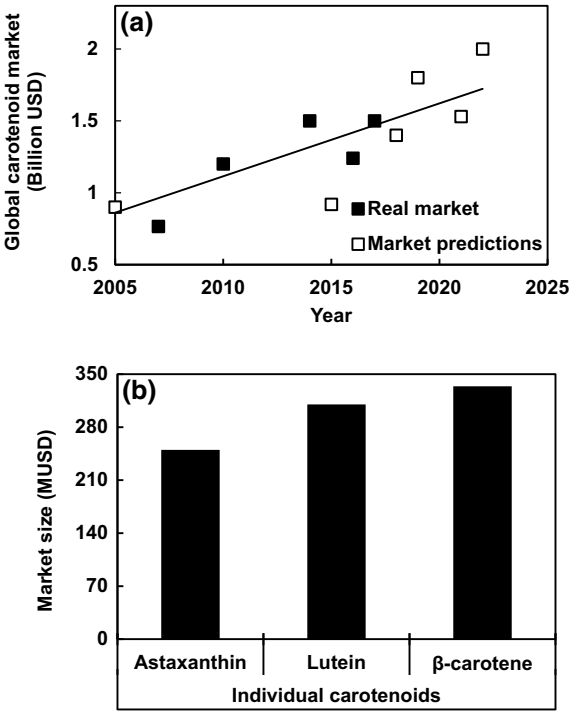

Fig. 5.1 Carotenoid market. **a** Time-course of the predictions for the carotenoid market and the actual carotenoid market. **b** Individual carotenoid market sizes. After http://www.bcresearch.com and Borowitzka (2013)

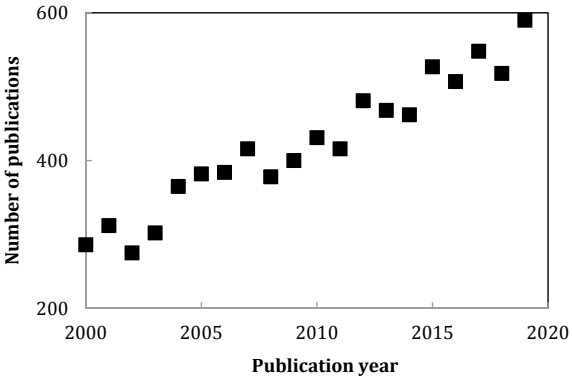

Fig. 5.2 Evolution of the number of publications with the word 'carotenoid*' in the title during the last 20 years. *Source* WOS. Search performed on 10 February 2020

et al. 2016; Mc Gee et al. 2018). Using such an approach, Mc Gee et al. (2018) screened 110 strains from West Ireland and discovered strains that can be considered as interesting alternative to traditional taxa for carotenoid production (Table 5.3). Selecting strains using literature data requires to take into account the continuous evolution of taxonomy, that generates different names for the same taxon (Schoefs et al. 2020) (Table 5.5).

Table 5.3 Comparison of the carotenoid productivity of different microalga species and strains

	Strain	Total Car	Astaxanthin	β-carotene	Canthaxanthin	Fucoxanthin	Lutein	Zeaxanthin
Amphora sp.	DMG_CW_23					3.3 mg g^{-1} DW (Mc Gee et al. 2018)		
Brachiomonas submarina	AP_SW_11						6.1 mg g^{-1} DW (Mc Gee et al. 2018)	
Chlorella variabilis	AP_OT_10						2.4 mg g^{-1} DW (Mc Gee et al. 2018)	
Chlorococcum citriforme	SAG62.80						7.2 mg g^{-1} DW (Del Campo et al. 2000)	
Chlorosarcinopsis sp.	PY02				1 mg g^{-1} DW (Cherdchukeattisak et al. 2018)			
Coccomyxa sp.	new species, not yest deposited in a collection						>5 mg g^{-1} DW (Vaquero et al. 2014)	
Coccomyxa sp.	DMG_FW_14						4.06 mg g^{-1} DW (Mc Gee et al. 2018)	
Dunaliella salina rubeus	CCAP 19/41	14.0 pg $cell^{-1}$ (Xu and Harvey 2019)	–	85% of TC (Xu and Harvey 2019)				
Dunaliella salina salina	PLY DF17	1.4 pg $cell^{-1}$ (Xu and Harvey 2019)	–					
Dunaliella salina	CCAP 19/40	2.3 pg $cell^{-1}$ (Xu and Harvey 2019)	–					

(continued)

Table 5.3 (continued)

	Strain	Total Car	Astaxanthin	β-carotene	Canthaxanthin	Fucoxanthin	Lutein	Zeaxanthin
Dunaliella salina bardawil	UTEX 2538	12.3 pg $cell^{-1}$ (Xu and Harvey 2019)	–					
Haemtococcus pluvialis	LA_FW_15		6.7–9.3 mg g^{-1} DW (Mc Gee et al. 2018)				2.1 mg g^{-1} DW (Mc Gee et al. 2018)	0.9 mg g^{-1} DW (Mc Gee et al. 2018)
Haemtococcus pluvialis	NIES-144		2.6 mg g^{-1} DW (Liu et al. 2020)					
Kirchneriella aperta	DMG_FW_21						4.0 mg g^{-1} DW (Mc Gee et al. 2018)	0.84 mg g^{-1} DW (Mc Gee et al. 2018)
Muriellopsis sp.							4.0 mg g^{-1} DW/35 mg L^{-1} (Del Campo et al. 2000)	
Nitzschia bizertensis	LA_CW_25						(Guiheneuf and Stengel 2015)	
Pyramimonas sp.	DMG_CW_37			1.6 mg g^{-1} DW (Mc Gee et al. 2018)			1.3 mg g^{-1} DW (Mc Gee et al. 2018)	
Rhodella sp.	AP_OT_15							0.80 mg g^{-1} DW (Mc Gee et al. 2018)

Table 5.4 Carotenoid distribution among the algal taxa. Neochrome is an artifact formed from neoxanthin that is present in the OT extract by the acid-catalyzed rearrangement during isolation (Britton et al. 2004)

	Cyanobacteria	Glaucophytes	Rhodophyceae	Chrysophyceae	Dinophyceae	Haptophyceae
α-carotene						
β-carotene	+	+	+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)
Alloxanthin						
Antheraxanthin			+ (Mc Gee et al. 2018)			
Canthaxanthin						
Crocoxanthin						
Cryptoxanthin			+ (Mc Gee et al. 2018)			
Diadinochrome				+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)
Diadinoxanthin				+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+(Mc Gee et al. 2018)
Diatoxanthin				+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)
Dihydrolutein						
Fucoxanthin				+ (Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	
Heteroxanthin						
Loroxanthin						
Loroxanthin-decenoate						
Loroxanthin-dodecenoate						
Lutein						
Micromonal						
Micromonol						

(continued)

Table 5.4 (continued)

	Cyanobacteria	Glaucophytes	Rhodophyceae	Chrysophyceae	Dinophyceae	Haptophyceae
Monadoxanthin						
Mutatoxanthin						
Neochrome						
Neoxanthin						
Peridinin and peridin isomers					+ (Mc Gee et al. 2018)	
Prasinoxanthin						
Siphonaxanthin and siphonaxanthin-like						
Uriolide						
Violaxanthin				+ (Mc Gee et al. 2018)		
Zeaxanthin			+ (Mc Gee et al. 2018)			

	Euglenophyta	Chlorophyceae	Bacillariophyceae	Prasinophyceae	Cryptophyceae	Mamiellophyceae
α-carotene		in 37 of the 67 taxa examined (Mc Gee et al. 2018)		+(Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	
β-carotene	+ (Mc Gee et al. 2018)	+ (Kopecky et al. 2000; Mc Gee et al. 2018)	+ (Mc Gee et al. 2018)	+ (Latasa et al. 2004)		
Alloxanthin					+ (Mc Gee et al. 2018)	
Antheraxanthin		+ (Kopecky et al. 2000; Mc Gee et al. 2018)	+ under very high light (Lohr and Wilhelm 1999)	(Mc Gee et al. 2018)		

(continued)

Table 5.4 (continued)

	Euglenophyta	Chlorophyceae	Bacillariophyceae	Prasinophyceae	Cryptophyceae	Mamiellophyceae
Canthaxanthin	+ (Mc Gee et al. 2018)					
Crocoxanthin					+ (Mc Gee et al. 2018)	
Cryptoxanthin						
Diadinochrome			+ (Mc Gee et al. 2018)			
Diadinoxanthin	+ (Mc Gee et al. 2018)		+ (Mc Gee et al. 2018)			
Diatoxanthin	+ (Mc Gee et al. 2018)		+ (Mc Gee et al. 2018)			
Dihydrolutein				+ (Latasa et al. 2004)		+ (Le Goff et al. 2020)
Fucoxanthin			+ (Mc Gee et al. 2018)	+ (Latasa et al. 2004)		
Heteroxanthin	+ (Mc Gee et al. 2018)					
Loroxanthin				+ (Mc Gee et al. 2018; Latasa et al. 2004)		
Loroxanthin-decenoate				in 44 of the 67 taxa examined (Mc Gee et al. 2018)		
Loroxanthin-dodecenoate				in 41 of the 67 taxa examined (Mc Gee et al. 2018)		
Lutein		+ (Kopecky et al. 2000; Mc Gee et al. 2018)		+ (Mc Gee et al. 2018; Latasa et al. 2004)		
Micromonal				+ (Latasa et al. 2004)		+ (Le Goff et al. 2020)
Micromonol				+ (Latasa et al. 2004)		

(continued)

Table 5.4 (continued)

	Euglenophyta	Chlorophyceae	Bacillariophyceae	Prasinophyceae	Cryptophyceae	Mamiellophyceae
Monadoxanthin					+ (Mc Gee et al. 2018)	
Mutatoxanthin		+ (Mc Gee et al. 2018)		+ (Mc Gee et al. 2018)		
Neochrome						+ (Le Goff et al. 2020)
Neoxanthin	+ (Mc Gee et al. 2018)	+ (Kopecky et al. 2000; Mc Gee et al. 2018)		+ (Mc Gee et al. 2018; Latasa et al. 2004)		
Peridinin and peridin isomers						
Prasinoxanthin				+ (Latasa et al. 2004)		+ (Le Goff et al. 2020)
Siphonaxanthin and siphonaxanthin-like	+ (Mc Gee et al. 2018)			+ (Latasa et al. 2004)		
Uriolide				+ (Latasa et al. 2004)		
Violaxanthin		+ (Kopecky et al. 2000; Mc Gee et al. 2018)	+ under very high light (Lohr and Wilhelm 1999)	+ (Mc Gee et al. 2018; Latasa et al. 2004)		
Zeaxanthin		+ (Mc Gee et al. 2018)	+ under very high light (Lohr and Wilhelm 1999)	+ (Mc Gee et al. 2018; Latasa et al. 2004)		

Table 5.5 Examples of some taxon synonymies

New name	Synonymous	Reference
Chromochloris zofingiensis	*Chlorella zofingiensis*	Fučíková and Lewis (2012)
Porphyridium purpureum	*Porphyridium cruentum*	Drew and Ross (1965)

5.3 Carotenoid Chemistry and Biochemistry

Carotenoids belongs to the family of terpenoid compounds and has more than 750 members (Langi et al. 2018; Novoveská et al. 2019; Gateau et al. 2017). From the chemical point of view, carotenoids are C30-C50 molecules characterized by an extended network of conjugated double bonds. Carotenoids range in two subfamilies i.e. carotene and xanthophyll. The members of the former subfamily lack oxygen atoms whereas the members of the latter present hydroxy groups (hydroxycarotenoids such as zeaxanthin and lutein), keto groups (keto-carotenoids such as canthaxanthin and echinenone), epoxy groups (epoxycarotenoids such as violaxanthin and diadinoxanthin). The structure of some xanthophylls is even more complex combining several types of side groups like in astaxanthin (keto- et hydroxy- groups), dinoxanthin and fucoxanthin (epoxy-, acetylated groups and allene linkage) (Dembitsky and Maoka 2007) or display an acetylene linkage such as monadoxanthin (Takaichi 2011).

The extended network of conjugated double bonds forms the chromophore, allowing the absorption of visible light in the violet-green region. The range of the absorbed wavelengths can be in first approximation determined by the amount of conjugated double bonds along the carbon backbone: the higher the number of conjugated double bonds the longer the wavelengths the pigment absorbs (Schoefs 2002). A consequence of the presence of double bonds is the abundant number of carotenoid isomers (Schoefs 2005a). Thus, carotenoids may adopt several 3D-configurations that are important for their biological properties. For instance, *cis*-isomers of fucoxanthin have been reported to be more valuable than all-*trans*-isomers in human cancer lines (Gateau et al. 2017). Another consequence of the presence of this extended conjugated double bonds is the antioxidant properties of carotenoids (Foo et al. 2017; Sahin et al. 2019).

The carotenoid diversity in microalgae is very large and in many cases specific of taxa (Mc Gee et al. 2018). For instance, lutein is only found in Chrysophyta, Euglenophyta, Chlorarachniophyta, Chlorophyta and some Rhodophyta (Table 5.4). In addition to the regular carotenoid content, some microalgae are able to produce one or several additional carotenoids with unique chemical structures as a stress response. A good example is the family of keto-carotenoids such as astaxanthin, canthaxanthin, fucoxanthin, peridinin and siphonaxanthin (Table 5.4).

Carotenoid biosynthesis occurs in chloroplasts (Han et al. 2013; Lichtenthaler 1999). The enzymes involved in the pathway are coded in the nucleus genome,

produced in the cytoplasm as pre-proteins before being imported into the chloroplast. Carotenoid biosynthetic pathway has been the topic of several recent review papers (Cherdchukeattisak et al. 2018; Gateau et al. 2017). In most microalgae, the carotenoid biosynthetic pathway is highly conserved thought that some species are able to accumulate unusual carotenoids via biosynthetic appendices such as the one resulting in the formation of keto-carotenoids and allelic carotenoids.

Carotenoids are synthesized from a C5 building block, the isopentenyl pyrophosphate (IPP) and its isomer, the dimethylallyl pyrophosphate (DMAPP). The isomerization step is catalyzed by IPP isomerase, coded by the gene *ipi*. Two pathways resulting in the production of these compounds have been described. The first one, denoted the mevalonate pathway occurs in animals, fungi, archaebacteria and certain bacteria. In eukaryotic cells, it is localized in the cytoplasm. The second pathway, the 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway, is active in many eubacteria, including *Escherichia coli*, apicomplexa, algae and land plants. In some taxa, both pathways can be active (Table 5.6). In eukaryotic organisms, MEP pathway is located in plastids. This pathway involves several enzymes namely 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductosiomerase (DXR), 2-C-methyl-D-erythritol 4 phosphate (ISPD), 2-methyl-D-erythritol 2,4-cyclodiphosphate synthase (ISPF), *E*-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), 4-hydroxy-3-methylbut-2-*en*-1-yl diphosphate reductase (HDR), isopentenyl diphosphate delta isomerase (IDI). The IPP building blocks are then assembled to yield geranyl-geranyl pyrophosphate. The condensation of two molecules of geranyl geranyl pyrophosphate (GGPP), catalyzed by the phytoene synthase (cyanobacteria: CrtB, microalgae: PSY) yields phytoene, a colourless compounds. CrtB/PSY enzyme is coded by a single gene or gene family (Meléndez-Martínez et al. 2015). Sequential desaturations catalysed by phytoene desaturase (cyanobacteria: CrtP, microalgae: PDS) and ζ-carotene desaturase (cyanobacteria: CrtQ, microalgae: ZDS) resulting in the formation of pro-lycopene. Inhibition of the PDS enzymatic activity by herbicides such as norflurazon (5-amino-4-chloro-2-[3-(trifluoromethyl)phenyl]pyridazin-3-one) and fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]pyridin-4-one) (Ben-Amotz et al. 1988) results in the accumulation of phytoene (Laje et al. 2019; Zhekisheva et al. 2005) and the absence of further carotenoids. Therefore, when applied in dividing microalgae, the carotenoid cellular quota decreases by dilution within the daughter cells (Laje et al. 2019).

Pro-lycopene is then isomerized by a carotenoid isomerase (CRTISO) into all-*trans*-lycopene that serves as a precursor for the cyclases. Lycopene can be either cyclized at both ends by lycopene β-cyclase (LCYB), yielding β-carotene with two β–ionone end groups or to the combined actions of LCYB and lycopene ε-cyclases (LCYEs) resulting in the formation of α-carotene. The amount of each carotenoid type is determined by the absolute activities of LCYE and LCYB. α-carotene and β-carotene are modified allowing the structural diversification of carotenoids.

Table 5.6 Occurrence of the different isopentenyl diphosphate (IPP) biosynthetic pathways in different microalgal taxa. (−) very weak if any contribution. The mevalonic acid (MVA) pathway is localized in the cytoplasm, while the methylerythritol phosphate (MEP) pathway is plastid located. Modified after Gateau et al. (2017)

Superphylum/Phylum	Genus species	Pathway used to synthesize IPP	
		MVA	MEP
Cyanobacteria	*Synechocystis*		+ (Cunningham et al. 2000; Disch et al. 1998; Lichthenthaler 1998)
Chlorophyta	*Scenedesmus obliquus*	− (Schwender et al. 1996)	+ (Schwender et al. 1996)
	Chlamydomonas reinhardtii	− (Disch et al. 1998; Lichthenthaler 1998)	+ (Disch et al. 1998; Lichthenthaler 1998)
	Chlorella fusca	− (Disch et al. 1998; Lichthenthaler 1998)	+ (Disch et al. 1998; Lichthenthaler 1998)
	Dunaliella salina	− (Capa et al. 2009)	+ (Capa et al. 2009)
Chrysophyta	*Ochromonas malhamensis*	+ (Maurey et al. 1986)	+ (Maurey et al. 1986)
Euglenozoa	*Euglena vulgaris*	+ (Disch et al. 1998) (Lichthenthaler 1998)	− (Disch et al. 1998; Lichthenthaler 1998)
Bacillariophyta	*Rhizosolenia setigera*	+ (Massé et al. 2004)	+ (Massé et al. 2004)
	Haslea ostrearia	− (Massé et al. 2004)	+ (Massé et al. 2004)
	Phaeodactylum tricornutum	+ (Cvejić and Rohmer 2000)	+ (Cvejić and Rohmer 2000)
	Nitzschia ovalis	+ (Cvejić and Rohmer 2000)	+ (Cvejić and Rohmer 2000)
Rhodophyta	*Cyanidium caldarium*	− (Disch et al. 1998; Lichthenthaler 1998)	+ (Disch et al. 1998; Lichthenthaler 1998)

5.3.1 Hydroxylated Carotenoid

Several unrelated types of carotenoid hydroxylases (CHY) have been identified so far among the different sequenced genomes. They are denoted as CrtR (cyanobacteria: Makino et al. (2008)), BCH/CHYB, CYP97A, CYP97B and CYP97C (microalgae and land plants: Kim et al. (2009), Cui et al. (2013)). BCH/CHYB enzymes are non-heme di-iron hydroxylase whereas CYP97 enzymes contain a hemic iron absorbing at 450 nm (cytochrome P450) (Yu et al. 2014; Cui et al. 2014; Kim et al. 2009). Several conserved amino acid residues can be used for identifying the subfamily to which they belong. Using these elements, Cui et al. (2019) ranged CYP97b in the CYP97 A/B/C subfamily.

β-carotene can be hydroxylated by a non-heme di-iron hydroxylase, called β-carotene hydroxylase (CHYB) to yield zeaxanthin. It is converted to violaxanthin by

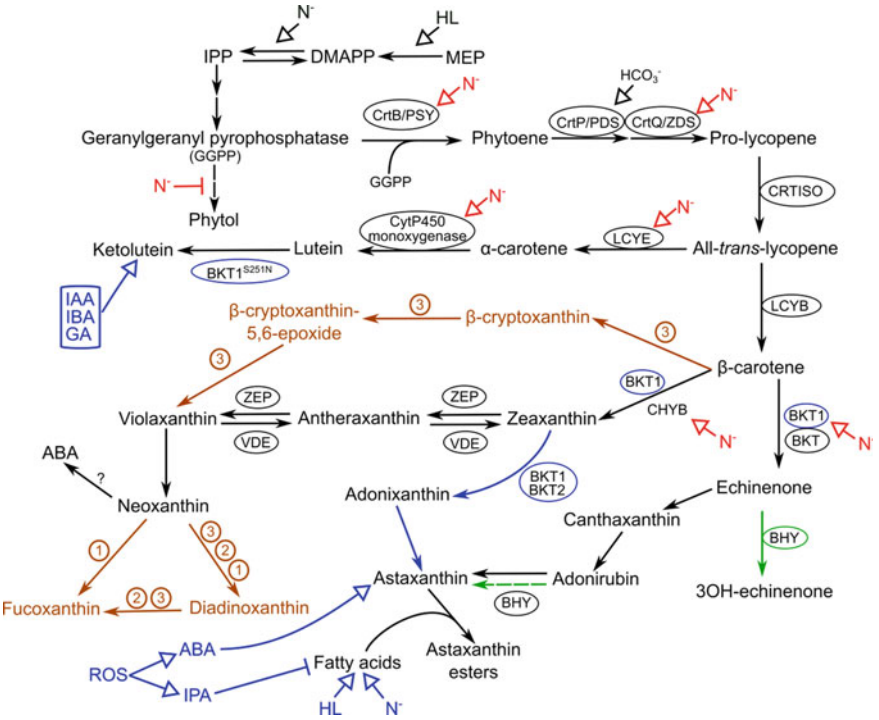

Fig. 5.3 Metabolic scheme of the carotenoid pathways in microalgae. Green, blue, red and brown designate reactions or regulation aspects specific of *Chlorosarcinopsis*, *Chromochloris zofigiensis*, *Haematococcus pluvialis* and Chl c containing algae, respectively. Elipses and arrows with open heads represent enzymes and regulatory factors, respectively

zeaxanthin epoxidase (ZEP), which inserts two epoxy groups at positions C-5,6 and C-5',6'.

α-carotene can also be hydroxylated by two heme-containing cytochrome P450 monooxygenases (namely, carotene β-hydroxylase and carotene ε-hydroxylase), which leads to the formation of lutein (Fig. 5.3).

5.3.2 *Astaxanthin and Other Keto-Carotenoids*

Besides their huge potential for health industries, keto-carotenoids are used as feed in fish (salmon, trout) and shrimp aquaculture (see Table 5.1). Therefore many studies have been dedicated to this family of xanthophylls, including analytical means and certification activities (for a review, see Lemoine and Schoefs (2010)). The latter domain is linked to the chemical tridimensional structure of astaxanthin regarding

the astaxanthin source: commercial synthetic astaxanthin is a mixture of the enantiomers (3*R*,3'*R* and 3*S*,3'*S*) and the meso compound (3*R*,3'*S*) in the ratio of 1:1:2, respectively (Visioli and Artaria 2017; Higuera-Ciapara et al. 2006) whereas astaxanthin extracted from *H. pluvialis* is only (3*S*,3'*S*) and mostly esterified (Visioli and Artaria 2017). Interestingly, the isomer conformation is not modified during astaxanthin metabolism in animal cells and therefore the conformation can be used for tracing the biological origin of astaxanthin found in fish for instance (Turujman et al. 1997). Keto-carotenoids derive from β-carotene. The capacity of keto-carotenoid biosynthesis has been described in one land plant petals, some yeasts and a few microalgal taxa (*Chromochloris zofingiensis*: Huang et al. (2006); *Tetradesmus*: Pirastru et al. (2011), *Chlorosarcinopsis* sp: Cherdchukeattisak et al. (2018), screening: Kopecky et al. (2000)), *Haematococcus pluvialis* being the most famous (Chen et al. 2020) (see Table 5.3 for the synonymous taxa).

Several pathways have been proposed for astaxanthin biosynthesis denoted as zeaxanthin and canthaxanthin pathways, respectively. They all involve β-carotene ketolase (β-carotene 4,4'-oxygenase) (BKT-microalgae: Lotan and Hirschberg (1995), Fraser et al. (1998); CRTW-cyanobacteria: Kajiwara et al. (1995), Choi et al. (2007)), the enzyme adding sequentially the keto groups in C4 and C4' positions on the β-ionone rings of β-carotene, yielding echinenone (4-monoketo-β-carotene) and canthaxanthin (4,4'-diketo-β-carotene), respectively (Breitenbach et al. 1996). BKT enzymes are characterized by conserved histidine motifs postulated as binding non-hemic iron required for enzymatic activity (Fraser et al. 1997) on the β-ionone rings of β-carotene. Interestingly, the S251W mutation reduced the capacity of BKT1 to catalyse β-carotene transformation, to canthaxanthin that instead catalyses the ketolutein production, suggesting that this region of the enzyme could be related to the α-ionone and β-ionone-ring selectivity (Ye and Huang 2019). This mutation is located at the C-terminus of the enzyme amino acid sequence.

Canthaxanthin is then sequentially hydroxylased by β-carotene hydroxylase (BHY) in C3 and C3' positions, resulting in the formation of astaxanthin (Varela et al. 2015; Sun et al. 1996; Schoefs et al. 2001). The production of keto-carotenoids can be restricted to part of the algal life cycle such as the zygospores (*Chlamydomonas*: Lohr et al. (2005)). Astaxanthin accumulates in the cytoplasm in lipid globules (Lemoine and Schoefs 2010).

A few microalgae such as *Chlorosarcinopsis* PY02 accumulate canthaxanthin rather than astaxanthin. The biosynthetic pathway is similar to that found in *Haematococcus pluvialis* i.e. β-carotene is first hydroxylated twice to yield echinenone and canthaxanthin, respectively by BKT enzymes. To explain the preferential accumulation of 3-OH-echinenone and canthaxanthin, Cherdchukeattisak et al. (2018) hypothesize that both hydroxylase and ketolase compete for β-carotene and suggest that the hydroxylase (BHY) is less active than the ketolase (BKT).

Two genes code for BKT enzymes, namely BKT1 and BKT2. BKT1, in addition to catalyse the hydroxylation of β-carotene to canthaxanthin can also convert zeaxanthin to astaxanthin (Huang et al. 2018; Wang and Chen 2008). A single nucleotide insertion (+G395) or substitution in highly conserved regions (H168R, 552K, P284L

or S251N) resulted in the non-functionality of BKT1 in *Chromochloris zofigiensis* (Ye and Huang 2019).

5.3.3 *Fucoxanthin, an Abundant Allelic Carotenoid*

Fucoxanthin is probably the most abundant carotenoid produced on Earth (Haugan and Liaaen Jensen 1994). It is characterized by the presence of an allelic bond. Beside its role in vivo as light-harvesting pigment, it shows a huge potential for medical treatment (Gateau et al. 2017). Despite its important potential, the biochemical pathways along which it is produced in vivo are not yet elucidated. Three pathways have been proposed. The first one involves neoxanthin, the end carotenoid product of the β-carotene to violaxanthin transformation. Neoxanthin would serve as a substrate for fucoxanthin, diadinoxanthin or/and abscisic acid syntheses (Gong and Bassi 2016). The second pathway is very similar to the first one but would use diadinoxanthin as the precursor of fucoxanthin (Coesel et al. 2008). The 3rd possibility differs from the second pathway by the steps transforming β-carotene to violaxanthin that would involve β-cryptoxanthin and β-cryptoxanthin-5,6-epoxide instead than zeaxanthin and antheraxanthin (Bertrand 2010) (Fig. 5.3). Cui et al. (2019) and Kuczynska and Jemiola-Rzeminska (2017) disagreed on the activity of the 3rd pathway in diatoms. The absence of this pathway is likely because when the genes encoding CYP97b1 and CYP97b2 were cloned in a strain of *Escherichia coli* producing β-carotene, only CYP97b1 showed an hydroxylase activity, producing zeaxanthin and no β-cryptoxanthin (Cui et al. 2019).

No gene coding for BCH enzyme was found in the genome of *Phaeodactylum tricornutum* CCAP 1055/1 and only a partial sequence was found in *Thalassiosira pseudonana* CCMP 1335 (Coesel et al. 2008; Gong and Bassi 2016). Interestingly, two genes coding for CYP97b or LUT-like hydroxylase P450 enzyme (CYP97b1/LUT-like 1 and CYP97b2/LUT-like 2) have been predicted in *Phaeodactylum tricornutum* and might be involved in either pathways (Gong and Bassi 2016; Coesel et al. 2008). Recently, Cui et al. (2019) cloned CYP97b1 and CYP97b2 genes to study their cellular localization. Both enzymes were targeted in the chloroplast but CYP97b2 could possibly also targeted in the cytoplasm.

5.4 Stress Controls Carotenoid Biosynthesis: Biochemical and Molecular Control

Carotenoids are crucial molecules for the development of microalgal cells. Their biosynthesis depends on the presence of adequate physico-chemical conditions in the cell environment. For instance, when ions serving as cofactor for enzymes involved in the carotenoid biosynthetic pathway are deficient, the carotenoid production is

reduced (e.g., Mn: phytoene synthase, Fe: β-carotene hydroxylase) (Lee and Schmidt-Dannert 2002; Dineshkumar et al. 2015). Other unfavourable conditions, such as cell aging (Gu et al. 2013), temperature (Tjahjono et al. 1994; Giannelli et al. 2015), irradiance level (Gu et al. 2014; Schoefs et al. 2001), UV-B irradiation, salinity (Gao et al. 2015), nutrient deprivation (Sampathkumar et al. 2019; Azadeh et al. 2017; Wu et al. 2013; Scibilia et al. 2015) and drought, alone or in combination (Hagen et al. 2000; Su et al. 2014; Zhekisheva et al. 2002; Lemoine and Schoefs 2010; Hong et al. 2015; Dominguez-Bocanegra et al. 2004) generate a stress against which the cells must react under penalty of dying. Among the different response mechanisms triggered by stress, the metabolic reorientation allows the accumulation of compounds such as lipids (Sayanova et al. 2017) and carotenoids (Lemoine and Schoefs 2010). If the metabolic network along which the carotenoids are synthesized is rather well established, the regulation pathways controlling the activity of the biochemical network remain mostly unknown. In this section, the impacts of stress on the carotenoid production are summarized as well as the signalling and some biochemical and molecular regulation aspects.

5.4.1 The Composition of the Growth Medium

In the natural environment, water evaporation triggers nutrient precipitation, reducing nutrient availability for the microalgae. Consequently, the concentration in sodium chloride increases and together with the decrease in nutrient availability triggers a stress. Simultaneously, the light intensity can increase, adding to the stress perception by the microalgae. These natural conditions to which each microalgal cell is exposed can be somehow mimicked by changing the culture environment, including the composition of the growth medium, to induce the carotenoid production by microalgae (Srinivasan et al. 2015, 2018; Sampathkumar et al. 2019).

5.4.1.1 The Ionic Composition

Ions are crucial for nearly all cellular activities. For instance, magnesium is vital for chlorophyll biosynthesis (Schoefs 2005b) and more generally for photosynthesis and other cell processes. Two types of ions can be defined: the essential and the nonessential ions. Essential ions are nitrate, phosphate (Boussiba et al. 1999) and sulfur ions (He et al. 2007) and nonessential are calcium, magnesium, chromium, cobalt and iodine ions (Fabregas et al. 2000). To ensure an adequate ion cellular homeostasis within the different cell compartments, each of them hosts an arsenal of ion transporters (Marchand et al. 2018, 2020). Unbalance of the ionic composition may promote stress effects (Masmoudi et al. 2013), impacting directly or indirectly the carotenoid production. For instance, a high magnesium concentration in the growth medium of the green alga *Chlorella vulgaris* promotes phytohormone production

(Ben Amor-Ben Ayed et al. 2015), that are thought to act as a cell stress signalling way involved in the carotenoid production.

Salinity occupies a particular place in the biology of living organisms because sodium are highly toxic to organisms that are not adapted to it (Masmoudi et al. 2013). Therefore, increasing the sodium concentration in the growth medium may constitute a stress able to trigger the accumulation of secondary carotenoids (astaxanthin-*Haematococcus pluvialis*: Gao et al. (2015), β-carotene-*Dunaliella*: Raja et al. (2007), Zarandi-Miandoab et al. (2019), lutein-*Chlorella sorokiniana*: Cordero et al. (2011)). Altogether, a careful look at the ionic composition of the growth medium is required when optimizing carotenoid production by microalgae.

5.4.1.2 The Nitrate and Phosphate Availability

Nitrogen availability is among the crucial component of the growth medium because it stimulates cell division. When the growth medium is depleted partially or totally in bioavailable nitrogen, cell replication is slowed down and the accumulation of secondary carotenoid is stimulated (Lemoine and Schoefs 2010). The gene coding the acetyl-CoA carboxylase, the enzyme catalysing the limiting step of fatty acid synthesis, is especially upregulated (Zhao et al. 2019). Actually, all the genes coding enzymes catalysing the transformation of IPI/DMAPP to astaxanthin were upregulated, the highest regulation being observed for *psy, zds, chyB* and *bkt* genes (Zhao et al. 2019). Geranylgeraniol diphosphate is at the crossroad of the chlorophyll (Schoefs and Bertrand 2000) and carotenoid biosynthetic pathways (Heydarizadeh et al. 2013). Nitrogen deprivation down-regulates the expression of genes coding enzymes involved in phytol production suggesting that the flux of IPP is oriented toward secondary carotenoid production. This is correlated with the overexpression of the PSY encoding genes (Zhao et al. 2019) and consistent with the disorganization of the photosynthetic apparatus (Tan et al. 1995; Lemoine et al. 2008). Interestingly, the genes involved in lutein biosynthesis were also upregulated. The genes coding for the enzymes involved in zeaxanthin and canthaxanthin pathways both reported to end with astaxanthin were upregulated. A similar upregulation was observed in *Haematococcus pluvialis* exposed to blue light (Gu et al. 2014) and exogenous phytohormone applications.

Recent data suggest that the level of nitrogen is probably not the sole factor acting on the activity of the secondary carotenoid pathway. Actually, it would be the C/N ratio that would regulate the secondary carotenoid production and not the actual carbon or nitrogen cellular quota. High C/N ratio would favour the production of these compounds (astaxanthin-*Haematococcus pluvialis*: Kang et al. (2007), Kakizono et al. (1992), astaxanthin-*Chromochloris zofingiensis*: Sun et al. (2008), cantaxanthin-*Chlorosarcinopsis* sp.: Cherdchukeattisak et al. (2018).

As nitrate starvation, phosphate starvation triggers astaxanthin accumulation in *Haematococcus pluvialis* (Fan et al. 1998).

5.4.1.3 The Carbon Source

Organic molecules, such as carotenoids are exclusively or mostly composed of carbon atoms. Microalgae being photosynthetic organisms, they need a carbon source to feed their metabolism with this compound (Schoefs et al. 2017). A few microalgae are able to use gaseous CO_2 and nearly all use predominantly the dissolved inorganic CO_2 (DIC), the availability of which is not only crucial for biomass production but also for the production of biomolecules, including carotenoids. DIC deficiency limiting growth and the production of biomolecules (e.g. Heydarizadeh et al. (2017), (2019)), a sufficient DIC availability is required for efficient growth and carotenoid production (Juneja et al. 2013; Gardner et al. 2013). Thanks to the carbon concentration mechanisms, microalgae are able to utilize up to 50 times more efficiently CO_2 that land plants (Anto et al. 2019). Optimizing DIC for lutein production by *Chlorella pyrenoidosa* triggered an upregulation of the *pds, lyc* and *chy* genes and resulted in the improvement of the production of carotenoid production by 300% (Sampathkumar and Gothandam 2019). A few studies have been dedicated to the effect of aeration on *Haematococcus pluvialis* (Göksan et al. 2011). Azizi et al. (2020) showed that cell growth under aeration with air at 380 ppm CO_2 at 0.5 vvm instead of 1 vvm favoured biomass production of *Haematococcus pluvialis* and astaxanthin production under high light. Other carbon sources than inorganic carbon can be used depending of the taxon. For instance, *Haematococcus pluvialis* can develop on different organic carbon sources (DOC) such as acetate, malonate and glycerol (Table 5.8). In the presence of DOC, microalgae are developing in mixotrophic conditions that may impact the production of secondary carotenoids (Orosa et al. 2001). Interestingly, the utilization of glycerol by the strain H_6 of *Haematococcus pluvialis* did not change either the biomass or the photosynthetic activity when grown under low light (150 μmol m^{-2} s^{-1}). In these conditions, the carbon flux was oriented toward the accumulation of pyruvate that in turn was transformed to acetyl CoA, a precursor of fatty acids. The formation of acetyl CoA occurred through the action of a glycerol dehydrogenase that transforms glycerol to dihydroxyacetone (DHA) and glycerol-3-phosphate dehydrogenase, respectively. DHA is then converted to DHA phosphate (DHAP) by a DHA kinase or through the formation of glyceric acid, that would be ultimately converted to DHAP, the precursor of acetyl CoA (Zhang et al. 2020). Such a reorientation of the carbon flux under low light was also observed in the diatom *Phaeodactylum tricornutum* grown under carbon starvation condition (Heydarizadeh et al. 2017, 2019). Altogether, this suggests that lipid accumulation constitutes a default answer to stress conditions in these organisms. It is well established that an active fatty acid biosynthesis is required for the accumulation of astaxanthin in *Haematococcus pluvialis*, the fatty acids being used for astaxanthin esterification (Lemoine et al. 2008; Schoefs et al. 2001). It is therefore not completely surprising that in the presence of exogenous glycerol, astaxanthin accumulated in *Haematococcus pluvialis* H_6 even under low light intensity, even though that in these conditions, astaxanthin cellular quota will not as high than under high-light conditions (Zhang et al. 2020). The ability of microalgae to use other carbon sources than DIC renders the development of biotechnological processes using heterotrophy and mixotrophic cultivation modes. Because

in these conditions, the competition for light is reduced or absent, respectively, a significant increase of the biomass productivity is allowed (Abreu et al. 2012).

5.4.1.4 Light Quantity and Light Quality for Enhancing Carotenoid Production

In the natural environment water evaporation i.e. a decrease of the height of the water column, is often accompanied by salt precipitation and crystallization. Consequently, light attenuation by the water column decreases (Kirk 1988) while the surface albedo increases (Varela et al. 2015). The upregulation of PtCyp97b1 and PtCyp97b2 by HL might be not as fast as *psy, pds, zep, vde* and *vdl* genes in the diatom *Phaeodactylum tricornutum* (Coesel et al. 2008) but it exhibits a similar transitory response that the P450 hydroxylase genes in the green algae *Haematococcus pluvialis* (Cui et al. 2014) and *Chlorella kessieri* (Yu et al. 2014). Actually, HL induces a burst of ROS that may trigger secondary carotenoid production such as astaxanthin (Zhekisheva et al. 2002; Lemoine and Schoefs 2010; Hong et al. 2015; Domínguez-Bocanegra et al. 2004) and is usually considered as a major factor triggering carotenoid production by microalgae (Lamers et al. 2010). All the genes but *ipi* coding for the enzymes involved in the MEP pathway are upregulated (Gwak et al. 2014). However, it is not always the case as demonstrated in Table 5.7 (see Table 5.5 for the synonymous

Table 5.7 Some examples of contrasted effects on HL on the accumulation of carotenoids

	Total carotenoids	Canthaxanthin	β-carotene	Lutein	Fucoxanthin
Chlorella fusca	+ (Duarte and Costa 2018)				
Synechococcus nidulans	+ (Duarte and Costa 2018)				
Acustodesmus sp.		+ (Grama et al. 2014)		− (Grama et al. 2014)	
Dunaliella salina			+ (Lamers et al. 2010)		
Tetradesmus obliquus	− (Gonçalves et al. 2019)			+ (Chan et al. 2013)	
Arthrospira platensis	− (Ajayan et al. 2012)				
Chlamydomonas reinhardtii	− (Wagner et al. 2016)				
Phaeodactylum tricornutum					− (Heydarizadeh et al. 2017, 2019)

Table 5.8 Rate limiting steps of the carotenoid biosynthesis. Both section of the biosynthetic pathways and the rate limiting steps can be visualized in Fig. 5.3

Organism	Biosynthetic section	Rate limiting step	Reference
Synechocystis sp. PCC 6803	β-carotene → zeaxanthin	β-carotene hydroxylation	Lagarde et al. (2000)
Synechococcus sp. PCC 7942	IPP → β-carotene	Phytoene desaturation	Chamovitz et al. (1993)
Haematococcus pluvialis	IPP → astaxanthin	Phytoene, lycopene and canthaxanthin formation	Gao et al. (2015), Su et al. (2014)

taxa). Recently, low light intensity has also been recognized as a mean to increase fucoxanthin accumulation in microalgae (Heydarizadeh et al. (2017), Heydarizadeh et al. (2019)) (Table 5.8).

Light intensity is certainly an important factor for controlling algae metabolism. Besides this characteristic, light quality is also a factor to consider because it reflects environmental modifications, to which photosynthetic organisms may react (Darko et al. 2014). The sensitivity to the wavelength requires sensors: pigments and photoreceptors (Hegemann 2008). Using specific illuminations, alone or in combination with white light, the synthesis of total carotenoids can be enhanced (Gonçalves et al. 2019). Blue light is activating the transcription of the genes involved in the carotenoid biosynthetic pathways (green algae: *Chlamydomonas*: Bohne and Linden (2002), *Volvox*: Kianianmomeni (2014); diatoms: Coesel et al. (2008)). Xu and Harvey (2019) have studied the impact of different LED lighting of the accumulation of β-carotene in different strains of *Dunaliella salina.* Beside the fact that the β-carotene accumulation capacity was strain dependent, the accumulation was the highest under red light (625–680 nm) or white light supplemented with red light. β-carotene increased transiently in blue-light grown cells transferred to red light (Xu and Harvey 2019). The cells accumulating the highest β-carotene concentrations were also those having the highest oxygen consumption and consequently the lowest photosynthetic activity (Xu and Harvey 2019). Interestingly, the trends of cellular phytoene, the β-carotene precursor, follows the same trends than β-carotene itself (Xu and Harvey 2019). The smaller rate of oxygen uptake compared to mitochondrial respiration suggests a function in directly coupling oxygen uptake and the exergonic reaction of plastoquinol oxidation with plastoquinone reduction by a phytoene/phytoene desaturase couple, to permit endergonic carotene desaturation without ATP involvement (Bennoun 2001). Bennoun (2001) and Salguero et al. (2003) suggested a connection between phytoene desaturation and chloroplastic oxygen dissipation through the plastoquinol:oxygen oxidoreductase. In *Dunaliella*, the connection would be controlled by the flux of red photons (Xu and Harvey 2019). The quality and quantity of the red photon would be sensed by phytochrome (Yokthongwattana et al. 2019; Schroeder and Johnson 1995; Bennoun 2001) and would involve the reduction of the transcription factor PIF1, an inhibitor of carotenoid biosynthesis (Yokthongwattana et al. 2019).

Beside the visible light, nonvisible light such as UV radiations, alone or/and as supplementing PAR can enhance carotenogenesis when compared with visible light. In *Dunaliella salina* and *Haematococcus pluvialis*, UV-C (200–280 nm) irradiation triggers the accumulation of secondary carotenoids, a phenomenon that is accompanied by the typical events of cell settlement i.e. detachment of the flagella and cell volume increase (Sharma et al. 2015). The studies performed with *Dunaliella salina* revealed that the UV-A (320–400 nm) effect might be related to the presence of a specific photoreceptor because neither UV-B (290–320 nm) nor blue light was as effective in triggering β-carotene accumulation (Jahnke 1999).

5.4.1.5 The Availability of Other Biochemical Pathways

As already explained, carotenoids are made of carbon atoms and therefore their accumulation requires a functional carbon metabolism. Indeed, this is crucial for the accumulation of astaxanthin molecules because nearly all the astaxanthin molecules that accumulate are esterified with one or two fatty acids (*Haematococcus pluvialis*: Lemoine et al. (2008); Schoefs et al. (2001); *Scenedesmus* sp.: Aburai et al. (2015), *Chromochloris zofingiensis*: Zhang et al. (2016)). Several reports have established that an active tricarboxylic acid cycle (Wang et al. 2014; Li et al. 2017; Wu et al. 2013; Recht et al. 2012, 2014; Zhekisheva et al. 2002) and an active biosynthetic pathway are *sine qua non* conditions for astaxanthin accumulation in *Haematococcus pluvialis* (Gwak et al. 2014; Chen et al. 2015; Lemoine et al. 2008; Schoefs et al. 2001; Hu et al. 2019). However, strains such as *Coelastrum* sp. HA-1 (Liu et al. 2013b) and *Ankistrodesmus* sp. (Kopecky et al. 2000) that accumulate 50% and nearly all their astaxanthin as a nonesterified form, respectively, have been described. This suggests that the mandatory character of the activity of the fatty acid biosynthetic pathway is relative. To get further insight in this biochemical control, Liu et al. (2020) studied the impact of externally added linoleic acid on the astaxanthin esterification in *Coelastrum* sp. HA-1. Interestingly, the presence of exogenously added linoleic acid downregulated the fatty acid biosynthetic pathway while stimulating the astaxanthin esterification. The authors concluded that the presence of a high level of nonesterified astaxanthin in this taxon results of a low activity of the fatty acid biosynthetic pathway and suggests that the regulation could operate through a fatty acid threshold that would be reached to allow astaxanthin accumulation. To obtain further insights on the involvement of the primary metabolism during the process of in vivo astaxanthin accumulation in *Haematococcus pluvialis*, Hu et al. (2020) used a metabolic approach that has revealed changes in the level of numerous metabolite including nucleotides, organic acids etc. (Table 5.9). In *Chromochloris zofingiensis*, another taxon accumulating astaxanthin, transcriptome analyses revealed the upregulation of the genes coding for astaxanthin biosynthesis enzymes whereas the genes coding enzymes involved in side pathways were down-regulated (Huang et al. 2016).

Table 5.9 Comparison of the metabolic changes occurring during the astaxanthin accumulation under high and low light (from (Hu et al. 2020)

Family of compounds	Compounds	Changes during astaxanthin accumulation	
		High light	Low light
Nucleotides	Most	↓	↓
	Inosine	No change	No change
Carbohydrates	Glucose, glucose-6-phosphate, glyceraldehyde, erythrose, xylose, deoxyribose, rhamnose	↑	↑
Organic acids	2-oxoglutarate, succinate	↓	↓
	Malate	No change	↓
Proteogenic amino acids	Alanine, arginine, asparagine, aspartate, isoleucine, leucine, methionine, proline, serine; tryptophane, tyrosine	↓	↓
	Glutamic acid	↑	↑
Nonproteogenic acid	Pyroglutamic acid, glutathione, pipecolic acid	↑	↑
Lipids	Traumatic acid, traumatin, sn-1 lysophosphatidylcholine, sn-1 lysophosphatidylethanolamine	↓	↓
	Decanoid acid, trihydroxy octadecadienoic acid	↑	↑

5.4.1.6 Signalling

In the above sections, the effects of various stresses are described. Once the stress is sensed by the dedicated receptors, the information must be sent to the targets concerned in order to trigger the appropriate response. This section is dedicated to the ways in which stress messages are conveyed.

Under nonstressful conditions, the rate of ROS formation and scavenging are in balance and ROS does not accumulate within cells (Dring 2005). This equilibrium is compromised under stress conditions while ROS accumulates intracellularly. There are several lines of evidence such as the inhibition of astaxanthin production by *Haematoccocus pluvialis* under high light in the presence of exogenously added glutathione, a strong antioxidant (Hu et al. 2020) that validate the hypothesis according to ROS, especially, $^1O_2$ are involved in the regulation of astaxanthin accumulation process (Fan et al. 1998; Lemoine and Schoefs 2010; Baxter et al. 2014).

The status of phytohormones is not clear in microalgae. Several receptors for auxins have been identified in microalgae (Alsenani et al. 2019) whereas others have established that the exogenous application of phytohormones impacts microalgal physiology (Li et al. 2015; Jiang et al. 2015). Unfortunately, the results are often

contradictory (Table 5.10), suggesting that the effects are taxon dependent. According to Chen et al. (2020) indole-3-propionic acid (IPA) was the most effective phytohormone to stimulate the accumulation of astaxanthin in *Chromochloris zofigiensis* (49% increase). Other phytohormones such as IPA and abscisic acid (ABA) had a similar effect but accompanied by a depressed biomass production. The fact that phytohormones such as gibberellic acid, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) promoted astaxanthin but not lipids suggests that these phytohormones may be used for enhancing free astaxanthin accumulation in the absence of the usually synergetically biosynthesis of lipids (Schoefs et al. 2001; Chen et al. 2020). To summarize, the real role of phytohormones in carotenoid accumulation would be more in coordinating the efficient production of astaxanthin and lipids in *Chromochloris zofigiensis* (Chen et al. 2020).

5.4.1.7 Elements of Regulation

A few data are available on the regulation mechanisms of the carotenoid biosynthesis. Classically, transcriptional, post-transcriptional and biochemical factors are all involved in the regulatory network. For instance, high levels of white light (1000 μmol m^{-2} s^{-1}) induced a contrasting modifications of the mRNA level corresponding to the carotenoid hydroxylase Cyp97a1 of *Chlorella kessleri*; it decreased at the early stage of the treatment before a strong increase when the treatment was continued after 10 h (Yu et al. 2014). The amount of carotenoids varied concomitantly with the mRNA level (Yu et al. 2014).

Red light lowers the concentration of PIF1 transcription factor, a repressor of carotenoid biosynthesis (Salguero et al. 2003).

The rate limiting steps of several sections of the carotenoid biosynthetic pathways have been identified (Table 5.8). For instance, in the cyanobacterium *Synechocystis* sp. PCC6803, β-carotene hydroxylase is the rate limiting step of the conversion of β-carotene to zeaxanthin, the regulation being exerted by the availability in the enzyme itself (Lagarde et al. 2000). Despite the fact that it was reported that phytoene desaturase constitutes the rate limiting step in *Synechococcus* sp PCC 7942 (Chamovitz et al. 1993), the heterologous overexpression of *crtP* in the cyanobacterium *Synechocystis* sp. PCC 6803 did not yield to an increase of the carotenoid cellular quota (Lagarde et al. 2000). However, the overexpression of both *crtP* and *crtB* resulted in an increase of the carotenoid content by 50% (Lagarde et al. 2000), suggesting that the two enzymes form a complex in which the product of the first reaction is funnelled to the second enzyme. The limiting steps of astaxanthin biosynthesis are phytoene, lycopene and canthaxanthin synthesis (Su et al. 2014; Gao et al. 2015).

Carotenoid accumulation under stress conditions may require de novo enzyme biosynthesis as in the case of astaxanthin accumulation in *Haematococcus pluvialis* (Gwak et al. 2014; Hu et al. 2019; Schoefs et al. 2001).

Table 5.10 Summary of the effects of phytohormones on carotenoid production and associated processes

		Phenomenon								
		Autofluorescence	Biomass	Carotenoid accumulation	Astaxanthin	Lipid accumulation				Transcription of astaxanthin genes
Auxin	Indole-3-acetic acid (IAA)	++	++		++					
	Indole-3-butyric acid (IBA)	++								
	2,4-dichlorophenoxy acetic acid (2,4D)	+								
	IPA	–	–	+	++	+	–		–	
	Kinetin (KT)	–								
	Ethanolamine (ETA)	–						+		
	Diethyl aminoethyl hexanoate (DA-6)	–								
Gibberelin	Gibberellic acid (GA)	+			+				–	+
	1-naphthoylacetic acid (NAA)	–								
	Abscissic acid (ABA)	+	–		++				–	
Ethylene	1-aminocyclopropane-1-carboxylic acid (ACC)	–		+		+	–			
	2-chlorobenzoic acid (CA)	–		+		+	–			
Taxon		*Chromochloris zofingiensis* (Chen et al. 2020)	*Chromochloris zofingiensis* (Chen et al. 2020)	*Scenedesmus quadricaula* livet (Liu et al. 2016)	*Chromochloris zofingiensis* (Chen et al. 2020)	*Crypthecodinium cohnii* (Li et al. 2015)	Cyanobacteria (Anahas and Muralitharan 2019)	*Crypthecodinium cohnii* (Cui et al. 2018)	*Chromochloris zofingiensis* (Chen et al. 2020)	*Haematococcus pluvialis* (Gao et al. 2013)

5.5 Genetic Engineering

The amount of carotenoid produced by microalgae can be also enhanced using genetic engineering. Theoretically, these methods allow to combine the quality aspects of carotenoid production i.e. the type of carotenoid and the quantity through cloning in adequate producing organisms (e.g. bacteria: Takemura et al. (2019), fungus: Misawa and Shimada (1998), Miura et al. (1998), microalga: Leon et al. (2007)). First trials to produce carotenoids using genetic engineering consisted in expressing genes various types of organisms. For instance, the β-carotene ketolase and β-carotene oxidase from *Haematococcus pluvialis* were expressed in a strain of *Escherichia coli* producing β-carotene and zeaxanthin (Harker and Hirschberg 1997), the cyanobacterium *Synechococcus* PCC7942 (Breitenbach et al. 1996) and the land plant *Arabidopis* (Zhong et al. 2011). In *Chlamydomonas reinhardtii*, the expression of the *Haematococcus pluvialis bkt* gene led to transgenic strains able to synthesize keto-carotenoids not present in the wild strain (Leon et al. 2007). The overexpression of a mutant version of the *pds* gene in *Chromochloris zofingiensis* and *Haematococcus pluvialis* led to increased levels of total carotenoids (+32% for *Chlorella*) and astaxanthin (+54% for *Chlorella* and +26% for *Haematococcus*) in transformants (Liu et al. 2013a, Steinbrenner and Sandmann 2006). Expression of the endogenous nuclear *pds* gene in the chloroplast of *Haematococcus pluvialis* showed up higher astaxanthin accumulation (Galarza et al. 2018). *Chlamydomonas* strains overexpressing exogenous *psy* genes (from *Dunaliella salina* and *Chromochloris zofigiensis*) were able to display a 2 fold increase in lutein level (Couso et al. 2011; Cordero et al. 2011). However, not all microalgal cloned genes are active in bacteria. For instance, PtZEP1, coding zeaxanthin epoxidase in the diatom *Phaeodactylum tricornutum* does not show any activity in *Escherichia coli* (Eilers et al. 2016). The homologous expression of cyanobacterial genes was also tested. For instance, Lagarde et al. (2000) reported the increase of zeaxanthin and other carotenoid production in engineered *Synechocystis* sp PCC6803. Recently, Ye and Huang (2019) reported the selection of mutants of *Chromochloris zofingiensis* ATCC 30412 using a random mutagenesis strategy. The mutants accumulate only traces of canthaxanthin and astaxanthin unless they are grown mixotrophically on glucose as an alternative carbon source. In these conditions, the content of astaxanthin was reduced when compared to the wild-type but the amount of zeaxanthin was 7–11 higher.

5.6 Conclusion and Perspectives

The production of carotenoids by microalgae requires an optimized growth medium that might be taxon specific and biotechnological processes for. For instance, the production of astaxanthin by *Haematococcus pluvialis* is a two-step process because the stresses triggering astaxanthin accumulation inhibit cell division. The biomass

that is produced during the first step is stressed for accumulating astaxanthin. Consequently, two growth media might be required. For instance Nahidian et al. (2018) optimized a growth medium for the biomass production of *Haematococcus pluvialis* TMU1 while Azizi et al. (2020) optimized a medium for astaxanthin production by *Haematococcus pluvialis* NIES 144 and *Haematococcus pluvialis* UTEX 2505. The optimization of the growth medium includes the selection of an adequate CO_2 source and/or aeration system. The optimization of all these parameters allowed Azizi et al. (2020) to increase significantly the astaxanthin production by *Haematococcus pluvialis.* It is generally assumed that sodium carbonate is more economically feasible because it is transported in an easier manner than gaseous carbon dioxide and it exhibits a higher solubility than carbon dioxide (Hsueh et al. 2007) allowing saturation of the growth medium in DIC (Sampathkumar and Gothandam 2019).

An often overlooked parameter in microalgal biotechnological at large scale is the changing weather conditions, not only the light-dark cycle and the seasonality but also the unpredictable and daily variations. Zhang et al. (2020) reported that in Yunnan Province (China) the light intensity can reach 2000 $\mu mol\ m^{-2}\ s^{-1}$ at noon but the frequent rains and cloudy conditions reduce it at 150 $\mu mol\ m^{-2}\ s^{-1}$ for long period, therefore affecting astaxanthin production. To overcome this difficulty, authors have suggested the use of organic carbon source as its utilization does not rely on the photosynthetic activity.

Carotenoids are mostly hosted in the photosynthetic membranes where they serve to harvest light and/or as antioxidant molecules. In eukaryotic organisms, the photosynthetic membranes are located in the chloroplasts (Solymosi 2012). When Cars accumulate under stress, they are associated with lipids and proteins into droplets that accumulate in the chloroplasts (β-carotene: Ben-Amotz et al. (1982); Derwenskus et al. (2019)) or in the cytosol as in *Haematoccoccus* sp. (Lemoine et al. 2008). The hydrophobic nature of carotenoids as well as their cellular localization lowers the efficiency of the extraction procedures. This, together with the high cost of the downstream processes (Vinayak et al. 2015; Postma et al. 2016) have slowed down the exploitation of microalgae as a source of carotenoids. To accelerate this development, alternative extraction methods are developed. Supercritical CO_2 extraction, sometimes using a co-solvent (e.g. Chronopoulou et al. (2019)), pressurized extraction (Derwenskus et al. 2019), ultrasound (Plaza et al. 2012), microwaves (Pasquet et al. 2011) and pulsed electric fields (Parniakov et al. 2015) are among these possibilities. Nevertheless, these methods are destructive for the biomass and energy consuming and the generated wastes need to be treated (Vinayak et al. 2015). To reduce the impact of the biotechnological processes on the environment, biorefinery and biocompatible extraction concepts have been proposed. Both propose a holistic view of blue biotechnology based on microalgae.

Clearly, carotenoids are among the molecules with an attractive future. Although carotenoids are common molecules found in microalgae, they can be over accumulated in cells using environment constraints. Despite of this potential, there are not enough data to make microalgae a real platform for the production of carotenoids. More studies should be dedicated to the different aspects of the regulation of

the carotenoid biosynthesis, this knowledge being crucial for the development of biotechnological processes aiming at carotenoid production by microalgae.

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Chapter 6
Analytical Protocols in Chlorophyll Analysis

Isabel Viera and María Roca

Abstract Due to the importance of chlorophyll in phytoplankton, there are a number of analytical protocols. In this regard, the current chapter aims to be a practical guide for scientists interested in the description of the methodologies applicable to studying chlorophylls in phytoplankton. The complex pigment profile and the intricacies of pigment extraction from phytoplankton are the two main challenges encountered during chlorophyll analysis. The cell walls of certain species hinder the solvent capacity. For instance, Cyanobacteria and certain chlorophytes are termed *recalcitrant.* The current chapter will review (a) the different techniques that have been applied to guarantee the exhaustive chlorophyll extraction from microalgae and cyanobacteria, (b) the methods employed for chlorophyll analysis: which are often analyzed by liquid chromatography coupled with diode array detector (DAD). Considering the different structure of the chlorophyll derivatives present in phytoplankton, diverse chromatographic methods have been optimized. A deeper analysis has been achieved through the mass spectrophotometric (MS) studies of chlorophyll compounds of main microalgae and cyanobacteria, including the analysis of the fragmentation pathways to increase consistency of the identification. However, the disposal of appropriate standards is essential in chlorophyll investigation irrespective of the employed method of analysis. Only few chlorophyll standards are commercially available, and consequently protocols for yielding of the others have been specifically developed. Finally, the chapter will summarize the main chromatographic, spectroscopic properties, and MS characteristics of all chlorophyll present in phytoplankton.

Keywords Bacteriochlorophylls · Chlorophylls · Chlorophyll extraction · HPLC · Identification chlorophylls · Mass spectrometry

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6.1 Introduction

Following the definition of Scheer (2006), chlorophyll can be defined as cyclic tetrapyrroles with a characteristic isocyclic five-membered ring and having an active role during photosynthesis: and/or in light harvesting or in charge separation. In addition, chlorophyll usually has a magnesium atom as the central metal and a phytol chain ($C_{20}H_{40}$) esterified propionic acid at C-17. However, this rule is not mandatory and in nature there are exceptions to both structural properties. This chapter follows the IUPAC-IUB numbering system showed in Fig. 6.1.

Phytoplankton is a broad term that includes taxonomically very diverse organisms. Consequently, under the point of view of chlorophylls, phytoplankton represent a highly diverse and rich group. In this sense, chemically, phytoplankton contain the three classes of macrocycle, in function of the different degree of unsaturation: porphyrin, chlorin, and bacteriochlorin. The porphyrin macrocycle (Fig. 6.2) is completely unsaturated and it is present in the different chlorophyll *c* of chromophyte algae and some prokaryotes. These chlorophylls exhibit an intense absorption in the blue spectral region (Soret band around 450 nm) and a relatively moderate absorption around the 620 nm (see below). Chlorophyll c_1, c_2, c_3, and [8-ethyl]-chlorophyll c_3 (previously known as MV-chlorophyll c_3) present a characteristic propionic acid at C-17. On the contrary, [8-vinyl]-phlide a (also known as MgDVP), [7-methoxycarbonyl]-8-vinyl-phlide a (former Chl-C_{CS170}), and MV-Pchlide exhibit an acrylic acid at C-17. Moreover, for chlorophyll c_3, [8-ethyl]-chlorophyll c_3 and [7-methoxycarbonyl]-8-vinyl-phlide *a*, an additional carbomethoxy substituent is observed at C-7 (Fig. 6.2). This six chlorophylls *c* are denominated acidics as the group at C-17 is not esterified in contrast with the chl *c*-monogalactosyldiacylglyceride esters (MGDG) (Garrido et al. 2000). In these compounds different chlorophyll *c* can be esterified with different fatty acids (FA). Until now, only two FA have been properly identified as 14:0/18:4 and 14:0/14:0 (Garrido et al. 2000; Zapata et al. 2001).

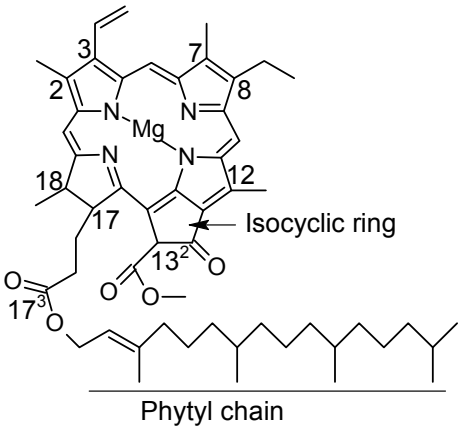

Fig. 6.1 Structure of chlorophyll *a* showing the numbering system for tetrapyrroles of the IUPAC-IUB

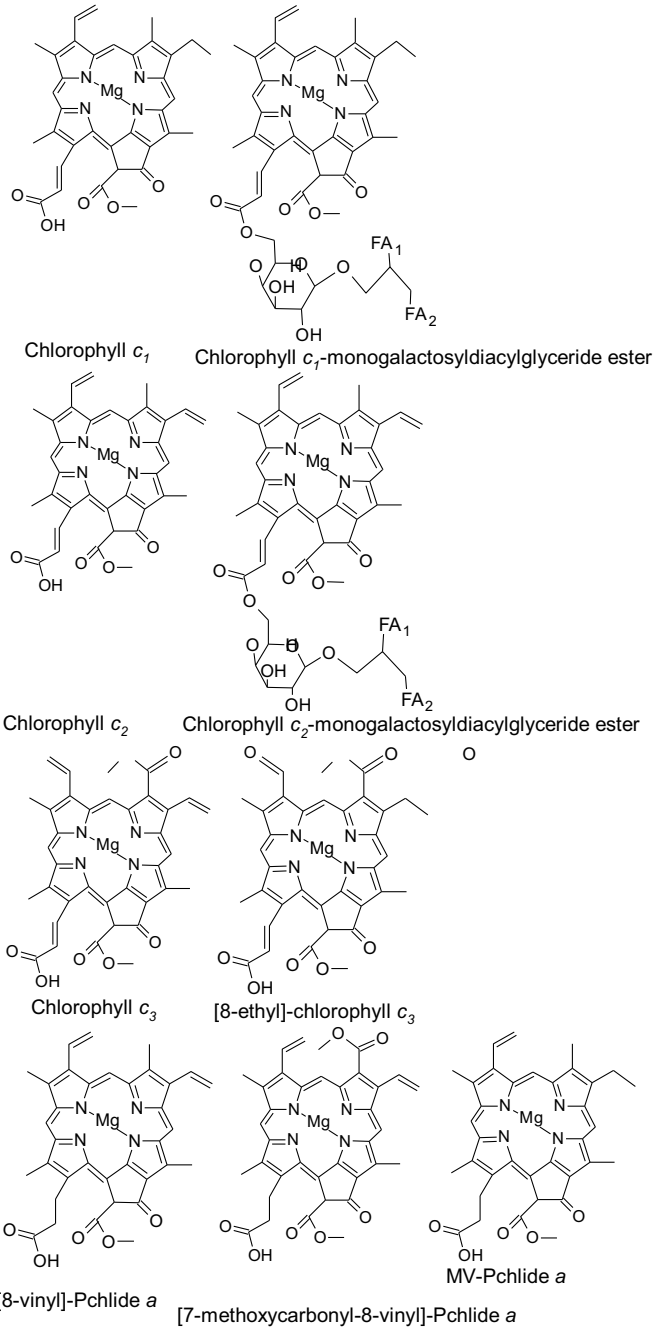

Fig. 6.2 Chlorophylls structures containing the porphyrin macrocycle present in phytoplankton. FA: fatty acid

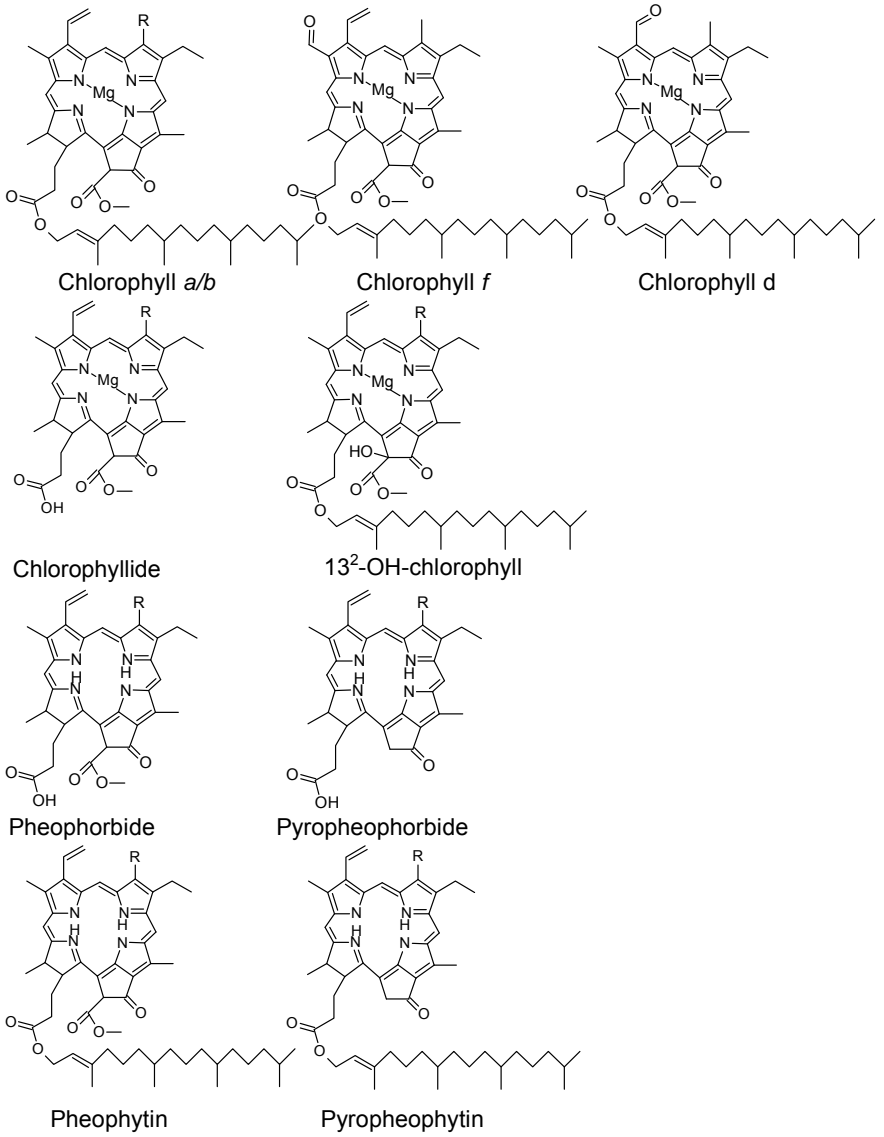

Fig. 6.3 Chlorophylls structures containing the chlorin macrocycle present in phytoplankton

The second type of macrocycle are the chlorins, chemically defined as 17,18-*trans*-dihydrophytoporphyrin (Fig. 6.1), which means an unsaturated bond exists between the C-17,18. Consequently, chlorophylls carrying a chlorin macrocycle typically presents two maxima, around 440 and 660 nm, with similar absorbance (Table 6.6). Chlorophylls *a*, *b, d,* and *f* (including their derivatives, Fig. 6.3) of oxygenic organisms as well as bacteriochlorophylls *c, d,* and *e* (Fig. 6.4) are formed with a chlorin

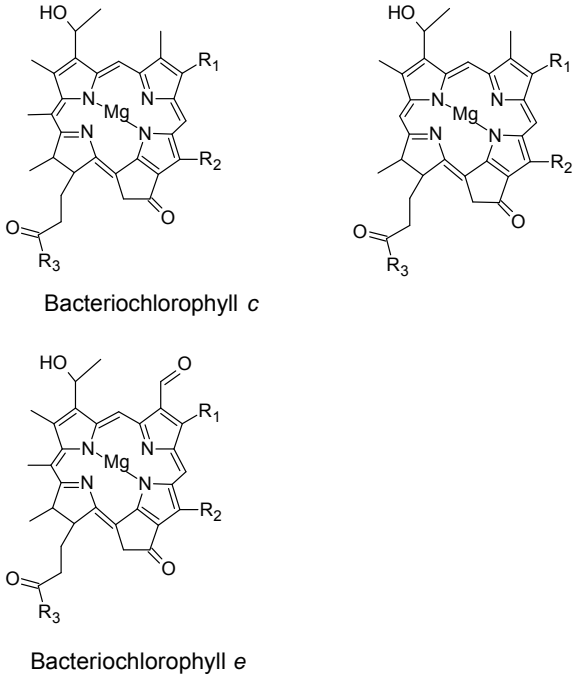

Fig. 6.4 Bacteriochlorophylls structures containing the chlorin macrocycle present in phytoplankton

macrocycle. Taking chlorophyll *a* as a model chlorophyll, it is seen that chlorophyll *b* differs from chlorophyll *a* by the presence of a formyl group at C-7, chlorophyll *d* exhibits the formyl group at C-3, and chlorophyll *f* contains the extra formyl group at C-2. Further, a number of other chlorophyll derivatives are formed in tissues as a result of natural metabolism. One of the main reactions is the de-esterification of the phytol chain at C-17. This is achieved enzymatically through chlorophyllase and/or pheophytinase. If the dephytylation takes place on chlorophyll, chlorophyllides are formed, but if the reaction takes place on pheophytin, then pheophorbides are obtained (Fig. 6.3). Other common reaction is the substitution of the central atom of magnesium of the tetrapyrrole by two atoms of hydrogen (Schwartz and Lorenzo 1990). This reaction takes place easily under acidic conditions, but also enzymatically (Shimoda et al. 2016). If the reaction proceeds from chlorophyll we have formation of pheophytins, while if it occurs from chlorophyllide then pheophorbides are formed. Pyro-derivatives (pyropheophorbides and pyropheophytins, Fig. 6.3) are formed by the loss of the carboxymethoxy group at C-13^2, probably due to the action of pheophorbidase enzyme (Suzuki et al. 2006). In addition, chlorophyll molecules are also prone to oxidation reactions, as the substitution of the hydrogen atom at $C13^2$ by a hydroxyl group, forming the 13^2-hydroxy derivatives (Fig. 6.3). The chlorin η-system is also present in bacteriochlorophylls *c*, *d*, and *e*, characteristics of green bacteria. These chlorins constituted a diversified group, as different substituents can

be found at C-8 (ethyl, n-propyl or iso-butyl), C-12 (ethyl or methyl group), and C-17[3] (farnesyl and other alcohols) (Fig. 6.4). All present a characteristic hydroxyethyl at C-3 and bacteriochlorophyll *e* an additional formyl group at C-7.

Finally, the third type of macrocycle is the bacteriochlorins or 7,8-*trans*, 17,18-*trans*-dihydrophytoporphyrin (Fig. 6.3). The double unsaturation (C-7,8 and C-17,18) determines the characteristic absorption spectrum with two high absorption maxima, one near UV (350–400 nm) and the second near IR (700–800 nm, approximately), and a lower band in the visible region (Table 6.6). This macrocycle can be found in bacteriochlorophylls *a, b,* and *g* of anoxygenic bacteria (Fig. 6.5). Bacteriochlorophyll *a* and *b* contain a characteristic acetyl group at C-3 and specifically bacteriochlorophyll *b* contains an ethylidene group at C-8. Bacteriochlorophyll *g*, characteristic of heliobacteria, present a characteristic farnesyl group esterified at C-17[3] instead of the typical phytol besides an ethylidene group at C-8 (Fig. 6.5).

Taking into account the extensive heterogeneity of chlorophylls in phytoplankton, chlorophyll determination has been always a primary necessity in oceanography. The Scientific Committee on Oceanic Research (SCOR) in conjunction with United Nations Educational, Scientific and Cultural Organization (UNESCO) created in 1966, the SCOR Working Group 17 aimed of the determination of photosynthetic pigments (UNESCO 1966). Later, in 1984 SCOR approved the working group 78

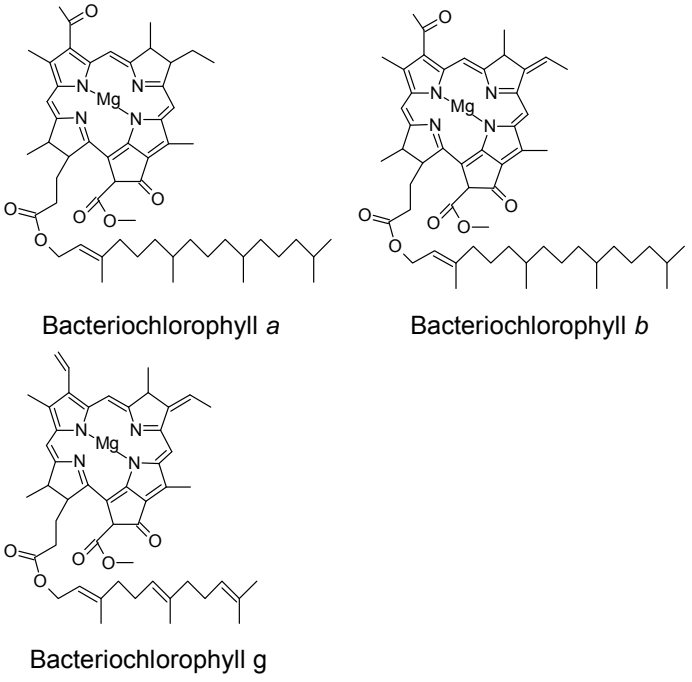

Fig. 6.5 Bacteriochlorophylls structures containing the bacteriochlorin macrocycle present in phytoplankton

(determination of photosynthetic pigments in seawater) which published in 1997 a monograph compiling the state of art, on determination of phytoplankton pigments (Jeffrey et al. 1997). In the recent times, the working group at SCOR (156) related to chlorophylls is dealing with the active chlorophyll fluorescence for autonomous measurements of global marine primary productivity (SCOR Working Group 156). Since then, different reviews or books have been published, compiling the knowledge on chlorophyll analysis in phytoplankton (Roy et al. 2011a, b).

6.2 Chlorophyll Extraction Methods from Microalgae and Cyanobacteria

Multiple methods have been proposed to extract and quantify chlorophyll pigments in phytoplankton, as no specific protocol is applicable to all the species known due to lack of applicability of a common protocol to all known species (Bowles et al. 1985; Cartaxana and Brotas 2003). The extraction efficiency depends on the resistance of the cell wall, the penetration power of the solvent and the solvation properties, the duration of the extraction, and the type of mechanical disruption used (Wright et al. 1997). Classically, the main and more generalized method used to extract intracellular chlorophyll from microalgae and cyanobacteria is the application of organic solvents. The organic solution penetrates through the cell membrane, dissolves lipids and lipoproteins of the chloroplast membranes, and facilitates the pigment extraction. Arnon (1949) was the first researcher to use acetone to extract chlorophyll *a* from higher plants for spectrophotometric measurements. Since then, as shown in Table 6.1, a variety of methods have been developed, each one for specific taxonomic groups.

Richards and Thompson (1952) successfully applied acetone to phytoplankton and provided the first equations for the spectroscopic quantification of this pigment from algae. These authors based their studies on the extraction of pigments in submerged algae and the excellent quantitative data obtained led to the recommendation of the method as a standard protocol by SCOR (UNESCO 1966). Since then acetone has become probably the most common solvent, although it is important to optimize the proportion of acetone:water (Mantoura and Llewellyn 1983). High amount of water during the extraction facilitates chlorophyllase activity (Wright et al. 1997), forming in vitro large proportions of chlorophyllides. This precaution should be taken with species with high chlorophyllase activity levels as in diatoms (Barrett and Jeffries 1971). In general, acetone is a better solvent to extract nonpolar chlorophylls.

Table 6.1 Different solvents options for chlorophyll extraction from phytoplankton

References	Solvents studied	Organism	Best option
Richards and Thompson (1952)	Acetone (90%)	Phytoplankton	Acetone
Mantoura and Llewellyn (1983)	Acetone/water 90:10 Acetone/water 1:1 Methanol (100%) Methanol/water 1:1	*Phaeodactylum tricornutum*	Acetone/water 90:10
Sartory and Grobbelaas (1984)	Ethanol (95%) Methanol Acetone (90%)	*Scenedesmus quadricauda, Selenastrum capricornutum, Microcystysis aeruginosa*	Methanol and 95% ethanol were superior to 90% acetone
Neveux (1988)	DMF Acetone	Phytoplankton	DMF
Suzuki and Ishimaru (1990)	DMF Acetone	Phytoplankton	DMF
Jeffrey et al. (1997)	Methanol (90%) Ethanol (90%) Ethanol (100%) DMF	Phytoplankton	DMF
Simon and Helliwell (1998)	Methanol Acetone	Freshwater algae *Selenastrum obliquus*	Methanol
Schumann et al. (2005)	Acetone, DMF	*Stichococcus Chlorella*	DMF
Hagerthey (2006)	acetone, (90%) methanol (90%) acetone/methanol/water (45:45:10) methanol/acetone/DMF/water (30:30:30:10)	Algae	Methanol/acetone/DMF/water
Macías-Sánchez et al. (2009)	DMF Methanol	*Dunaliella salina*	DMF

As an alternative, methanol has also been utilized for chlorophyll extraction. Different authors have found that methanol or ethanol (95%) when combined with different extraction methods such as homogenization, sonication, or boiling is more efficient than acetone (90%) alone (Sartory and Grobbelaas 1984). Advantages for the use of methanol (Wright et al. 1997): include, more acute HPLC peaks for polar chlorophylls and is greater efficiency for green algae (although less appropriate for freshwater cyanobacteria or diatoms). However, its use is restricted for it tends to degrade chlorophyll (Jeffrey et al. 1997; Mantoura and Llewellyn 1983). Different reactions over the chlorophylls in methanol-based solvents have been specifically

discussed in the work of Hynninen and Elfolk (1973): transesterification by chlorophyllase, epimerization reactions to form chlorophyll *a,* and if large amounts of algae are used, rapid formation (up to 40%) of allomeric forms and probably dichloric forms of chlorophyll *a* are produced.

Finally, several studies have explored the use of dimethylformamide (DMF) as an extraction solvent superior to methanol, ethanol, or acetone (Neveux 1988; Suzuki and Ishimaru 1990; Jeffrey et al. 1997). In this sense, DMF has been shown to be an efficient extractant for both phytoplankton (Neveux 1988; Suzuki and Ishimaru 1990; Suzuki et al. 1993; Furuya et al. 1998) and intertidal sediments (Honeywill et al. 2002). Specifically, DMF is recommended for cyanobacteria and coccoid green algae that are recalcitrant (Neveux 1988). Another advantage of DMF is that chlorophylls are highly stable in this solvent for weeks, allowing appropriate storage in the dark (Heyward 1991; Jeffrey et al. 1997; Schumann et al. 2005).

Independent of the solvent selection, the mechanical disruption of the cells significantly enhances the extraction in comparison with the simple soaking or immersion for several hours. Different cell disruption methods have been employed such as grinding (Schumann et al. 2005; Simon and Helliwell 1998), homogenization (Sartory and Grobbelaas 1984), or sonication (Simon and Helliwell 1998; Sartory and Grobbelaas 1984). Additional studies assess the convenience of freeze drying (lyophyllization) prior to solvent extraction (Hagerthey 2006). However, probably the conventional method prior to chlorophyll extraction is filtering the biomass followed by freezing. Although multiple types of filters can be used, the glass filter is most commonly used. Selection of pore size and quality is dependent on the phytoplankton characteristics.

Use of organic solvents for lipophilic extraction poses a serious threat to the environment. For example, the Montreal Protocol in 1987 proposed to restrict or to eliminate the manufacture and the use of ozone depleting solvents such as chlorofluorocarbons (CFCs). Since then, an increasing trend with high impact in the past few years is in the application of "green extraction techniques" that avoid the use of solvents (Table 6.2): supercritical fluid extraction (SFE), microwave assisted extraction (MAE), ultrasound-assisted extraction (UAE), assisted by pulsed electric field extraction (PEF), or extraction assisted by enzymes (EAE). Although these techniques have been applied preferentially for carotenoid extractions, several assays have been developed for chlorophyll extraction. SFE is a popular method with several advantages as the high purity of the extracting, the extraction requires less processing steps, is significantly safer than extraction with organic solvents and it can be operated at moderate temperatures to minimize the extract degradation. However, the chlorophyll extraction from microalgae using SFE depends on the fluid density which is a function of the operating pressure and temperature. Consequently, prior to its application it is necessary to optimize the conditions for a specific sample (Nobre et al. 2013). For example, the optimum conditions for *Nannochloropsis gaditana* (Macías-Sánchez et al. 2005) were 60 °C and 400 bar while for *Synechococcus sp.* (Macías-Sánchez et al. 2007) are 60 °C and 500 bar. MAE is an efficient method that takes advantage of microwave irradiation to accelerate the elimination of a variety of natural matrix compounds, causes direct generation of heat within the matrix, by friction

between polar molecules (Kaufmann and Christen 2002). The first assays showed that SFE is comparable with MAE, when methanol is used as a solvent (Macías-Sánchez et al. 2009), although the use of ultrasound facilitates the penetration of DMF in the cell membrane of microalgae, increasing the recovery of the pigments present in the raw material (Pasquet et al. 2011). UAE significantly reduces the extraction time and increases the extraction yields of many natural matrices, due to the production of cavitation bubbles in the solvent (Zou et al. 2013). PEF can be used to improve mass transfer processes, destroying cell membranes. Depending on the intensity, amplitude, duration, number, and frequency of repetition of the external in the membranes electric pulses, reversible or irreversible pores are produced. Formation of irreversible pores is of great importance for the extraction of bioactive compounds from natural matrices (Zbinden et al. 2013).

6.3 Methods for Chlorophyll Analysis

Certain investigations do not require the exhaustive determination of a complete profile or exact composition of chlorophyll derivatives. Sometimes it is necessary only to determine the amount of chlorophylls (*a, b, c,* and/or *d*). For such purpose, rapid spectrometric methods have been developed to calculate photosynthetic, metabolically active biomass, productivity of aquatic ecosystems, or the amount of chlorophyll per unit of protein (Ritchie 2006). Multiple algorithms and equations based on spectrophotometric and spectrofluorimetric techniques have been developed for routine assays based on up to three wavelengths (Jeffrey and Humphrey 1975; Porra et al. 1989, Rowan 1989), and reviewed by Porra (2006). More recently, the application of chemometric methods have allowed to use the complete spectrum (Neveux et al. 2011) to differentiate from the di- or trichromatic methods. This multivariate analysis enables the determination of a greater number of chlorophyll pigments. Another step is the application of artificial neural networks (Franco et al. 2019) to analyze microalgae spectral signatures from light absorption measurements with the aim to identify the prevailing strains. Re-training this promising methodology will reinforce the model.

However, the individual determination of chlorophylls requires the utilization of separation techniques, reverse phase HPLC coupled with DAD detector and less frequently with fluorescence detector, is the most commonly used application. A complete review of the HPLC methods for pigment analysis of phytoplankton was compiled by Garrido et al. (2011) and consequently this section mainly focuses on the innovations developed since then. Besides the new methods (Table 6.3), we also included the pioneering and original methods because the utility of these methods has been assayed for decades and also because they constitute the basis of the modern protocols. A practical protocol for the application of HPLC for characterization of pigments in phytoplankton has been recently published (Garrido and Roy 2015). Nevertheless, as stated before (Garrido et al. 2011) there is no perfect method of HPLC, and it is essential to adapt the method to the properties of the sample and the

Table 6.2 Green protocols for chlorophylls extraction from phytoplankton

Organism	Extraction method	References
Cylindrotheca closterium	MAE	Pasquet et al. (2011)
Dunaliella tertiolecta	MAE	Pasquet et al. (2011)
Cylindrotheca closterium	UAE	Pasquet et al. (2011)
Dunaliella salina	UAE	Macías-Sánchez et al. (2009)
Dunaliella tertiolecta	UAE	Pasquet et al. (2011)
Nannochloropsis gaditana	UAE	Macías-Sánchez et al. (2005) Macías-Sánchez et al. (2008)
Synechococcus sp.	UAE	Macías-Sánchez et al. (2007)
Chlorococcum littorale	SFE	Ota et al. (2009)
Dunaliella salina	SFE	Jaime et al. (2007) Macías-Sánchez et al. (2009) Macías-Sánchez et al. (2008)
Nannochloropsis sp.	SFE	Nobre et al. 2013
Nannochloropsis gaditana	SFE	Macías-Sánchez et al. (2005) Macías-Sánchez et al. (2008)
Synechococcus sp.	SFE	Montero et al. (2005) Macías-Sánchez et al. (2007) Macías-Sánchez et al. (2008)
Chlorella vulgaris	PLE	Santoyo et al. (2010)

aim pursued. Fundamentally, the analysis of chlorophylls from phytoplankton faces three problems: separation of monovinylic chlorophylls (chlorophyll *a*, chlorophyll *b*, chlorophyll c_1, and MV-chlorophyll c_3) from their counterparts divinyl chlorophylls (DV-chl *a*, DV-chl *b*, chl c_2, and chl c_3), separation of highly polar chlorophylls (chlorophyllide, pheophorbide, and different chlorophyll *c*), and separation between bacteriochlorophylls (mainly, bacteriochlorophyll *c*, *d*, and *e*).

Table 6.3 HPLC methods for chlorophyll analysis from phytoplankton

Authors	Column type	Particle size (μm)	Time (min)	Mobile phases	Detectors
Wright et al. (1991)	C18	5	20	A: methanol: ammonium acetate. B: acetonitrile:water C: ethyl acetate	DAD Fluor[a]
Garrido and Zapata (1997)	C18	5	25	A: methanol: acetonitrile: aqueous pyridine B:acetone	DAD Fluor
Zapata et al. (2000)	C8	3.5	40	A: methanol: acetonitrile: aqueous pyridine B: methanol: acetonitrile: acetone	DAD Fluor
Van Heukelem and Thomas (2001)	C8	3.5	30	A: methanol: tetrabutylammonium acetate B: methanol	DAD
Jayaraman et al. (2011)	C16	5	40	A: methanol. B: acetonitrile C: ammonium acetate	DAD Fluor
Sanz et al. (2015)	C18	3	40	A: methanol: ammonium acetate. B: ethanol	DAD
Zhang et al. (2016)	C8	1.8	15	A: ammonium acetate B: acetonitrile C: methanol	DAD
Suzuki et al. (2015)	C8	1.8	7	A: methanol: tetrabutylammonium acetate B: methanol	DAD Fluor

To address these problems, partly, different stationary phases are employed, classically the monomeric C8 (mainly for separation of chlorophylls with similar polarities) or the polymeric C18 (for chlorophylls with different molecular arrangements) is most commonly used (Table 6.3), with relatively good resolution. However, continuous developments are aimed to achieve complete separation. Jayaraman et al. (2011) for example, applied a C16 column, initially developed for pharmaceutical products, to improve the resolution. The palmitamidopropylsilane bonded phase column, less hydrophobic than the C18 columns, is compatible with aqueous mobile phases and suitable for the separation of MV and DV chlorophyll *a* and *b*. And more recently, Sanz et al. (2015) with a pentafluorophenyl column, instead of the alkyl-bonded

stationary phases, has resolved completely the separation between all the known MV and DV chlorophylls. In addition, there is a continuous reduction on the particle size of the columns, from the initial 5 μm, through the 3.5 μm to the present sub-2 μm particles. This reduction obeys to an increasing necessity to shorten the analysis time and increase the data throughput. Decreasing particle size implies increase in back-pressure, substituting the HPLC by UHPLC (ultra-HPLC), able to deliver pressures above 40 MPa, and generally preferred when coupled with mass spectrometry.

In relation with the mobile phases, the use of an ion pair agent is required to improve the separation between acidic chlorophylls (very polar dephytylated chlorophylls). Between the different ionic pairs, ammonium acetate and more recently, tetrabutylammonium acetate are the most popular reagents included in the different gradients (Table 6.3).

Additionally, advancements have been developed over the classical HPLC methods. As an example, Latasa (2014) has increased the sensitivity in reverse phase HPLC (RP-HPLC) by adding an extra loop to the HPLC system that should be equipped with an autosampler able of mixing and repeat injections. The idea is to pre-load the extra loop with alternative injections of sample and water and ejected into seat, repeating the process as many times required until the complete volume is injected (around 2400 μL). Applied in several investigations, authors advised increasing in the elution gradient and the importance of the mixing in the loop and not in separate vials. Other alternative to resolve pairs of chlorophylls that co-eluted in one peak is to use the first derivative spectrum technique (Suzuki et al. 2015). This method is based on the differences in pigment absorption spectra and it has been successfully applied to differentiate chlorophyll c_2 from MgDVP and chlorophyll *b* from DV chlorophyll *b*.

As certain chlorophylls exhibit the same absorption spectra, other chlorophylls are difficult to separate, and chlorophyll standards are scarce, the combination of HPLC with MS is a helpful tool that has been used for many years during the analysis of chlorophylls. Airs and Garrido (2011) compiled the main MS techniques employed to obtain the MS and MS^2 of the main chlorophylls in phytoplankton. Recently, this information has been updated in a special thematic issue of mass spectrometry of chlorophylls derivatives, specifically devoted to chlorophylls *c* (Gavalás-Olea et al. 2018), chlorophylls of phototrophic prokaryotes (Airs 2018), and chlorophyll *a* and *b* and its derivatives (Hynninen 2018; Viera et al. 2018).

For MS chlorophyll analysis different ionization techniques have been applied. Initially, fast atom bombardment (FAB) was a breakthrough methodology during the 1980s–90s, providing the first insights in the MS characterization of phytoplankton chlorophylls (Garrido and Zapata 1996). However, this technique has been overcome with softer ionization modes, with better signal performance. Although the matrix-assisted laser desorption (MALDI) spectrometry was employed during a long period of time, the nature of the matrix influenced the results. Recently, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the

more successful ionization modes applied to chlorophylls. In general, APCI shows better performance for phytylated chlorophylls, as the gas efficiency is higher for nonpolar compounds (Chen et al. 2015a), while ESI is more appropriate for polar (dephytylated) chlorophyll (Chen et al. 2015b). Chorophyll *c* is a special group that generates low-quality mass spectra, probably due to the inferior ionization efficiency of porphyrins compared to chlorins (Airs and Garrido 2011).

Similarly, different mass analyzers have been used successfully for chlorophyll elucidation: quadruples are often applied for the determination of known compounds, ion trap is highly suitable to elucidate new structures through MS^n analysis (Airs et al. 2014), and time of flight (TOF), whose excellent resolution properties makes it the ideal mass analyzer for accurate mass determinations in high-resolution MS (Gavalás-Olea et al. 2018). More recently, hybrid mass spectrometers allow to combine the advantages of two mass analyzers, as triple quadrupole (Zhang et al. 2016), QTOF (Maroneze et al. 2019), or quadrupole-orbitrap mass spectrometer (Freitas et al. 2019).

The application of the hyphenated HPLC-MS on chlorophylls has allowed not only the determination of the accurate mass and elemental composition of the corresponding different compounds (see below) but also to describe characteristic ion products that facilitate a rapid identification. Table 6.4 describes the main product ions described for chlorophylls present in phytoplanktons, associated with the corresponding structural assignment when known. A detailed description of the fragmentation during the MS and MS^2 studies can be found in the works of Gavalás-Olea et al. (2018), Airs (2018), Hynninen (2018), Viera et al. (2018). Besides specific fragmentations as the CO loss in chlorophyll derivatives from *b* series (Chen et al. 2015a), chlorophylls are commonly fragmented by the phytyl chain (or the alcohol esterifying at $C17^3$) followed by successive fragmentations through the propionic chain and at the carboxymethyl group at $C13^2$ level. Further fragmentations that implied the opening of the macrocycle have been detailed by Chen et al. (2015a, b).

6.4 Chlorophyll Standards: Obtaining Protocols and Commercial Suppliers

Few chlorophyll compounds are available commercially, marketed by five principal companies (Table 6.5). Frontier Scientific offers a specific section of porphyrins with more than 600 different compounds, while DHI lab is specialized on phytoplankton pigments. In general, working with chlorophylls requires a special attention for the presence or formation of chlorophyll allomers.

Alternatively, chlorophyll compounds can be isolated from natural sources, higher plants or phytoplankton. Specifically, standards of chlorophyll can be obtained from strains of reference algal cultures. Roy et al. (2011a, b) described an updated list of laboratory suppliers of SCOR reference cultures besides a detailed reference of

Table 6.4 Main characteristic product ions (*m/z*) of the chlorophylls present in phytoplankton

Chlorophyll	Product ions	Structural assignments
Chlorophyll c_1	$[M+H-22]^+$	$[M+H-Mg]^+$
Chlorophyll c_2	$[M+H-60]^+$	
Chlorophyll c_3	$[M+H-18]^+$	$[M+H-H_2O]^+$
[8-ethyl]-chlorophyll c_3	$[M+H-44]^+$	$[M+H-CO_2]^+$
	$[M+H-77]^+$	$[M+H-H_2O-CO_2CH_3]^+$
[8-vinyl]-phlide *a*	$[M+H-22]^+$	$[M+H-Mg]^+$
[7-methoxycarbonyl]-8-vinyl-phlide *a*	$[M+H-60]^+$	
	$[M+H-42]^+$	$[M+H-CH_2CO]^+$
DV chlorophyll *a*	$[M+H-278]^+$	$[M+H-phytyl]^+$
	$[M+H-278-60]^+$	$[M+H-phytyl-COCH_3OH]^+$
	$[M+H-278-32]^+$	$[M+H-phytyl-CH_3OH]^+$
Chlorophyll *d*	$[M+H-22]^+$	$[M+H-Mg]^+$
	$[M+H-22-278]^+$	$[M+H-Mg-phytyl]^+$
	$[M+H-22-278-60]^+$	$[M+H-Mg-phytyl-CO_2CH_3]^+$
Chlorophyll *f*	$[M+H-278]^+$	$[M+H-phytyl]^+$
Bacteriochlorophyll *c* Bacteriochlorophyll *d* Bacteriochlorophyll *e*	$[M+H-18]^+$	$[M+H-H_2O]^+$
	$[M+H-44]^+$	$[M+H-CH_3COH]^+$
	$[M+H-204]^+$	$[M+H-farnesyl]^+$
	$[M+H-272]^+$	$[M+H-geranylgeranyl]^+$
Bacteriochlorophyll *a*	$[M+H-278]^+$	$[M+H-phytyl]^+$
	$[M+H-60]^+$	$[M+H-CH_3HCO_2]^+$
	$[M+H-42]^+$	$[M+H-CH_2CO]^+$
Bacteriochlorophyll *g*	$[M+H-22]^+$	$[M+H-Mg]^+$
	$[M+H-204]^+$	$[M+H-Mg-farnesyl]^+$
Chlorophyll *a* Pheophytin *a*	$[M+H-278]^+$	$[M+H-phytyl]^+$
	$[M+H-46]^+$	$[M+H-HCO_2H]^+$
	$[M+H-60]^+$	$[M+H-CH_3HCO_2]^+$
Chlorophyll *b* Pheophytin *b*	$[M+H-278]^+$	$[M+H-phytyl]^+$
	$[M+H-46]^+$	$[M+H-HCO_2H]^+$
	$[M+H-60]^+$	$[M+H-CH_3HCO_2]^+$
	$[M+H-28]^+$	$[M+H-CO]^+$
Pyropheophytin *a*	$[M+H-278]^+$	$[M+H-phytyl]^+$
	$[M+H-46]^+$	$[M+H-HCO_2H]^+$
Chlorophyllide *a* Chlorophyllide *b*	$[M+H-60]^+$	$[M+H-CH_3HCO_2]^+$
Pheophorbide *a*	$[M+H-32]^+$	$[M+H-CH_3OH]^+$

(continued)

Table 6.4 (continued)

Chlorophyll	Product ions	Structural assignments
	$[M+H-58]^+$	$[M+H-CH_2CO_2]^+$
	$[M+H-76]^+$	$[M+H-CH_3CH_2HCO_2]^+$
Pyropheophorbide *a*	$[M+H-46]^+$	$[M+H-HCO_2H]^+$

Table 6.5 Commercial suppliers of chlorophyll standards

Chlorophyll standard	Commercial supplier
Bacteriochlorophyll *a*	Frontier Scientific Sigma Parchem
Chlorophyll *a*	Sigma DHI Wako Parchem
Chlorophyll *b*	Sigma DHI
Chlorophyll c_1	DHI
Chlorophyll c_2	DHI
Chlorophyll c_2-MGDC	DHI
Chlorophyll c_3	DHI
Chlorophyll *d*	Parchem
Chlorophyllide *a*	DHI
Divinyl chlorophyll *a*	DHI
MgDVP	DHI
Pheophorbide *a*	DHI Sigma Frontier Scientific
Pheophytin *a*	DHI Wako
Pyropheophytin *a*	Frontier Scientific
Pyropheophorbide *a*	Frontier Scientific

microalgal cultures recommended for new algal classes and prochlorophytes. Isolation employs an extraction followed by a separation. Chlorophyll standards can be segregated from other pigments, including epimers and allomerized counterparts, either by thin-layer chromatography (TLC) or by semipreparative HPLC.

For chlorophylls not commercially available or present in living organism in low amounts, it is necessary to develop specific protocols. For example, the preparation of dephytylated chlorophylls (chlorophyllide or pheophorbide) is based on an enzymatic de-esterification using partially purified chlorophyllase from high-activity fruits, as orange or pepper (Chen et al. 2015a). Magnesium-free derivatives (pheophytin and pheophorbide) are easily obtained from the counterpart chlorophylls in ethyl ether by acidification with two or three drops of 5 M HCl (Sievers and Hynninen 1977). Allomerized chlorophylls can be formed by oxidation of the corresponding parents with selenium dioxide (SeO_2) in pyridine (Laitalainen et al. 1990) at 70 °C for 3 h (Chen et al. 2015a). The reaction of decarbomethoxylation for pyro-derivatives production has been developed in pyridine at temperatures higher than 100 °C (Pennington et al. 1964). Although recently specific and smoother temperatures have been set up for different chlorophyll compounds (Chen et al. 2015a). After obtaining the standard, isolation of the chlorophyll compound of interest is an essential step which is achieved, either by TLC or chromatography, by open column or by HPLC.

6.5 Chlorophyll Identification: Chromatographic and Spectroscopic Properties of the Chlorophylls Present in Microalgae

The development or selection of a specific protocol for chlorophyll analysis requires the knowledge of the chromatographic and spectroscopic properties of the different chlorophylls. Table 6.6 shows, as example, the relative chromatographic peak elution order and absorption maxima for the main chlorophylls in phytoplankton. The data is an approximation and the exact information will depend on the chromatographic characteristics: HPLC column, solvents, gradient, etc., but in any case, it will help during the analysis.

Table 6.6 Identification of chlorophylls according to their elution order and spectral maxima absorption

Peak	Chlorophyll compound	Absorption (nm)	Elemental composition	Exact mass $[M+H]^+$
1	Chlorophyllide *b*	470 654	$C_{35}H_{32}MgN_4O_6$	629.2245
2	Chlorophyll c_3	460 638	$C_{36}H_{28}MgN_4O_7$	653.1881
3	[8-ethyl]-chlorophyll c_3	450 585	$C_{36}H_{30}MgN_4O_7$	655.2038
4	[7-methoxycarbonyl]-8-vinyl-phlide *a*	448 579	$C_{36}H_{30}MgN_4O_7$	655.2038
5	Chlorophyllide *a*	432 666	$C_{35}H_{34}MgN_4O_5$	615.2452
6	Chlorophyll c_2	450 630	$C_{35}H_{28}MgN_4O_5$	609.1983
7	Chlorophyll c_1	446 630	$C_{35}H_{30}N_4O_5$	611.2139
8	[8-vinyl]-phlide *a*	438 628	$C_{35}H_{30}MgN_4O_5$	611.2139
9	Pheophorbide *a*	410 666	$C_{35}H_{36}N_4O_5$	593.2759
10	Pyropheophorbide *a*	410 666	$C_{33}H_{34}N_4O_3$	535.2704
11	MV Protochlorophyllide *a*	435 631	$C_{35}H_{32}MgN_4O_5$	613.2296
12	Bacteriochlorophyll *a*	364 770	$C_{55}H_{74}MgN_4O_6$	911.5532
13	DV Chlorophyll *b*	480 655	$C_{55}H_{68}MgN_4O_6$	905.5062
14	13^2-OH-chlorophyll *b*	466 650	$C_{55}H_{70}MgN_4O_7$	923.5168
15	Chlorophyll *b*	466 651	$C_{55}H_{70}MgN_4O_6$	907.5219
16	Chlorophyll *d*	447 690	$C_{54}H_{70}MgN_4O_6$	895.5219
17	Chlorophyll *f*	406 705	$C_{55}H_{70}MgN_4O_6$	907.5219
18	Chl c_2-MGDG [18:4/14:0]	455 630	$C_{76}H_{96}MgN_4O_{14}$	1313.6846
19	Chl c_2-MGDG [14:0/14:0]	460 637	$C_{72}H_{96}MgN_4O_{14}$	1265.6846
20	Chl c_1-MGDG[a]	457 637		
21	13^2-OH-chlorophyll *a*	430 666	$C_{55}H_{72}MgN_4O_6$	909.5375
22	DV Chlorophyll *a*	442 666	$C_{55}H_{70}MgN_4O_5$	891.5269
23	Chlorophyll *a*	432 666	$C_{55}H_{72}MgN_4O_5$	893.5426
24	Pheophytin *b*	436 650	$C_{55}H_{72}N_4O_6$	885.5525
25	Pheophytin *a*	410 666	$C_{55}H_{74}N_4O_5$	871.5732
26	Pyropheophytin *a*	410 666	$C_{53}H_{22}N_4O_3$	813.5677
	Bacteriochlorophyll *b*	368 796	$C_{55}H_{72}MgN_4O_6$	909.5375
	Bacteriochlorophyll *c*[b]	432 666		
	Bacteriochlorophyll *d*[b]	427 665		
	Bacteriochlorophyll *e*[b]	469 654		
	Bacteriochlorophyll *g*	365 766	$C_{50}H_{58}MgN_4O_5$	819.4330

[a]At the moment no fatty acid has been identified. [b]Bacteriochlorophyll *c, d,* and *e* exhibit a diverse elemental composition and polarity in function of the substituents, but usually they are less polar than chlorophyll *a*

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Chapter 7
Analytical Protocols in Carotenoid Analysis

Antonio Pérez-Gálvez and Javier Fontecha

Abstract Carotenoids are key pigments in microalgae organisms where they develop significant functions related with the capture of light and other metabolic processes. Indeed, considering the actions carotenoids perform in mammals, with potential benefits to human health, the concept of microalgae as carotenoid food sources deserves a growing attention. Therefore, a great diversity of analytical protocols is available, although the striking structures that carotenoids arrange in microalgae, and the complex profile these organisms often show, still demand the application of *state-of-the-art* techniques for their isolation and identification. This chapter is focused on the analytical techniques applied for the exhaustive extraction of pigments from microalgae and, thereafter, the LC methods that allow the acquisition of the individual chromatographic and spectroscopic properties of the carotenoids. At this point the reader realizes that the identification of carotenoids requires further independent and complementary physicochemical properties through hyphenated LC systems with hybrid mass spectrometers.

Keywords HPLC-MS · Exhaustive extraction · Isolation · Identification · Analytical protocols

7.1 Introduction

There is a vast and unexplored diversity of valuable natural products that accumulate in marine organisms, which are becoming an attractive research subject at many different levels. Although marine resources have traditionally been exploited

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by many cultures, current demands of an increasing human population to face challenges related with new food supplies, climate change, health claims, and sustainable economy have driven the traditional use of marine stores to the application of *state-of-the-art* analytical procedures and technologies for (i) systematic screening of marine organisms to yield unraveled novel compounds, (ii) new drugs development, and (iii) *green* production of crude biomass for fractionation in target bioactive compounds. Among marine resources with a high socioeconomic impact factor, microalgae comprise a large group of species with enormous biochemical diversity and high plasticity and adaptability to different cultivation conditions and biological stimuli. Hence, microalgae-based products encompass food applications as sources of proteins (Becker 2007), polyunsaturated fatty acids (Singh et al. 2005), algal pigments (Prasanna et al. 2007), and minerals (Gouveia et al. 2009), or directly as dietary supplements; animal feed (Borowitzka 1997); fertilizers and soil conditioners; cosmetics (Bedoux et al. 2014); and health products (Cornish and Garbary 2010).

The isoprenoids represent more than 50% of the total secondary metabolism in algal organisms, including terpenes, steroids, prenylated quinones and hydroquinones, and carotenoid pigments. However, the key role of some of the latter as accessory pigments in photosynthesis makes them become primary metabolites associated with the cellular photosynthetic apparatus, while other carotenoid species are molded by the local environment or cultivation conditions and even some of them are biosynthesized to counteract external harmful stimuli. These different biosynthetic aims are determining factors for both the intracellular distribution and accumulation rate of primary and secondary carotenoids (Orosa et al. 2000; Huang et al. 2017). The occurrence and biosynthesis of carotenoids are out of the scope of this chapter and the reader is referred to the chapters in this handbook dealing with this topic (Chaps. 2, 4, and 5). Nevertheless, some points related to microalgae as source of carotenoids deserve attention in this chapter focused in the analytical protocols. The first one is the feasibility of microalgae to biosynthesize a relatively simple carotenoid profile under certain culture conditions. This is the case of the unicellular microalgae *Dunaliella salina* and *Dunaliella bardawil*, which are the common sources of β-carotene that reaches almost 12% in dry cell weight (Saini and Keum 2018). Other examples are the freshwater green algae *Haematococcus pluvialis* that in environmentally stressful conditions produces astaxanthin, reaching 5% in dry weight (Boussiba et al. 1999), and *Coelastrella striolata* and *Coelastrella zofingiensis* that under salt stress and nitrogen-deprivation cultivation conditions accumulate canthaxanthin (Abe et al. 2007). In these examples, the processing of the biomass for extraction concentrates most of the effort, either for analytical or commercial purposes, while the strains accumulating a complex carotenoid profile would require a comprehensive strategy for both processing for exhaustive extraction, and application of an analytical method for pigment characterization. Another issue with a potential impact on the performance of the analytical protocols is the intracellular location of carotenoids in microalgae. Those primary pigments accumulating in subcellular structures, i.e., light-harvesting complexes or membranes, are firmly attached to the membrane (Okulski et al. 2000; Sujak et al. 2000) performing

a rivet-locking function, while the secondary pigments accumulate in cytosolic lipid droplets, so that the method applied for exhaustive extraction would require different strategies. Analysis of carotenoids in microalgae encompasses several aims including microalgae chemotaxonomy—some pigments are characteristic of a strain, species, class or taxon-assessment of ocean productivity and changes in marine ecosystem, control of the targeted metabolic or genetic engineering approach for enhancing the production of a pursued bioactive compound, monitoring of the pigment metabolism and biosynthetic responses to different cultivation conditions, and high-throughput screening to unravel unknown or unprecedent carotenoid structures in microalgae species (Serive et al. 2017). Therefore, the comprehensive description of the analytical procedures applied in analysis of carotenoids in microalgae is a key issue with multidisciplinary objectives and the present chapter has been outlined according to this characteristic.

7.2 Analysis

7.2.1 Extraction

An efficient extraction of carotenoids from microalgae is achieved by considering the different problems that the isolation of the pigment (lipophilic) fraction from aquatic environments must face. Usually, a protocol based in just one single strategy for extraction is not successful because of the wide range polarity that carotenoid pigments display and the different opposition of the cellular environments to the force that drives extraction. Indeed, carotenoids are prone to oxidation mediated by temperature, light, and oxygen, as well as those degradative processes that may occur when the cellular content, grouped in subcellular structures, is mixed and homogenized in a single environment. Thus, acids, enzymes, and other compounds originally inert in the cells may promote transformation and/or degradation of the initial carotenoid content. Therefore, minimization of these processes and situations is advised to reduce the generation of artifacts and lack of correspondence with the natural carotenoid profile. To achieve this objective, the following good laboratory practices are recommended. Extraction time should be short, and the temperature should be monitored, controlling unnecessary excessive heating. Direct exposure of samples and extracts to sunlight must be avoided by working in a lab with diminished light or illuminated with yellow-light bulbs. Effect of cellular acids is neutralized by adding calcium carbonate to the extracting solvents, which might be supplemented with synthetic antioxidants (butylated hydroxytoluene, pyrogallol, ascorbyl palmitate) as well, at the 0.1–0.2% (w/v) range.

Another issue to consider in the extraction protocol is the application of a pretreatment step that may considerably facilitate the subsequent extraction activities.

Here, the objective is to disrupt the natural barriers (cell walls and organellar lipoprotein associations) to enhance the transfer of the lipids to the solvent(s) phase. Disruption could be performed by physical, chemical, or enzymatic methods and the choice is made according to the strength of the natural barrier, which is species-dependent. Hence, those microalgae with trilayered cell walls (*Haematococcus pluvialis*) need intensive, and high energy cost, procedures to produce successful disruption, while softer treatments are enough to achieve a high extraction yield in other species. According to literature, most of the disruption methods applied in the extraction of lipids from microalgae are physical as the use of chemical protocols involve the application of strong acidic or basic solvents that are unappropriated for carotenoid analysis, while the use of enzyme-based technologies is not cost-effective at the lab scale. Indeed, the reader should keep in mind that the techniques applied in the downstream processing for carotenoid isolation from algal biomass are not necessarily suitable for analysis at the lab.

Mechanical grinding is among the most commonly used techniques for cell disruption (Hu et al. 2013; Taucher et al. 2016). The solid shear produces mechanical damage in the cell tissues by collisions with the mortar side walls and among cells. This pre-treatment increases its efficiency when the biomass has been previously dehydrated (by filtering or freeze-drying). The disruption yielding of this procedure has been rated to 93% of cells (McMillan et al. 2013). Solid shear could be performed with a blender, rendering a fast disruption of the cells by physical collisions of the cells with the blades and among them, and when the blend is made in a stainless-steel flask, disruption is extended to the collisions with the side walls. This method is easily scaled-up for semi-pilot and industrial processing of biomass (McMillan et al. 2013). Mechanical grinding could be complemented at the lab with alternating cycles of ultrasonication, so that the extraction is even faster but monitoring of the temperature should be made at the ultrasonication step (Halim et al. 2013).

Other pre-treatment alternatives are available for routine in the lab, but their application is aimed for processing of the biomass at the industrial scale. Pulsed and moderate electric fields and high-pressure homogenization are physical methods for disruption of the cells and assist the extraction of lipophilic algal compounds. The technologies based on the application of electric fields require optimization of the conditions according to the cell characteristics (Vito et al. 2008) and even the intensity could be tailored to promote the selective extraction of the intracellular compounds (Carullo et al. 2018) and retention of their bioactivity value (Grimi et al. 2014; Sánchez-Moreno et al. 2005), although the extraction rates are lower in comparison with other pre-treatment alternatives (Lai et al. 2014). This is the case of high-pressure homogenization, a technology with a significant relevance for large-scale disruption of cells, and that yields a non-selective release of the cellular contents and small debris. This fact means a handicap for subsequent processing stages at the industrial level because purification of added-value compounds will require further separation processes (Balasundaram et al. 2009). Operating pressure, nozzle diameter, and number of passes are conditions that should be optimized to reduce the energy requirements and increase the performance of the disruption process, while the monitoring of the temperature is mandatory to avoid undesirable degradation

processes (Halim et al. 2013). Microwave is another technology applied for disruption of cells that reduces the amount of solvent required for extraction, but caution must be considered because of the heat that may cause degradation (Pasquetet al. 2011). Finally, the reader should notice that though modern processing technologies considerably reduce the amount of solvent required for efficient extraction, they still need revision of several key operative conditions (Kim et al. 2016), and that their use is not intended for the lab scale.

Once the pre-treatment process has been performed, extraction with organic solvent(s) is the next step where other critical factors should be considered. Selection of solvent or solvents mixture is made according to the frequently wide polarity range of the carotenoid profile. Hence, the occurrence of polar xanthophylls, and apolar carotenes and xanthophyll esters in the cellular contents demands the use of solvents combinations made with hexane, diethyl ether, ethanol, acetone, and others (Alfonsi et al. 2008; Soares et al. 2016). For industrial processing of biomass, other factors are considered such as sustainability and eco-friendly production, as well as health and safety issues. At the lab, ethyl acetate either alone or in combination with apolar solvents (hexane) fulfills the wide polarity range requirements for successful extraction of carotenoids (Strati and Oreopoulou 2011). Solvent mixtures from the protocols designed by Folch et al. (1957), Bligh and Dyer (1959), and Christie (1993) are equally recommended for lipid extraction from tissues including microalgae (Axelsson and Gentili 2014). These protocols are based in solvent mixtures of chloroform, methanol, and water, while the solvent to sample ratio could be adjusted considering the effectivity of the pre-treatment process applied for cell disruption.

Supercritical fluid extraction is a technology designed for extraction of valuable compounds from different raw materials including microalgae (Liau et al. 2010; Patil et al. 2013). Although its application is aimed at the semi-pilot and industrial scales, it is interesting for the reader to have a brief summary of this eco-friendly technique for extraction. The driving force of the supercritical fluid extraction is the application of high pressure to convert a gas (CO_2) into a fluid that still shows a viscosity as gas. In these conditions, the fluid penetrates the raw material achieving a high efficiency of mass transfer in a short time. Although supercritical CO_2 is an apolar fluid, this feature can be tailored by adding co-solvents (ethanol) that widen the polarity range of the extraction. Other conditions that are extremely favorable for carotenoid extraction are the low temperature conditions and the short time of the operation, while the final extract is completely free of extractant fluid as CO_2 backs to its gas state at normal pressure conditions (Zaghdoudi et al. 2016). Indeed, optimization of these conditions may produce a selectivity of the extracted compounds from the raw material and research activities have been focused in this aim (Macías-Sánchez et al. 2007; Kitada et al. 2009; Guedes et al. 2013; Goto et al. 2015; Reyes et al. 2016). This possibility points to the supercritical fluid extraction as a technology that potentially yields extracts with a high purity of the selected target compounds from microalgae. Another advantage is that the extraction procedure could be coupled online with the analytical technique, liquid or gas chromatography and even supercritical fluid chromatography, so that the extract is immediately analyzed and after data interpretation, the information could be feedbacked to optimize extraction conditions, allowing a

high-throughput screening. The features of this online coupling are detailed in the chromatographic analysis section.

Finally, a common derivatization process applied to the extract is chemical hydrolysis with ethanolic or methanolic KOH, in order to hydrolyze the xanthophyll esters to the corresponding free forms, reduce the content in triacylglycerides that interfere in the subsequent chromatographic analysis as well as the chlorophyll pigments, which are transformed to acyclic degradation products. Chemical hydrolysis is not suitable for screening the presence of xanthophyll esters and the determination of chlorophyll and carotenoid pigments in the same measurement.

To summarize this topic, extraction with solvent is the choice for lab processing of microalgae samples while the application of cold pre-treatment for disruption of cells is always recommended. In our laboratory the routinely applied protocol consists of filtering an aliquot of the biomass using a 47 mm glass fiber filter (Zhang et al. 2016; Maroneze et al. 2019). The filter containing the biomass is weighed and immediately froze at −80 °C. When it is grinded in a mortar with liquid nitrogen, the crushed filter material significantly increases the solid shear forces, while the liquid nitrogen releases any heat produced by the mechanical process and contributes to the disruption process. Subsequently, the powder is mixed with 10 mL of extraction solvent (9:1, *N*,*N*-dimethylformamide:water) under continuous stirring for 15 min at 4 °C, and then centrifuged (10,000 rpm, 4 min). The organic layer is accumulated in a decanting funnel and the solid residue is re-extracted with 10 mL hexane mixed in an ultrasonic bath (5 min, 720 W), vortexed (5 min), and after the addition of 10 mL NaCl solution (10% w/v) the mixture is centrifuged (10,000 rpm, 5 min). The supernatant is added to the funnel and the pellet is re-suspended with 10 mL diethyl ether, mixed in an ultrasonic bath (5 min, 720 W), vortexed (5 min), and finally centrifuged (10,000 rpm, 5 min) after the addition of 10 mL NaCl solution (10% w/v). The combined solvent fractions in the funnel are extracted with diethyl ether and NaCl solution (10% w/v). The upper phase is isolated and concentrated to dryness in a rotary evaporator. The residue is dissolved in acetone and stored at − 20 °C.

7.2.2 Spectroscopic Measurement

The use of spectrophotometry as a simple, fast, and low-cost technique for quantification of the pigment content in extracts is still a suitable analytical procedure recommended for some experiments. Hence, phytoplankton pigments in samples from sediments, natural lake aliquots, pure, or single species cultures can be spectrophotometrically monitored to estimate productivity and physiological and toxicological studies, as these measurements do not usually require an exact resolution of the pigment content (Kobayashi et al. 1993; Sairam et al. 2002; Marschall and Proctor 2004). Spectrophotometric methods are available in several fashions considering alternatives of solvents for the measurement and equations for determination

(Jeffrey and Humphrey 1975; Wellburn 1994; Porra 2006; Picazoet al. 2013). Basically, these methods determine the absorbance value at some wavelengths that are pigment(s)-specific peaks and taking the zero value from the region at 700–750 nm of the spectrum. The measured values are introduced in equations obtained empirically to determine the pigment content. Alternatively, some methods apply a refined-strategy to rebuild the total spectrum as a weighted sum of all the individual spectra (Naqvi et al. 2004; Küpper et al. 2000, 2007). This approach contains several biases as the peak-width variations and pigment peak positions, as well as the contribution of the background spectra to the total determination. These approximations could be improved by the application of alternative parametrization and fitting of the boundaries, and Monte-Carlo based simulations of the pigment composition to avoid aliasing of the samples, so that the predictions are closer to the real values (Thrane et al. 2015). The reader should evaluate whether these methods are appropriate to solve the scientific question they face. Sometimes it is convenient to sacrifice exhaustive description to gain in a fast comprehension and acquisition of analytical data, while statistical power could be increased by introducing many replicates, and still this technique is cost-effective. However, if the specific pigment composition is required to answer questions regarding biosynthesis, new or unprecedent structural arrangements arising from environmental conditions, and exhaustive identification and quantification, liquid chromatographic techniques are the gold standard procedures.

7.2.3 Liquid Chromatography (LC)

LC is the gold standard analytical technique for separation of the individual chlorophylls and carotenoids contained in the bulk pigment biomass, allowing their identification and quantification by examination of the data obtained from the observed chromatographic, spectroscopic, and mass properties, once the chromatographic method is coupled to UV-visible detection, and hyphenated with mass analyzers and/or nuclear magnetic resonance spectrometers. The development of the chromatographic method is made according to the polarity range of the pigments aimed to be individually isolated, so that a combination of stationary phase properties and both eluent composition and elution program is made to achieve a clear-cut separation and, subsequently, an unequivocal identification. Hence, reversed-phase stationary phases, i.e., C_{18} and C_{30} and modern C_{18} products (particles produced with "core-shell" morphology, or through organo-silica grafting processes, technologies that increase the performance of the separation) are the common choice for carotenoid analysis by HPLC. Indeed, a wide arrange of up-to-date column designs (combining different internal diameter and length of the column, and particle size of the stationary phase) allows the acquisition of chromatographic peaks with a high resolving power, which is a tremendous feature for hyphenation of HPLC with mass spectrometry. The reader should note that the extracts obtained from phytoplankton biomass, and particularly from cyanobacteria, contain chlorophylls and carotenoids and that both

families of pigments are biological markers of a wide array of essential indicators. Consequently, their simultaneous analysis is a fundamental piece for acquisition of useful data. However, the amount of chlorophyll derivatives and carotenoid types that potentially may co-exist in cyanobacteria, as well as their coincident polarity properties complicate their simultaneous analysis by HPLC. Coelution is a latent source of artifacts and equivocal identification and quantification. To avoid this problem several alternatives are available, although the shortcut solution is to hydrolyze the extract when the focus is solely the carotenoid profile (see Sect. 7.2.1). The analyst faces a different context when the target of the study is the chlorophyll profile. In that case, no technique has been developed so far to obtain a chlorophyll extract free of carotenoids, and the HPLC method must finely resolve the separation of both families of pigments (see Chap. 6).

The method developed by Wright et al. (1991) has been the benchmark in the measurement of carotenoids and chlorophylls from phytoplankton materials and the starting point for establishing alternative HPLC conditions with the same aim. Almost 50 different pigments may potentially separate in a C_{18} column using a ternary gradient system in a 30 min run. The quantification is made through the internal standard method, adding canthaxanthin to the extract. This is a handicap of the procedure as the internal standard co-elutes with zeaxanthin, when this xanthophyll is present in the carotenoid profile. However, the ability of this method to separate the substantial amount of pigments deserves the attention of the reader.

Table 7.1 contains the description of several HPLC approaches applied for separation of pigments from phytoplankton. These methods may be used by the reader as a reference for direct application, or to introduce improvements in analytical studies. The progress to be noted in this section is the progressive increasing number of procedures reporting the use of alternative columns to the classical octadecyl (C_{18}) packing material. Thus, napthylethyl or cholesteryl bonded silica (Indriatmoko et al. 2015) are recent stationary phases applied to the separation of photosynthetic pigments. Different interactions between analytes and stationary phase in pentafluorophenyl C_{18} silica column have been used as an advantage in the simultaneous analysis of chlorophylls and carotenoids, what it is very convenient in the analysis of green plankton populations and cyanobacteria (Sanz et al. 2015). Methods based in octyl silica (Zapata et al. 2000) and C_{30} columns (Guarantini et al. 2009) are also remarked considering their ability to produce chromatograms where carotenoid isomers and other carotenoids with similar structural arrangements are separated. Peak resolution of polar compounds is also improved with the application of a palmitamidopropylsilane bonded column that it is practical in the separation of complex pigment profiles (Jayaraman et al. 2011). The reader is also referred to the optimized method published by van Leeuwe et al. (2006), which was tested with a broad range of phytoplankton species to obtain a high sensitivity and reproducibility. Improvement of the chromatographic resolution was also the aim of the method published by Airs et al. (2001), particularly useful in the resolution of complex pigment profiles. Within all this milieu, the reader should clearly note that other methodological factors beyond the chromatographic conditions have a direct consequence in the ability of

Table 7.1 Selected chromatographic methods applied for the analysis of phytoplankton pigments

Sample type	Targeted pigments for analysis	Stationary phase	Mobile phase	Detection/Identification	References
Unialgal cultures	Carotenoids and chlorophylls	C_{18}-5 μm, 250 × 4.6 mm	Gradient; A: 80:20 methanol: 0.5 M aqueous ammonium acetate, pH 7.2 (v/v); B: 90:10 acetonitrile:water (v/v); 1 mL/min	UV/Vis: 436 nm	Wright et al. (1991)
Marine phytoplankton	Carotenoids and chlorophylls	C_8-3.5 μm, 150 × 4.6 mm	Gradient; A: 50:25:25 methanol:acetonitrile:0.25 M aqueous pyridine solution (v/v/v); B: 20:60:20methanol:acetonitrile:acetone (v/v/v); 1 mL/min	UV/Vis: 350–720 nm	Zapata et al. (2000)
Microbial mat and *Chlorobium phaeobacterioides* culture	Carotenoids and chlorophylls	Two in-line SpherisorbODS2-C_{18}-3 μm colums,150 × 4.6 mm	Gradient; A: acetonitrile; B: methanol; C: 0.01 M aqueous ammonium acetate; D: ethyl acetate; 0.7 mL/min	UV/Vis: 300–800 nm—coupled to ion trap and APCI[a] source	Airs et al. (2001, 2005)
Microalgae and cyanobacteria samples	Carotenoids and chlorophylls	C_{18}-3.5 μm, 150 × 4.6 mm	Gradient; A: 70:30 methanol:28 mM aqueous ammonium acetate (v/v); B: methanol; 0.8 mL/min	UV/Vis: 450 nm—coupled to Q-ion trap and APCI source/ESI interface	Frassanito et al. (2005, 2008)
Natural phytoplankton populations and microalgal cultures	Carotenoids and chlorophylls	C_{18}-5 μm, 150 × 3.9 mm	Gradient; A: 85:15 methanol:0.5 M aqueous ammonium acetate (v/v); B: 90:10 acetonitrile:water (v/v); C: ethyl acetate; 0.8 mL/min	UV/Vis: 400–750 nm	van Leeuwe et al. (2006)
Chlorella pyrenoidosa	Carotenoids	C_{30}-5 μm, 250 × 4.6 mm	Gradient; A: 84:14:2 methanol:acetonitrile:water (v/v/v); B: methylene chloride; 1 mL/min	UV/Vis: 450 nm	Inbaraj et al. (2006)

(continued)

Table 7.1 (continued)

Sample type	Targeted pigments for analysis	Stationary phase	Mobile phase	Detection/Identification	References
Phytoplankton monocultures	Carotenoids and chlorophylls	C_{16}-Amide-5 μm,150 × 4.6 mm	Gradient; A: methanol; B: acetonitrile; C: 0.5 M aqueous ammonium acetate; 1 mL/min	UV/Vis: 440, 450 nm—coupled to fluorescence: ex., 440 nm, em., 660 nm	Jayaraman et al. (2011)
Chlorococcum humicola	Carotenoids	C_{18}-5 μm, 250 × 4.6 mm	Isocratic; 20:70:10 dichloromethane:acetonitrile:methanol (v/v/v)	UV/Vis: 450 nm—coupled to Q and APCI source	Sivathanu and Palaniswamy (2012)
Dunaliella salina	Carotenoids and chlorophylls	C_{18}-1.8 μm, 150 × 2.1 mm	Gradient; A: 70:20:10 acetonitrile:methanol:*tert*-butyl-methyl ether; B: 10 mM aqueous ammonium acetate; 0.5 mL/min	UV/Vis: 450 nm—coupled to Q-TOF and ESI interface	Fu et al. (2012)
Isochrysis spp.	Carotenoids	C_{30}-5 μm, 250 × 3 mm	Gradient; A: methanol; B: 1:3 isopropanol:hexane (v/v); 0.5 mL/min	UV/Vis: 447 nm—coupled to ion trap and ESI interface	Crupi et al. (2013)
Scenedesmus protuberans	Carotenoids	C_{30}-5 μm, 250 × 4.6 mm	Gradient; A: methanol; B: methyl-tert-butyl ether; C: water; 1 mL/min	UV/Vis: 450 nm—coupled to Q^b-ion trap and APCI source	Erdogan et al. (2015)
Porphyridium purpureum	Carotenoids and chlorophylls	C_{18}-1.7 μm, 50 × 2.1 mm	Gradient; A: 0.01% formic acid in water; B: 0.01% formic acid in methanol; 0.4 mL/min	Q-TOF[c] and ESI[d] interface	Juin et al. (2015)

(continued)

Table 7.1 (continued)

Sample type	Targeted pigments for analysis	Stationary phase	Mobile phase	Detection/Identification	References
Microalgae samples	Carotenoids and chlorophylls	C_{18}-1.8 μm, 150 × 2.1 mm	Gradient; A: 70:20:10 acetonitrile:methanol:*tert*-butyl-methyl ether; B: 10 mM aqueous ammonium acetate; 0.5 mL/min	UV/Vis: 450 nm—coupled to TWIM[e]-Q-TOF and ESI interface	Pacini et al. (2015)
Selected marine phytoplankton cultures	Carotenoids and chlorophylls	C_{18}-Pentafluorophenyl-3 μm, 150 × 4.6 mm	Gradient; A: 82:18 methanol: 225 mM aqueous ammonium acetate (v/v); B: ethanol; 1 mL/min	UV/Vis: 320–720 nm	Sanz et al. (2015)
Selected marine phytoplankton cultures	Carotenoids and chlorophylls	UHPLC: C_8-1.8 μm, 50 × 4.6 mm HPLC: C_8-3 μm, 150 × 4.6 mm	Gradient; A: 70:30 methanol:28 mM aqueous tetrabutylammonium acetate, pH 6.5(v/v); B: methanol; 2 mL/min (UHPLC), 1.2 mL/min (HPLC)	UV/Vis: 350–700 nm	Suzuki et al. (2015)
Microalgae samples	Carotenoids	1-aminoanthracene-1.7 μm, 100 × 3 mm	Gradient; A: CO_2; B: methanol; 2 mL/min	UV/Vis: 430 nm—coupled to Q-ion trap and ESI interface	Jumaah et al. (2016)
Seawater samples	Carotenoids and chlorophylls	C_8-1.8 μm, 100 × 2.1 mm	Gradient; A: 0.03 M aqueous ammonium acetate; B: acetonitrile; C: methanol; 0.3 mL/min	UV/Vis coupled to triple Q and ESI interface	Zhang et al. (2016)
Microalgae and cyanobacteria samples	Carotenoids	C_{30}-5 μm, 250 × 4.6 mm	Gradient; 95:5 0.1% triethylamine[f] in methanol:*tert*-butyl-methyl ether (v/v); 0.9 mL/min	UV/Vis: 450 nm—coupled to ion trap and APCI source	Patias et al. (2017)
Biomass from microalgae strains	Carotenoids	C_{18}-5 μm, 150 × 4.6 mm	Gradient; A: 1:4 water:methanol (v/v); B: 1:1 acetone:methanol (v/v); 1 mL/min	UV/Vis: 360–700 nm	Cerón-García et al. (2018)

(continued)

Table 7.1 (continued)

Sample type	Targeted pigments for analysis	Stationary phase	Mobile phase	Detection/Identification	References
Microalgae and cyanobacteria samples	Carotenoids	C_{30}-3 μm, 250 × 4.6 mm	Gradient; A: 81:15:4 methanol:*tert*-butyl-methyl ether:water (v/v/v); B: 7:90:3 methanol:*tert*-butyl-methyl ether:water (v/v/v); 1 mL/min	UV/Vis: 450 nm—coupled to qQ-TOF and APCI source	Maroneze et al. (2019)

[a]*APCI* atmospheric pressure chemical ionization. [b]*Q* quadrupole. [c]*TOF* time of flight. [d]*ESI* electrospray ionization. [e]*TWIM* traveling wave ion mobility. [f]Triethylamine is included in the mobile phase when the analysis is performed with uncoupled UV/Vis detection

the method to reach high analytical standards. Indeed, several good laboratory practices are extremely recommended in the application of quality-assurance procedures, because this practice reduces the number of factors introducing uncertainty in the analytical data. Hence, the use of integrated extinction coefficients, application of batch quantification limit, statistical procedures applied for estimation of linearity of the calibration curves, and purity of the standards significantly increase the quality of the method and deserve attention of the analyst as well (Claustre et al. 2004).

The recent evolution of the column packings from spherical particles with diameter of 3–5 μm to sub-2 μm particles has produced an extraordinary increment in the efficiency of the liquid chromatography regarding time and cost of the analysis, and greater separation power and performance of the results. The design of such column products has been tied with the manufacture of instruments able to deliver pressures higher than the conventional 400 bar (Mazzeo et al. 2005). Some of the methods detailed in Table 7.1 operate under UHPLC conditions, while those annotated with HPLC settings are susceptible to be transferred to the UHPLC procedure with the use of convenient tools (most of them are available in the web sites of column dealers). The reader is also referred to specific publications regarding the theory and equations applied in the transfer of methods from HPLC to UHPLC conditions (Guillarme et al. 2007, 2008). As it was mentioned above, these developments are convenient in the hyphenation of liquid chromatography with mass analyzers to take full advantage of the resolving power and high sensitivity that features mass spectrometry. Some methods that were not specifically developed for carotenoid analysis in phytoplankton biomass should be considered by the reader, such as Guzman et al. (2012) aimed for determination of the photosynthetic pigments in *Brassica oleracea* vegetables, the chromatographic separation established by Li et al. (2012) that provides a complete scenario of different carotenoid isomers in tomatoes, and the method published by Chauveau-Duriot et al. (2010) that achieves the simultaneous determination of carotenoids and vitamins A and E in different food sources. Although the advances in UHPLC have attractive characteristics for the high-throughput screening of carotenoids, still the high selectivity of C_{30} HPLC columns deserves attention for thorough analysis of undetermined carotenoid profile (Bijttebier et al. 2014), which may occur in unraveled phytoplankton species. Nevertheless, column packings with C_{30} material developed for UHPLC conditions are also available (Zoccali et al. 2017). The reader will find valuable information in the work published by Turcsi et al. (2016) where a database containing the separation characteristics of carotenoids in C_{18} and C_{30} stationary phases is provided. Hence, the retention and elution order of *ca.* 90 carotenoids is detailed considering their structural features for hydrocarbons and polar carotenoids, optical isomers, geometrical isomers, and epoxy-carotenoids.

Application of multidimensional LC (LC × LC) has been proposed as an excellent alternative to analyze the carotenoid profile in complex extracts, particularly in the study of carotenoid esters. In this set-up, the extract is analyzed in the 2-D independently, by using a switching valve as a transfer system between the columns (Dugo et al. 2006, 2008a). Moreover, the number of analytical conditions increases as the set-up may include normal-phased columns coupled to reverse-phased ones,

C_{18} columns coupled to C_{30}, and different sequence of the columns for the first and second dimension, respectively. The LC × LC set-up is also very convenient for hyphenation with MS as demonstrated by Dugo et al. (2008b) whose were the first authors to couple LC × LC with MS detection for the analysis of carotenoids. Thus, the increased resolution of the LC × LC set-up is fully arranged with the identification power of the mass spectrometry (Giuffrida et al. 2018a, b).

To finalize this section, it is challenging to recommend a LC method among the different available alternatives. If the reader faces an unknown profile, the application of the reference method described by van Leeuwe et al. (2006) is advised, with the first screening of a pigments standard mix for an initial comparison between the known carotenoid profile and the one of the target sample. Thereafter, the application of a C_{30}-based method is recommended to ascertain the presence of geometric and *cis-trans* isomers.

7.2.4 Supercritical Fluid Chromatography (SFC)

Supercritical fluids have a pivotal role in different areas of chemistry, from operation units in sample preparation and isolation, to production processes and organic synthesis, although their application in analytical chemistry has experimented a discontinuous progress since the interest in the 80s decade of the last century and the decline by the start of the new millennium. The commercially available equipment capable of operating at high temperatures and pressures has not succeeded in routine chromatography in most of the labs where liquid chromatography systems, either GC, HPLC, or UHPLC are the standards for analytical separation. However, SFC has found specific niches where it shows potential advantage over LC systems, considering the faster analysis and the universal coupling with detectors, particularly with mass analyzers. Indeed, SFC has outstanding applications in the enantioseparation of chiral analytes and isomers, and in the effective separation of complex mixtures of structurally closely apolar compounds. Separation of *cis*/*trans* isomers of β-carotene has been achieved with this technique (Lesellier et al. 2003). Epoxy-carotenoids are difficult to isolate and distinguish from the corresponding hydroxy-carotenoids isomers, and some references have reported the successful application of SFC with that aim (Abrahamsson et al. 2012; Matsubara et al. 2012). Apocarotenoids also deserve attention, as they represent a current challenge in chromatographic studies (Zoccali et al. 2018). Last but not least, the reader should note the case of pigment extracts containing xanthophyll esters. Although the esterification takes place with different fatty acids for different xanthophylls, the amount of resulting combinations does not yield a wide range of polarity but a very narrow one, so that coelution and peak resolution are the absolute challenges for the analyst (Mariutti and Mercadante 2018; Zoccali et al. 2018). Indeed, experimental set-ups that include supercritical fluid extraction coupled directly to SFC and analysis by spectrophotometry and mass spectrometry have been developed for the analysis of carotenoids in complex matrices (Giuffrida et al. 2018a, b). These issues are also related with the complex carotenoid

profiles that potentially exist in phytoplankton biomass, and SFC may face the test to resolve the separation of the individual carotenoids (including xanthophyll esters) in such mixtures. Hence, some methods have been published dealing with SFC and analysis of carotenoids in microalgae (Abrahamsson et al. 2012; Jumaah et al. 2016). A suggestion for the reader is to consider the advantages of the SFC when coupling this separation technique with mass analyzers and nuclear magnetic resonance spectroscopy. The evaporation of the supercritical mobile phase containing the analytes is easily accomplished in the MS source, while the absence of protons in carbon dioxide yields "clean" NMR spectra.

7.2.5 Detection Methods and Quantification

7.2.5.1 UV-Visible

Spectrophotometric detection means the first look at the pigment profile. Independently of whether the separation has been fully achieved (coelution), UV-visible spectrophotometry is an elemental LC-based detection method. The features of the carotenoid spectrum mainly arise from the polyenoic conjugated chain yielding three peaks with characteristic λ_{max} values that allow the annotation of some structural arrangements of the carotenoid molecule. Thus, the number of conjugated double bonds is correlated with the location of the λ_{max} values with shorter wavelengths for lower conjugated systems of double bonds and vice versa. Subsequently, hypsochromic shift occurs when the spectra of acyclic and cyclic carotenoids with the same configuration of the polyenoic system are compared. Additionally, deflection of one of the maximum values arises with the consequent partial loss of fine structure (peak definition of the spectrum). Further modifications of the carotenoid structure, such as the introduction of hydroxyl groups, do not introduce changes in the spectra of carotenoids with the same polyenoic system, so that the distinction of carotenoids such as β-carotene (carotene), β-cryptoxanthin (monohydroxylated xanthophyll), and zeaxanthin (dihydroxylated xanthophyll) are equivalent). The esterification of the hydroxylated xanthophylls with fatty acids does not affect the spectrum of the parent free xanthophyll. The same "no-effect" in the spectra of carotenoids with carbonyl groups not conjugated with the polyenoic system is observed, but those structures where the conjugation takes place show a bathochromic shift of the spectrum and loss of the fine structure, so that a typical single broad curve is representative of the carotenoids with conjugated-carbonyl group(s), such as the spectra of canthaxanthin or capsanthin. The introduction of epoxide or furanoid groups produce a hipsochromic shift of the spectrum as the length of the polyenoic system is decreased. Table 7.2 contains the absorption maxima for selected carotenoids that appear in the pigment profile of phytoplankton biomass. The reader should take note that the UV-visible spectrum provides basic structural information but it does not assist in the identification of geometrical isomers or carotenoids with the same configuration of the polyenoic system, so that the information obtained from the UV-visible spectrum

Table 7.2 Product ions observed in the analysis by tandem MS-APCI source of representative carotenoids of phytoplankton biomass

Carotenoid	λ_{maxima} (nm) in ethanol[a]	Elemental composition	Molecular weight[a] $[M + H]^+$	Product ions grouped by fragmentation process[b, c]		
				In-chain	Polyene chain and terminal group	Decyclization
Astaxanthin	478	$C_{40}H_{52}O_4$	597.3938	505.3312	579.3830 561.3730	-[a]
Canthaxanthin	474	$C_{40}H_{52}O_2$	565.4040	473.3414	–	
α-Carotene	423, 444, 473	$C_{40}H_{56}$	537.4455	445.3829	413.3200	481.3830
β-Carotene	450, 478	$C_{40}H_{56}$	537.4455	445.3829	413.3200	481.3830
γ-Carotene	440, 460, 489	$C_{40}H_{56}$	537.4455	445.3829	467.3670	–
β-Cryptoxanthin	428, 450, 478	$C_{40}H_{56}O$	553.4404	461.3780	535.4300	495.3990
5,8-epoxy-Diadinoxanthin	447, 476	$C_{40}H_{54}O_3$	583.4157	491.3531	565.4040 221.1540	–
Diatoxanthin	425, 449, 475	$C_{40}H_{54}O_2$	567.4197	475.3571	549.4090	551.4250 533.4140
Fucoxanthin	426, 449, 475	$C_{42}H_{58}O_6$	659.4306	567.3680	641.4200 623.4090 599.4090 581.3990 221.1540	–
Lutein	442, 445	$C_{40}H_{56}O_2$	569.4353	477.3571	551.4250 533.4140 429.3150	–
Lycopene		$C_{40}H_{56}$	537.4455	467.3670 445.3829	413.3200	-[b]

(continued)

Table 7.2 (continued)

Carotenoid	λ_{maxima} (nm) in ethanol[a]	Elemental composition	Molecular weight[a] $[M + H]^+$	Product ions grouped by fragmentation process[b, c]		
				In-chain	Polyene chain and terminal group	Decyclization
Neoxanthin	418, 442, 471	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4150 565.4040 221.1540	393.2790
Violaxanthin	419, 440, 470	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4150 565.4040 221.1540	–
Zeaxanthin	428, 450	$C_{40}H_{56}O_2$	569.4353	477.3571	551.4250 533.4140 429.3150	–

[a]Maxima detailed in Britton et al. (2004). [b]product ions not yet experimentally observed. [c]in the acyclic carotenoids the fragmentation process involves the end-group. The product ions have been detailed by Airs and Llewellyn (2005), de Rosso and Mercadante (2007), van Breemen et al. (2012), Crupi et al. (2013), Rivera et al. (2014), Rodrigues et al. (2015), Maroneze et al. (2019)

must be paired with other physicochemical characteristics observed from the chromatographic analysis (retention time) and from the analysis of other spectral features (mass spectrometry or nuclear magnetic resonance).

One of the main applications of the UV-visible detection coupled to LC is the quantification of the pigment profile once the identification and acquisition of the adequate peak isolation have been secured. Quantification is made according to the external calibration procedure by the analysis of the corresponding standards that are present in the pigment profile samples. Calibration curves are built for each carotenoid as the spectrum features, extinction coefficient, and response in the chromatographic conditions are different, and this is a common source of error in the quantification methodology. It is frequent that some standards are not commercially available, or with expensive procedures for the isolation at the lab, so that quantification is made by using a calibration curve made for a different available carotenoid but resembling most of the spectral features of the unavailable one. Indeed, carotenoid standards are not necessarily pure and may contain isomers or impurities. Therefore, they should be conveniently checked before calibration and even re-purified by thin-layer chromatography or open-column chromatography (Kimura et al. 2007). The reader should refer to some laboratory guidelines for validation of the analytical procedures applied for calibration, quantification, and analytical methodology (U.S. Department of Health and Human Services 2001; ICH Expert Working Group 2005) and that we use in our lab. The calibration curves should be linear in the concentration range, which depends on the intended application, but must include the expected concentrations in the sample, while the correlation coefficients, y-intercepts, slopes, and residual sum of squares should be noted. The reader must consider that the concentration range of different carotenoids occurring in the same sample is generally wide, and calibration curves should be built according to this feature, while the statistical analysis must provide the corresponding significance power for each pigment. The limit of detection (LOD) is established on the signal-to-noise approach with LOD between 3 or 2:1 generally considered acceptable, while the limit of quantification (LOQ) is measured by the same approach and considering a 10:1 LOQ ration as acceptable.

As it was noted above, some carotenoid standards are commercially available and even can be obtained upon by-request if the targeted pigment does not appear in the catalogue of the dealer. Basically, the common carotenoids amarouciaxanthin A, astaxanthin, canthaxanthin, capsanthin, α-carotene, β-carotene, γ-carotene, β-cryptoxanthin, β-apo-8'-carotenal, crocetin, diadinoxanthin, diatoxanthin, fucoxanthin, isofucoxanthinol, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin are affordable as they occur in common sources or are reasonably produced by synthesis. Other less-frequent carotenoids are also available in specialized dealers (www.carotenature.com), including *cis-trans* isomers, apocarotenoids, stereoisomers, and esters. Additionally, some reference materials of mixed pigments are commercially available (www.c14.dhigroup.com) for controlling the performance of the HPLC/UHPLC method, for identification purposes, and validation of the technique.

7.2.5.2 Electrochemical Detection (ED)

ED is a detection technique suitable for those compounds with electroactivity and carotenoids are electroactive and HPLC-ED systems are an alternative for their analysis. The application of an electrical potential modifies the oxidation state of the analytes, yielding hydrodynamic voltammograms, which are featured for each compound. This technique is more sensitive than UV-visible detection but the number of artifacts and interferences (background noise produced by solvent phase) considerably increases. Analytes are destroyed during the analysis, so that coupling with a subsequent detection method is not allowed. Guaratini et al. (2009) revealed the carotenoid profile in phytoplankton biomass (microalgae and cyanobacteria) by HPLC-ED, and successfully compared the performance of this technique with UV-visible detection.

7.2.6 Identification Methods

7.2.6.1 Mass Spectrometry (MS)

MS is an analytical technique where the analyst must develop the *know-how* in different areas (hardware electronics, chemistry, experimental design, computing, and big data management), and apply the best laboratory practices to fully take advantage of the potential capabilities of this tool. In the field of carotenoids analysis, MS is mainly focused in the identification when spectrophotometry does not allow distinction between carotenoid structures with the same UV-visible spectra and similar chromatographic behavior, or when the acquisition of high-quality UV-visible spectrum is not possible in low concentrated samples. Indeed, those carotenoid profiles where unprecedent carotenoid structures or *unknowns* appear, and consequently the UV-visible spectrum does not provide enough information, MS is required for structural elucidation. The reader is referred to excellent revisions dealing with the fundamentals of MS and the progress of the hardware and *state-of-the-art* set-ups currently available, including those exclusively focused in carotenoids (Enzell and Wahlberg 1980; Britton et al. 1995; Dole 1997; de Rosso and Mercadante 2007; Rezanka et al. 2009; Rivera et al. 2011; van Breemen et al. 2012; Amorim-Carrilho et al. 2014), while this section is dedicated to the identification strategy.

The first information contained in MS spectra acquired through soft-ionization techniques (atmospheric pressure ionization) is the molecular weight of the protonated ion, denoted as $[M+H]^+$, which allows to filter the elemental composition among a huge amount of alternatives, *the candidates*, with the application of two filtering rules: mass accuracy and isotopic pattern. Thus, when the mass accuracy level is increased, the number of candidates is reduced, but still the list could be reduced by the application of additional physicochemical information such as the isotopic pattern. The experimental and theoretical isotopic patterns are compared, and a correlation value is obtained for each candidate. Subsequently, the analyst must

establish the threshold values for each filtering rule (mass error and isotopic pattern) and the first list of *potential candidates* is obtained. The lower the threshold values, the lower the number of potential candidates. The second dimension of data is then applied to filter that list, and to obtain structural information of the compounds. This is made by means of the tandem MS spectra that include product ions isolated from the original protonated ion. The same filtering rules (mass error and isotopic pattern) are applied to check the consistency of the product ions with the protonated ion, thus increasing the reliability of the annotated tentative identification and reducing the number of potential candidates initially included in the list, so that the second list of *filtered candidates* is obtained. Indeed, the product ions yield information regarding the structural features of the parent protonated ion, once their structure has been predicted for each one of the potential candidates included in the list of filtered candidates (Pérez-Gálvez et al. 2018). In this step, the use of predictive software has become a key tool to improve the accuracy of the formulations, and to reduce the labor time in the analysis of data. Hence, the list of theoretical product ions (qualifiers) predicted by the software for each of the filtered candidates included in the second list is compared with the experimental product ions. Therefore, this theoretical/experimental screening contributes to refine the number of filtered candidates included in the second list and to assign structure(s) for each protonated ion measured in the initial MS spectra. The third dimension of physicochemical data that should be applied to constrain the list of filtered candidates is that contained in the UV-visible spectra and the chromatographic behavior, increasing the reliability of the identification. Table 7.2 contains the common product ions observed for the selected carotenoids that appear in the pigment profile of phytoplankton biomass. The reader should consider that the occurrence of these product ions is not a *sine qua non* condition, but helpful for the identification (some of the product ions may not appear, other could constitute the base peak of the MS pattern, and even new product ions not previously denoted may become as qualifiers). Indeed, the instrumental configuration and conditions for acquisition of the MS spectra may produce changes in the MS pattern. Therefore, the use of experimental data obtained from authentic standards analyzed with the same MS conditions used for the sample, should be considered a golden rule for reliability of the identification (Sumner et al. 2007).

Application of MS techniques to the analysis of carotenoids in phytoplankton has enlarged the knowledge in carotenoid composition and metabolism for taxonomic screening (Frassanito et al. 2005), identification of novel carotenoids with fucoxanthin-related structures (Airs and Llewellyn, 2005; Crupi et al. 2013), screening the isomers and esters of astaxanthin (Holtin et al. 2009; Frassanito et al. 2008), monitoring of the bioavailability of carotenoids after microalgal biomass ingestion in animal models (Rao et al. 2010), high-throughput paired identification of chlorophylls and carotenoids species with the application of tandem MS (Fu et al. 2012; Juin et al. 2015; Zhang et al. 2016), or unraveled carotenoid esters (Maroneze et al. 2019). The reader should note that different hardware configurations and experimental approaches are described in literature, but they succeed in achieving the aim(s) proposed by the authors. This fact demonstrates the high plasticity of the MS

technique and its powerful capability in selectivity, limit of detection, and strategies applied for structural elucidation. However, the reader should consider that the best LC-MS configuration for MS analysis of phytoplankton pigments may include UHPLC-DAD system hyphenated with APCI source and Q-TOF mass analyzer, to acquire MS spectra with full detailed information in different dimensions (MS with mass accuracy and isotopic pattern of protonated ions, and subsequently tandem MS with mass accuracy and isotopic pattern of product ions).

7.2.6.2 Nuclear Magnetic Resonance (NMR)

The progress in the hardware developments achieved in recent years has finally allowed the hyphenation of LC with fine structural spectrometric analysis such as ^{1}H NMR, which were technically banned for hyphenation. Now it is possible to build metabolomic platforms that join the separation power of LC, the online acquisition of UV-visible and MS spectra and the final structural confirmation by NMR of the individual analytes annotated previously. Hence, the intense labor work of isolation and purification of the individual compounds to proceed with the NMR spectrometry has been released, and the high-throughput analysis providing definitive structural identification of the analytes is a reality. However, still these advances are constrained to large research facilities aimed to resolve metabolomic issues from different fields than phytoplankton profiling. Several studies have been published dealing with NMR of carotenoids in microalgae by using the classical routine of isolation and purification of the target compound, and subsequent acquisition of the NMR spectra either in 1D or 2D (Holtin et al. 2009; Sivathanu and Palaniswamy 2012). The development of probes and the increase of the magnetic field have decreased the handicaps of this technique, requiring less quantities of material and the option of performing sequences on many nuclei. Even the option of acquiring NMR spectra directly from tissue is available (Gaysinski et al. 2015) by means of the development of high-resolution magic-angle spinning probes, a technique at the interface of the liquid phase and the solid-state NMR, which is successfully applied to the analysis of interest compounds in phytoplankton biomass (Simon et al. 2015).

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Chapter 8
Analytical Protocols in Phycobiliproteins Analysis

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Abstract The aim of this chapter is to review and discuss methodology and protocols in the analysis of phycobiliproteins (phycocyanins, allophycocyanins, and phycoerythrins) and their chromophores. Due to the presence of multiple covalently bound open-chain tetrapyrrole chromophores, phycobiliproteins are colored and strongly fluorescent molecules, with high absorption coefficients (10^5 to 10^6) and excellent fluorescent quantum yield (0.51 up to 0.98). Therefore, a vast number of methods for phycobiliproteins analysis is based on these spectral characteristics, whereas assessment of their bioactivity is related to their exceptional redox and metal-chelating properties. This chapter is dedicated to methods used for isolation and purification, structure analysis, physicochemical properties and stability characterization, quantification, as well as in vitro and in vivo biological activities evaluation. In addition, emerging approaches related to phycobiliproteins analysis are also reviewed including interactions with other biomolecules and ions and identification of phycobiliprotein (chromo)peptides by mass spectrometry.

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8.1 Introduction

Phycobiliproteins (PBPs) are a family of water-soluble, highly fluorescent bioactive molecules composed of apoproteins and phycobilin open-chain tetrapyrrole chromophores covalently bound via cysteine amino acid. There are three PBP classes in cyanobacteria and red algae: phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), containing phycocyanobilin (PCB) and phycoerythrobilin (PEB) as pigments that differ in their spectral properties. Given its increasing application in the various fields, the aim of this chapter is to review and discuss methodology and protocols in PBPs research focusing on recent and the most relevant literature data.

8.2 PBPs Isolation and Purification

PBPs are produced by the photoautotrophic, mixotrophic, or heterotrophic cultivation of cyanobacteria and red algae.

Isolation of PBPs in high yield requires en efficient extraction process. There are several effective approaches used for their extraction: freezing and thawing, sonication, microwave assisted extraction, pulsed electric field extraction, high-pressure homogenization, osmotic shock, acid treatment, enzymatic treatment, organic solvent extraction, etc. (reviewed in Bleakley and Hayes 2017). Extraction of algal proteins could be very challenging due to the presence of mechanically robust, multilayered cell wall, and application of different polysaccharides-hydrolyzing enzymes (xylanase, cellulase, etc.) significantly increases PBPs extraction (Dumay et al. 2013).

Procedures for PBPs purification use single or combination of several chromatographic steps (ion-exchange chromatography, hydrophobic chromatography, gel filtration, hydroxyapatite chromatography, and expanded bed adsorption chromatography), as well as preparative electrophoresis or two-phase aqueous extraction (Sonani et al. 2016). One of the main challenges during the purification of PBPs is the separation of individual PBP from PBPs mixture because of their similar properties (pI, molecular mass, chromophore spectral properties). Recently, ultrasound assisted three-phase partitioning was employed for efficient extraction and purification of PC from *Arthrospira platensis* (Zhang et al. 2017b).

PBPs purity is evaluated using the ratio between absorbance in the visible region (652 nm, 620 nm, 565 nm, or 540 nm for APC, PC, R-PE, and B-PE, respectively) and the absorbance at 280 nm. PC preparations with $A_{620}/A_{280} > 0.7$ are considered as food grade, while those with $A_{620}/A_{280} > 4.0$ have an analytical grade of purity (Vernès et al. 2015). Absorbance ratio $A_{652}/A_{280} > 4.0$ means analytical purity

grade of APC (Yan et al. 2011) whereas the commonly accepted criterion for PE purity is when A_{565}/A_{280} ratio reaches 3.2 (Galland-Irmouli et al. 2000). An additional approach for evaluation of PBPs purity is determination of the ratios between absorption maxima of individual PBPs. Ratio $A_{650}/A_{620} < 0.3$ means that PC preparation contains negligible contamination from APC, while the ratio $A_{650}/A_{620} > 1.5$ indicates that APC is pure relatively to PC (McGregor et al. 2008). The purity of R-PE is also estimated by means of following indexes: $A_{565}/A_{498} \leq 1.5$ and $A_{565}/A_{620} \leq 0.005$, indicating minimal contamination by B-PE and PC, respectively (Niu et al. 2006). However, these absorbance ratios could sometimes give false interpretations. The pure APC in trimeric form has a peak at 652 nm, but monomers strongly absorb at 620 nm, preventing discrimination of PC from APC by visible absorption measurements (MacColl et al. 2003). In these cases, additional analytical methods, such as SDS-PAGE, are needed to obtain reliable results regarding PBPs purity. Induction of fluorescence after incubation of SDS-PAGE gels with Zn^{2+} ions specifically marks PBPs bands (Berkelman and Lagarias 1986), giving the possibility to make a distinction between PBP and other proteins by comparing electrophoretic gels after Zn^{2+} and CBB staining. The molecular weights of PBPs are determined by gel filtration and electrophoretic techniques (SDS-PAGE and PAGE), as well as by mass spectroscopy (MS) (Chen et al. 2006).

Isolation of tetrapyrrole chromophores requires cleavage of thioether bond between apoprotein and chromophore by acid hydrolysis, enzymatic cleavage, or extensive refluxing in alcohols. Although the most common procedure for the chromophore cleavage is still conventional reflux in methanol (Fu et al. 1979), it has been shown that performing ethanolysis in the sealed vessel at 120 °C decreases reaction time to 30 min and obtained PCB has higher purity in comparison to conventional reflux method (Roda-Serrat et al. 2018). Purification of released tetrapyrroles is usually performed by reversed-phase HPLC (Roda-Serrat et al. 2018).

Although recombinant production of PBPs is demanding, as a complete synthesis of PBPs depends on co-expression of α- and β-chains, in parallel with the synthesis of chromophores and their covalent attachment to protein, recombinant APC/PC were successfully produced in *E. coli* (Chen et al. 2015a; Cherdkiatikul and Suwanwong 2014). Tetrapyrrole chromophores could also be produced recombinantly. PCB was produced in mammalian cells by metabolic engineering introducing genes for heme oxygenase-1 and PCB:ferredoxin oxidoreductase with simultaneous knock-down of biliverdin reductase A to prevent PCB reduction to phycocyanorubin (Muller et al. 2013).

8.3 PBPs Structure and Physicochemical Properties Evaluation

Early studies based on PBPs enzymatic or CNBr hydrolysis and products detection after Edman degradation of chromopeptides by MS, revealed that the side chain (at position 2) of tetrapyrrole ring A is covalently bound to the cysteine residues of

apoprotein (Williams and Glazer 1978). The real progress in PBPs research has been enabled by resolving the crystal structures of several PBPs in last three decades (Li et al. 2019), providing valuable information about amino acid sequence(s), oligomerization state, subunit interactions, shapes of PBPs, as well as tetrapyrrole interactions with apoproteins and chromophore conformations in binding pockets. Details about the spatial arrangement of chromophores and modulations of their conformations enabled the study of mechanisms of energy transfer between chromophores in phycobilisomes (Jiang et al. 2001). While X-ray crystal structures have provided molecular details on the isolated PBPs, relatively high-resolution images (3.5 Å) of the overall architecture of phycobilisome assembly were obtained by single-particle cryo-electron microscopy (Zhang et al. 2017a). SDS-PAGE, HPLC, and MS confirmed the existence of at least two different types of γ subunit in some PEs which exist within the central cavity of $[(\alpha\beta)_3]_2$ hexamers (Isailovic et al. 2004; Wang et al. 2015).

While crystallographic studies have a big impact on the understanding of the structure and function of PBPs, they do not provide answers on proteins 'behavior in solution. Small angle X-ray and small angle neutron scattering techniques were employed for the determination of dimensions, aggregation state, and shapes of PBPs in solution (Golub et al. 2017). Special software (CRYSOL) was used for comparison between experimentally obtained scattering curve and theoretical curve based on the PDB file of the crystal structure of PC, enabling comparison of the structure of PBPs in crystal and solutions (Golub et al. 2017). PC dynamics per se, as well as the importance of hydration (interfacial water), have been studied by elastic incoherent neutron scattering (Combet and Zanotti 2012). In another approach, the structure and dynamics of chromophore binding pocket in PC were investigated by Heteronuclear Multiple-Quantum Correlation (HMQC) ^{15}N NMR. HMQC spectra unequivocally confirmed that all four nitrogen atoms of PCB in α subunit of PC are protonated (Hahn et al. 2007).

Optical spectroscopic properties of covalently bound tetrapyrrole chromophores alter in response to changes in conformation and oligomerization state of PBPs, which makes UV/VIS absorption, fluorescence, and CD spectrometry very convenient for studying PBPs properties (Thoren et al 2006). An important finding is that these spectroscopic methods are useful for the characterization of PBP chromophores and their conformers in free and protein-bound form (Minic et al. 2015; Minic et al. 2018a). Due to high absorption coefficients (10^5 to 10^6 M^{-1} cm^1) and excellent fluorescence quantum yields (from 0.51 to 0.98) these techniques have high sensitivity toward PBPs (Hermanson 2013). Based on absorption spectra, it is possible to estimate the chromophore content of PBPs and make a distinction between different PBPs. Moreover, the same chromophore molecules, bound at different sites in PBPs do not have the same spectroscopic properties, i.e., they show different absorption maxima. These differences may not be obvious in the raw absorption spectra due to broadness of peaks, but application of deconvolution method, using a Gaussian model analysis of the components, allows peaks to be resolved into several peaks that arise from the same types of chromophores bound at different regions on PBPs (Sepúlveda-Ugarte et al. 2011).

Study of energy transfer between tetrapyrrole chromophores during photosynthesis is an important topic in PBPs 'research. The energy transfers in PBPs have been investigated by steady-state fluorescence and fluorescence polarization spectroscopy, as well as by fluorescence lifetime and kinetic absorption spectroscopy (Li et al. 2019). Single-molecule fluorescence spectroscopy was used for analysis of switching of PC between different conformations and the role of this process in energy transmission to photosystem I (Gwizdala et al. 2018). UV/VIS absorption, fluorescence anisotropy, and CD spectroscopy are also useful tools for studying the effects of ionic strength, pH, and protein concentration on oligomerization states of PBPs (Thoren et al. 2006). Additionally, analytical ultracentrifugation enables analysis of PC at smaller concentrations ($\leq$0.01 mg/mL), and the determination of equilibrium constants between different oligomerization states (monomers, trimers, and hexamers) of PC (Berns and MacColl 1989).

8.4 PBPs Quantification and Stability Measurements

Tetrapyrrole chromophores are responsible for the typical color of various PBPs and have characteristic light absorption properties: PE, pink-purple, λ_{max} = 540–570 nm; PC, blue, λ_{max} = 610–620 nm, and APC, bluish-green, λ_{max} = 650–655 nm (Dufossé 2018). The quantity of PBPs/chromophores in solution is routinely determined by measurements of absorption in the visible spectral region (usually at the wavelength(s) of PBSs maximum absorption). In general, four sets of equations, combining the extinction coefficients, are used for the estimation of PBPs concentrations. The first was described by Bennett and Bogorad (1973) and the concentrations are given in mg/mL:

$$\begin{aligned}
[\mathrm{PC}] &= (A_{615} - 0.474\, A_{652})\,/5.34;\\
[\mathrm{APC}] &= (A_{652} - 0.205\, A_{615})\,/5.09;\\
[\mathrm{PE}] &= (A_{562} - 2.41[\mathrm{PC}] - 0.849\,[\mathrm{APC}])\,/9.62.
\end{aligned}$$

The second set was proposed by Kursar et al. (1983) with concentrations expressed as μg/mL:

$$\begin{aligned}
[\mathrm{APC}] &= 181.3\, A_{651} - 22.3\, A_{614};\\
[\mathrm{PC}] &= 151.1\, A_{614} - 99.1\, A_{651};\\
[\mathrm{PE}] &= 155.8\, A_{498.5} - 40.0\, A_{614} - 10.5\, A_{651}.
\end{aligned}$$

For expression of the hexameric form concentration in mol/L, the authors assumed the molar mass of 210 kDa (APC), 225 kDa (PC), and 250 kDa (for PE $[(\alpha\beta)_6\gamma]$). For expression of chromophores concentration in mol/L, the number of tetrapyrroles per hexamer was assumed to be 12 (APC), 18 (PC), and 40 (for PE $[(\alpha\beta)_6\gamma]$).

Beer and Eshel (1985) proposed equations for calculation of concentration (mg/mL), which are not affected by the concentration of interfering components, and which are presently mainly used for determination of PBPs from red algae:

$$[\mathrm{PE}] = ((A_{564} - A_{592}) - (A_{455} - A_{592})\,0.20)\,0.12;$$
$$[\mathrm{PC}] = ((A_{618} - A_{645}) - (A_{592} - A_{645})\,0.15)\,0.15.$$

Finally, Sampath-Wiley and Neefus (2007) described equations for estimation of PBPs content in aqueous extracts (mg/mL), which are, according to the authors, more accurate than previously published methods:

$$[\mathrm{R-PC}] = 0.154(A_{618} - A_{730});$$
$$[\mathrm{R-PE}] = 0.1247((A_{564} - A_{730}) - 0.4583(A_{618} - A_{730})).$$

For quantification of PCB obtained by methanolysis of purified PC, the extinction coefficient of 37900 M^{-1} cm^{-1} in MeOH/HCl solution (Cole et al. 1967) is frequently used.

While chromophore is a light-sensitive part of PBPs, apoprotein part confers the stability with respect to pH and temperature. Typically, the thermostability, the effect of pH itself, or the effect of pH on thermal stability of the PBPs are measured by incubating protein samples at chosen temperatures/pH values, followed by measurements of the characteristic absorbance maximum at regular time intervals, and calculation of the remaining concentration of PBP (C_R, %) relative to the initial concentration (e.g., Rahman et al. 2017; Wu et al. 2016; González-Ramírez et al. 2014; Liu et al. 2009). Differences in denaturation midpoint, defined as the temperature (Tm) at which 50% of the protein still remains in solution (C_R = 50% value), the purity ratio (e.g., A_{620}/A_{280} for PC), and the half-life value ($t_{1/2}$, the time taken for the initial protein concentration to be reduced by half) are also used to compare the stability of PBPs from various algal species. The examination of conformational state and functional dynamics of PBPs by measurements of optical properties of chromophores have been recently applied to compare the stability of the full length and truncated α-subunit of cyanobacterial PE. Urea-induced denaturation α-subunit transitions were monitored and the role of the truncated region in PE stability was also investigated by molecular dynamics simulations (MDS) (Anwer et al. 2015).

Improving the stability of PBPs is an important goal for their practical application as natural colors in the food and cosmetic industry and as a fluorescent probe and analytical reagent (Stanic-Vucinic et al. 2018). PBPs spectroscopic properties, including degradation kinetics with determination of degradation rate constant (k_d), are wildly used to examine improvement of PBPs storage stability by encapsulation (Suzery et al. 2015; Purnamayati et al. 2018), nanofiber encapsulation, and/or in the presence of preservatives (Braga et al. 2016; Bhattacharya et al. 2018).

8.5 Evaluation of Biological Activities of PBPs

The exceptional redox and metal-chelating properties of PBPs, and especially their chromophores, are proved by various biochemical assays for determination of their antioxidant potential: oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), β-carotene or crocin bleaching, ferric ion reducing antioxidant power (FRAP), lipid peroxidation inhibition, thiobarbituric acid reactive substances (TBARS), Fe^{2+} ions chelating, copper ion reducing antioxidant capacity (CUPRAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) or 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging, hydroxyl radical absorbance capacity (HORAC), etc. (reviewed in Kenny et al. 2015).

An increasing number of studies, performed in different in vivo and in vitro model systems, show that purified PBPs, PBSs-enriched extracts, recombinant, and even encapsulated PBSs, as well as their chromophores, exhibit a plethora of biological activities with substantial health benefits (summarized in Table 8.1). In vivo, in rat, mouse, and hamster models with induced disorders, PBPs were tested for their anti-cancer, anti-inflammatory, neuroprotective, nephroprotective, hepatoprotective, antihyperlipidemic, and antioxidative effects, while in *Caenorhabditis elegans* PC's anti-aging action was examined. In vitro, in healthy cells, such as human erythrocytes and rat cardiomyocytes, PBPs were examined for their protective antioxidant effects during induced oxidative stress. In cancer cell lines, anti-cancer activity of PBPs was investigated mainly by testing cell viability, cycle, and apoptosis, while mechanisms of PBS's anti-cancer action were examined by monitoring of mRNA and protein expression of genes and signaling pathways involved in cell death. Photodynamic cytotoxicity on cancer cell lines was tested upon PBPs treatment followed by laser irradiation (Table 8.1).

8.6 PBPs Interactions with Other Biomolecules and Ions

Although PBPs and their chromophores are prone to interact (non)covalently with other molecules and ions, there is limited literature data related to this topic. In the majority of the studies, fluorescent properties of PC and PCB are exploited for the determination of their noncovalent interactions and the most frequently used method is fluorescence quenching. In several studies, fluorescence quenching of a chromophore was monitored upon its titration with tested molecule or ion. The interactions of PCB with proteins were also followed by fluorescence quenching of protein Trp residue(s) upon titration with PCB. Gelagutashvili et al. (2013) compared three methods for monitoring of heavy metal binding to PC: equilibrium dialysis, fluorescence, and absorption titration. Heavy metal binding to PC was even exploited for the creation of fluorescence chemosensor for Cu^{2+} and mercapto biomolecules (Puangploy et al. 2015). Fluorescence quenching was used for monitoring of PC interactions

Table 8.1 Selected examples of evaluation of biological activities of PBPs in vitro and in vivo. AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, Tumor necrosis factor

Disorder/disease model system	PBP source	PBP activity	Experimental approach	References
In vivo				
Murine sarcoma 180-bearing mice	PE	Anti-cancer	Tumor inhibitory rate and organ index, SOD activity in liver and serum, splenic lymphocyte proliferation, natural killer cells activity, and TNF secretion capacity, histopathology of tumors	Pan et al. (2013)
Caenorhabditis elegans/C. elegans CL4176 transgenic model for Alzheimer's disease	PE	Antioxidant and anti-aging	Life span and stress resistance assays, locomotion and paralysis assays, expression of signaling pathways involved in aging; aggregation and proteotoxicity-mediated paralysis phenotype	Sonani et al. (2014a, b)
Acetic acid-induced colitis (Sprague-Dawley rats)	PC extract	Anti-inflammatory	Myeloperoxidase activity, histopathology, and electron microscopy of colonic tissue	González et al. (1999)
Pancreatic adenocarcinoma PANC-1 based tumor xenograft (Mice)	PC	Anti-cancer	Tumor growth and weight, body weight	Liao et al. (2016)
Kainic acid-induced hippocampus neuronal damage (Sprague-Dawley rats)	PC	Neuroprotective	Neurobehavioral activities, body weight, expression of markers of microglia and astroglia	Rimbau et al. (1999)

(continued)

Table 8.1 (continued)

Disorder/disease model system	PBP source	PBP activity	Experimental approach	References
Oxalate-induced renal injury (Wistar rats)	PC	Nephroprotective	Histopathology, oxalate content and lipid peroxidation of renal tissues, stone forming, biochemical analysis of urine	Farooq et al. (2004)
Thioacetamide-induced hepatic encephalopathy (Wistar rats)	PC	Hepatoprotective	Levels of NH_3 in serum, liver, and brain; prothrombin time and albumin levels in plasma; lipid peroxidation, Trp, antioxidant enzymes activity in the brain; histopathology and electron microscopy of brain tissue	Sathyasaikumar et al. (2007)
CCl_4 and R-(+)-pulegone-induced hepatotoxicity (Albino rats)	PC	Hepatoprotective	Serum GPT activity; liver microsomal cytochrome P450, glucose-6-phosphatase, aminopyrine-*N*- demethylase activities	Vadiraja et al. (1998)
Atherogenic diet-induced oxidative stress (Golden Syrian hamsters)	PC	Antihyperlipidemic and antioxidative	Plasma lipid concentrations and antioxidant capacity; liver antioxidant enzymes activity; aortic fatty streak area; cardiac superoxide anion production and expression of NADPH oxidase	Riss et al. (2007)

(continued)

Table 8.1 (continued)

Disorder/disease model system	PBP source	PBP activity	Experimental approach	References
Hypercholesterolemic diet-induced hyperlipidemia and oxidative stress (Golden Syrian hamsters)	PC	Antihyperlipidemic and antioxidative	Plasma lipid concentrations, GOT/GPT activity and lipid peroxidation in serum; antioxidant enzymes activity in the liver; mRNA levels of LDL receptor, HMG-CoA reductase of HepG2 cells	Sheu et al. (2013)
Selenite-induced cataractogenesis (Wistar rat)	PC	Anticataractogenic	Antioxidant enzymes activity, lipid peroxidation, and glutathione lens tissue levels	Kumari et al. (2013)
Encephalitogen-induced autoimmune encephalomyelitis (Lewis rats)	C-PC/PCB	Anti-multiple sclerosis	Balance and motor coordination; oxidative stress markers in brain and serum; cytokine levels in the brain; electron microscopy of brain tissue	Cervantes-Llanos et al. (2018)
In vitro				
Colon cancer SW480 cells	PE	Anti-cancer	Cell viability, cycle and apoptosis assays; electron microscopy; proteomics analysis of cell lysate; assays for signaling pathways involved in cell death	Li et al. (2016)

(continued)

Table 8.1 (continued)

Disorder/disease model system	PBP source	PBP activity	Experimental approach	References
A549 human lung carcinoma cells	PE	Anti-cancer	Cell viability, cycle and apoptosis assays; mitochondrial membrane potential and intracellular ROS generation	Madamwar et al. (2015)
Cervical carcinoma HeLa cells	PE	Anti-cancer	Cell viability and apoptosis assays after laser irradiation (photodynamic cytotoxicity); assays for signaling pathways involved in cell death	Pan et al. (2013)
Human hepatocellular carcinoma SMMC-7721 cells	PC and PE	Anti-cancer	Cell viability and apoptosis assays after laser irradiation (photodynamic cytotoxicity)	Cai et al. (2014)
AAPH-induced oxidative stress in human erythrocytes	PC-enriched algae extract	Antioxidant	Cytosolic glutathione content, lipid peroxidation, and hemolysis assay	Benedetti et al. (2004)
Pancreatic cancer Capan-1, PANC-1, and BxPC3 cell lines	PC	Anti-cancer	Cell viability, cycle, apoptosis, autophagy, and autosis assays, assays for signaling pathways involved in cell death	Liao et al. (2016)
Lung cancer NCI-H1299, NCI-H460, and LTEP-A2 cell lines	PC	Anti-cancer	Cell viability, cycle, apoptosis, migration, and colony formation assays; assays for signaling pathways involved in cell death	Hao et al. (2018)

(continued)

Table 8.1 (continued)

Disorder/disease model system	PBP source	PBP activity	Experimental approach	References
Breast cancer MDA-MB-231 cell lines	PC	Anti-cancer	Cell viability, cycle, apoptosis, migration, and colony formation assays; assays for signaling pathways involved in cell death	Jiang et al. (2018)
Breast cancer MDA-MB-231, MCF-7, SKBR-3, BT-474, and HBL-100 cell lines	PC	Anti-cancer and anti-angiogenic	Cell viability, cycle, apoptosis, migration, and colony formation assays; angiogenic assay; assays for signaling pathways involved in cell death	Ravi et al. (2015)
Doxorubicin-induced oxidative stress in rat cardiomyocytes	PC	Cardioprotective	Cell viability and apoptosis assays; intracellular ROS generation; assays for signaling pathways involved in cell death	Khan et al. (2006)
B16F10 murine melanoma cells	PC	Anti-melanogenic	Cell viability assay, tyrosinase activity, and melanin content; assays of signaling pathways involved in melanogenesis	Wu et al. (2011)
AAPH-induced oxidative stress in human erythrocytes	Natural PC and recombinant apo-PC	Antioxidant	Hemolysis assay, electron microscopy	Pleonsil et al. (2013)
Human colon T116 cancer cell line	Encapsulated PC	Anti-cancer	Cell viability, cycle, and apoptosis assays; assays for signaling pathways involved in cell death	Wen et al. (2019)

with lectins, such as jacalin (Pandey et al. 2009a), concanavalin A and peanut agglutinin (Pandey et al. 2009b), and PCB interactions with human serum albumin (HSA) (Minic et al. 2015), bovine serum albumin (BSA) (Kathiravan et al. 2009; Minic et al. 2018a), and beta-lactoglobulin (BLG) (Minic et al. 2018b). For identification of PCB binding site on HSA, competition experiments with site markers, as well as the determination of binding constants in the presence of site markers were exploited (Minic et al. 2015). Fluorescence microscopy has shown that PC penetrates into nucleated cells and stains the nucleus. High affinity of PC for DNA was confirmed by agarose gel electrophoresis, suggesting that PC can be used as a natural non-toxic replacement for ethidium bromide for specific detection of genomic DNA and as a marker of various blood cells/molecules (Singh et al. 2011; Paswan et al. 2016). On the other hand, the microscale thermophoresis, a new method for characterization of ligand-macromolecule binding, was used for characterization of PCB-BSA interactions (Fig. 8.1; Minic et al. 2018a), while isothermal titration calorimetry and scanning electron microscopy-energy dispersive X-ray spectrophotometry were used for characterization of PC interactions with Hg^{2+} (Bhayani et al. 2016). PC interactions with lipid monolayers were investigated by surface pressure measurements (Almog et al. 1988). PCB interactions and its potential binding sites to proteins were also studied by molecular docking (Minic et al. 2015, 2018a, b). Covalent binding of PCB to proteins was investigated by fluorescence and absorption spectrometry, MS and electrophoretic techniques, supported by computational methods (Isailovic et al. 2006; Minic et al. 2018a).

Several studies have also investigated the consequences of PC and PCB interactions with other molecules/ions on the conformation of interacting species. PCB-induced thermal stability and conformational changes of HSA, BSA, and BLG were monitored by synchronous fluorescence, CD spectroscopy, and FT-IR, while chromophore conformational changes were detected by CD spectroscopy in the visible region (Kathiravan et al. 2009; Minic et al. 2015, 2018a). Heavy metal ions-induced conformational changes of PC were observed by FT-IR and CD spectroscopy

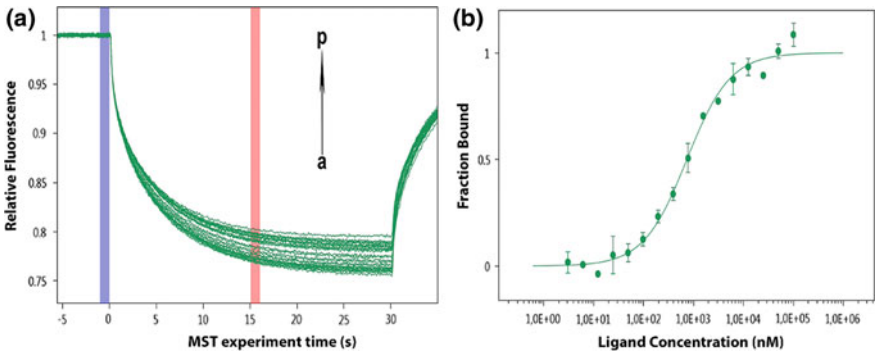

Fig. 8.1 Microscale thermophoresis was used for the determination of PCB-BSA interactions. Reprinted from Minic et al. (2018a), Copyright (2018), with permission from Elsevier

(Bhayani et al. 2016). The role of trehalose on the dynamics and structural stabilization of PC was investigated by neutron scattering (Koeper et al. 2008). In addition, Minic et al. (2018a) demonstrated a mutual protective effect of complexed PCB and BSA against free radical attack using antioxidant assays.

8.7 PBPs-Derived Natural and Synthetic (Chromo)Peptides

Early studies of structure and/or configuration of PBP chromophores were performed on chromopeptides and provided the means for the development of methods for their purification. Rabier and Vijayalakshmi (1983) digested PC with pepsin and obtained good separation of chromopeptides on histidyl-Sepharose gels based on histidine-tetrapyrrole interactions. Chromopeptides from pepsin digests of PC were purified by chromatography and isoelectrofocusing and, after photoisomerization, chromophore configuration was characterized by ‘H NMR spectroscopy (Thümmler and Rüdiger 1983). In the study of Wedemayer et al. (1992), PBPs were digested by pepsin, chromopeptides were purified by chromatography, and bilin groups were identified by absorption, fluorescence, ^{1}H NMR, and MS.

Although multiple studies investigated biological activities of peptides produced by enzymatic hydrolysis of whole algal biomass or isolated whole algal proteins (Ovando et al. 2018), only a few of them focused on the bioactivities of (chromo)peptides obtained from purified PBPs. In several studies, in addition to the identification of bioactive peptides, some of the peptides were synthesized and tested for bioactivity. Kim et al. (2018) identified PBPs by proteomics and, based on obtained amino acid sequences, synthesized 13 PBPs-derived peptides which were tested for their anti-cancer activity effect in vitro. Minic et al. (2016) digested PC by pepsin in simulated gastric fluid, separated chromopeptides by chromatography, and analyzed chromopeptides by MS. Sequences of chromopeptides were determined by manual de novo sequencing and confirmation of sequences was done by analysis of MS2 and MS3 spectra of parent ions and pure PCB. They also tested chromopeptides for antioxidant and metal-chelating activities, as well as for the protection of human erythrocytes from free radical-induced hemolysis and cytotoxic effect on HeLa and Caco-2 cells. Oh et al. (2018) separated whole algal proteins on 2D PAGE, digested PE band by trypsin, and after identification by MS, synthesized PYP peptide and tested its effect in hippocampal neuron cell culture. Wu et al. (2017) hydrolyzed PE by pepsin and purified the hydrolysate by gel permeation and reversed-phase chromatography. Two peptides with the highest angiotensin-converting enzyme inhibitory activity were determined with the Edman degradation method, followed by the synthesis of peptides with the same sequences. Xu et al. (2018) generated PC-derived peptides by limited trypsin hydrolysis, grafted hydrolysate to N-succinyl chitosan by microbial transglutaminase and tested them for antioxidant and anti-cancer activity on HeLa and L929 mouse fibroblast cell lines.

8.8 PBPs Identification by Proteomics

Using emerging proteomic methodologies, the most recent studies used global, and especially differential proteomic analysis of cyanobacteria/algae, for investigation of their circadian rhythm, cellular differentiation or acclimation to external/stress factors and starvation focusing on the expression of PBPs. Proteomics was used to investigate the link between light adaptation responses and phylogeny (different strains) and pigmentation (different PBPs ratio) (Mackey et al. 2017), as well as to monitor photo-acclimation by proteomics identification of expressed genes for PBPs (Herrera-Salgado et al. 2018). PBPs degradation was followed by proteomics analysis during an organism's adaptation to nitrogen depletion (Deschoenmaeker et al. 2014) and desiccation/rehydration (Xu et al. 2016). In an attempt to understand the toxicity of herbicide butachlor, a proteomic approach was helpful for detection of butachlor-induced down-regulation of PBPs (Kumari et al. 2009). Semiquantitative proteomics was used to monitor the expression level of each of 20 PE subunits, depending on light intensity during algal growth (Kieselbach et al. 2018). For quantitative proteomics, isobaric tags for relative and absolute quantitation (iTRAQ) were exploited to reveal the capacity for transfer of light energy and expression of PBPs during high-temperature stress and tolerance (Shi et al. 2017), as well as for monitoring of PBPs abundances in response to phosphate acclimation (Fuszard et al. 2013). In addition to label-free proteomics, quantitative proteomics with $^{14}N/^{15}N$-labeled proteins was used for investigation of temperature-induced remodeling of the photosynthetic machinery, where cells grown at high temperature were metabolically labeled with ^{15}N (Nikolova et al. 2017). Phosphoproteomics, with the additional step of phosphopeptides enrichment by TiO_2 chromatography, was exploited for detecting how phosphorylation status of PBPs affects the energy transfer and state transition of photosynthesis (Angeleri et al. 2016; Chen et al. 2015b). Spat et al. (2018) used quantitative phosphoproteomics to describe the proteomic of a dormant cyanobacterium and its dynamics during the transition to vegetative growth, in order to find the link between hyper-phosphorylation and the lifespan of PBPs during chlorosis.

On the other hand, high-resolution native mass spectrometry (NMS) which preserves noncovalent interactions, enabled better insight into the adaptation of the algal light-harvesting system to a wide range of environmental conditions via oligomerization of PBPs (Eisenberg et al. 2017). The combination of NMS and fluorescence spectroscopy was used to characterize the (dis)assembly of the PE protein complex regarding species contributing to color and highly fluorescent properties of the complex (Leney et al. 2018). The major challenge in working with the proteome of cyanobacteria is the high abundance of PBPs which affects the dynamic detection range and therefore suppresses the MS identification of other proteins. Matallana-Surget et al. (2014) successfully improved the cyanobacterial proteome coverage using 3D LC-MS/MS approach. They introduced a immobilized Cu(II)-affinity chromatography separation step to eliminate PBPs as the most abundant

proteins, and therefore improved access to additional low-abundance proteins. Ultimately, complete or near-complete sequences of novel PBPs could be deduced by combined proteomics and de novo sequencing approaches (Nair et al. 2018).

8.9 Computational Studies of PBPs' Structural and Dynamics Properties

MDS and quantum mechanics/molecular mechanics (QM/MM) are valuable tools for investigation of PBPs and their chromophores. These methods are useful for explanation of spectroscopic and structural properties found by experimental data, in addition to uncovering still controversial molecular mechanisms of energy transport in light-harvesting complexes. Several studies applied MDS to uncover the effects of bound solvent molecules on the conformation of PCB and dynamic of PC (Adir et al. 2002; Bellissent-Funel 2004). Waterman et al. (2014) investigated the conformational response of PCB to the ability of solvents to form hydrogen bonds using ab initio MDS of PCB in different solvents and ab initio calculations of NMR chemical shift patterns. In order to understand long-lived quantum coherences in PE, MDS, combined with quantum chemistry calculations, was employed to study the coupling between the biological environment and the vertical excitation energies of chromophores of PE antenna system (Aghtar et al. 2014). MDS was exploited for monitoring of PCB conformational changes and HSA overall and individual domain flexibility upon PCB binding to any of the two found binding sites on the protein. MDS enabled refining these binding sites and supported experimental data which demonstrate PCB-induced stabilization of HSA (Radibratovic et al. 2016). MDS was a tool for comparison of conformational flexibility of PC from Arctic cyanobacterial strain and mesophilic *Arthrospira platensis* in relation to cold adaptation (Su et al. 2017). The solvation dynamics of individual pigments in PC was quantified using ab initio QM/MM nuclear dynamics (Blau et al. 2018), demonstrating how the molecular motion of PBP antennae funnel excitations to low-energy pigments. QM/MM method was established for calculating the Raman spectra of protein-bound chromophores and revealed the potential and limitations of QM calculations on isolated tetrapyrroles for determining the chromophore structures which are not available (Mroginski et al. 2007). Elgabarty et al. (2013) presented hybrid ab initio QM/MM MDS and theoretical NMR chemical shift calculations of PCB in the binding pocket of the α-subunit of PC, unraveling the existence of dynamic water channels in light-harvesting proteins.

8.10 Conclusion

A recent increased interest in the use of PBPs for various industrial, biotechnological, pharmaceutical, and clinical applications demands reliable experimental protocols for their comprehensive analysis. The overview of traditional methods, as well as the most recent experimental advances used for PBPs' analysis given in this chapter, may be useful for further scientific research of the role of PBPs in photosynthesis. In addition, it may also serve as a literature guide for assembly of analytical protocols for PBPs analysis prior to commercial and/or medical use.

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Chapter 9
Analytical Protocols in Antioxidant Capacity Measurement

Tatiele Casagrande do Nascimento, Renan Campos Chisté, and Leila Queiroz Zepka

Abstract Microalgae have been recognized as promising sources of carotenoids, chlorophylls, phycobiliproteins, and phenolic compounds, which are bioactive compounds widely investigated in the decrease of oxidative damage. The ability of these structures to scavenge reactive chemical species occurs by different mechanisms and can be determined by several assays. Among the existing protocols, there are non-competitive [DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (Ferric Reducing Antioxidant Power), and RC (Reducing Capacity by Folin-Ciocalteu assay)] and competitive methods [ORAC-H (Oxygen Radical Absorbance Capacity for hydrophilic antioxidants), ORAC-L (Oxygen Radical Absorbance Capacity for lipophilic antioxidants) and PRSC (Peroxyl Radical Scavenging Capacity)]. Each protocol has a specific reaction medium, which can lead to some limitations, and therefore, its use cannot be generalized. Considering that the microalgae biomass has compounds with different structural characteristics for the solubility (hydrophilic and lipophilic), the compatibility of the reaction medium with the structure must be considered for an unequivocal measurement. Based on this context, this chapter presents a comprehensive description of the most used protocols to determine the in vitro antioxidant capacity of microalgae compounds, including the structural characteristics of the bioactive compounds, reaction medium, principles, mechanisms, and limitations of existing protocols, as well as a database of existing measurements.

Keywords Microalgae · Bioactive compounds · Antioxidant capacity

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9.1 Introduction

Over the past few years, the need to discover sources of bioactive compounds with potential to scavenge reactive chemical species has become increasingly common, as these reactive species induce oxidative damages, from technological losses in food matrices to structural modifications in biological components, causing irreversible damage to human health (Ahmed et al. 2014; Ayala et al. 2014; Gaschler and Stockwell 2017). Oxidative stress has been strongly associated with numerous chronic degenerative diseases and ageing processes (Bhat et al. 2015; Peña-Bautista et al. 2019), which justifies the intensified search for antioxidant compounds capable of delaying or inhibiting these oxidative processes.

In this context, microalgae (including cyanobacteria) have become the target of much research as they are recognized for producing diverse biologically active compounds including carotenoids, chlorophylls, phycobiliproteins, and phenolic compounds (Borowitzka 2018; Goiris et al. 2015; Hossain et al. 2016; Jacob-Lopes et al. 2019). Carotenoids, chlorophylls, and phycobiliproteins are the main classes of microalgae pigments (Fernandes et al. 2017; Patias et al. 2017; Rodrigues et al. 2015; Zepka et al. 2019; Jacob-Lopes et al. 2019). On the other hand, although some colored flavonoids have been reported in microalgae, the most common phenolic compounds are colorless and structurally acidic (Mahmood et al. 2019; Goiris et al. 2014). These bioactive compounds have in common the ability to scavenge reactive oxygen species (ROS), and their antioxidant potential is mostly related to their structures.

The first step in investigating the antioxidant potential of these compounds occurs via in vitro methodologies. Approximately 19 in vitro assays are used for antioxidant evaluation (Alam and Bristi 2013). Among them, the most relevant for the assessment of microalgae extracts include ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), DPPH, (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), RC (Reducing Capacity), ORAC-H (Oxygen Radical Absorbance Capacity for hydrophilic antioxidants), ORAC-L (Oxygen Radical Absorbance Capacity for lipophilic antioxidants), and PRSC (Peroxyl Radical Scavenging Capacity) (Brand-Williams et al.,1995; Benzie and Strain 1996; Ou et al. 2001; Re et al. 1999; Rodrigues et al. 2012).

In general, the objective of these assays is to promote the contact of the antioxidant compound (isolated or present in extracts) with some kind of reactive species under specific reaction conditions that allows measuring directly or indirectly the antioxidant capacity in comparison with a reference standard (Alam and Bristi 2013).

Although the antioxidant assays serve the same purpose to estimate the antioxidant capacity, they have different reaction media, which may be competitive or not during the mechanisms of electron transfer (Single-Electron Transfer, SET) or a hydrogen atom (Hydrogen Atom Transfer, HAT). While in ORAC-H, ORAC-L, and PRSC assays there is a competition between an antioxidant and an oxidizable substrate (probe), in ABTS, FRAP, DPPH assays, and RC this competition does not happen (Apak et al. 2016; Huang et al. 2005). The competition characteristic of

competitive trials puts them in advantage as compared to other methods as it confers close representation to biological mechanisms (Apak et al. 2016).

According to Rodrigues et al. (2012), to ensure an unequivocal measurement, it is essential to guarantee the solubilization of the antioxidant compound in the medium since, among the existing protocols, there is variability in terms of polarity. Consequently, the structural characteristics of the compound to be analyzed are taken into account since some are lipophilic, such as carotenoids and chlorophylls, and others are water-soluble, such as phycobiliproteins and phenolic compounds.

All of these details culminate in several analytical factors before choosing the protocol to be carried out on microalgae extracts. In this sense, this chapter provides a comprehensive description of the main protocols used to investigate the in vitro antioxidant capacity of microalgae compounds, including the characteristics bioactive compounds to be analyzed, reaction medium, principles, mechanisms involved, limitations and advantages of the protocols used, as well as a database with existing measurements.

9.2 Microalgae Bioactive Compounds: Structure and Antioxidant Capacity

Over the years, microalgae have become the focus of scientific exploration as they consist of different metabolites with significant bioactive potentials, such as carotenoids, chlorophylls, phycobiliproteins, and phenolic compounds (Raposo et al. 2013; Fernandes et al. 2017; Jacob-Lopes et al. 2019; Nascimento et al. 2019).

In terms of carotenoids, several compounds have been structurally defined and reported in the literature. Britton et al. (2004) report over 750 structures, whereas in the last compilation by Yabuzaki (2017), approximately 1181 were reported. In microalgae, a range of these pigments has been isolated, the most common being β-carotene, α-carotene, lutein, zeaxanthin, astaxanthin, violaxanthin, neoxanthin, crocoxanthin, fucoxanthin, canthaxanthin, echinenone, and myxoxanthophyll (Rodrigues et al. 2014; Jacob-Lopes et al. 2019), and their structures, as well as their preferred antioxidant mechanisms, are illustrated in Fig. 9.1.

Carotenoids are fat-soluble natural pigments that may impart yellow, orange, or red color due to their basic chemical structures consisting of a linear and symmetrical skeleton with a series of conjugated double bonds (CDBs) (Rodriguez-Amaya 2001). This series of CDBs generates a resonance system of π electrons moving along the entire polyenic chain; so these compounds are very reactive and absorb light in the visible region of the spectrum (around 450 nm) (Mercadante et al. 2008).

Structurally, they are grouped into carotenes and xanthophylls: carotenes are formed only by carbon and hydrogen (e.g., β-carotene and α-carotene), while xanthophylls contain oxygen-containing substituents (e.g., lutein, zeaxanthin, astaxanthin, violaxanthin, neoxanthin) (Fernandes et al. 2018).

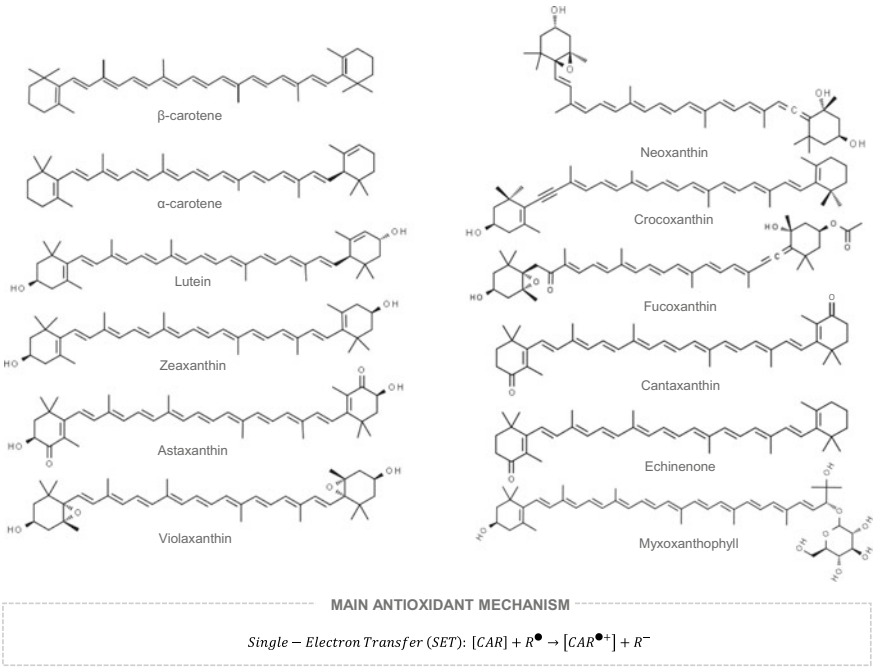


Fig. 9.1 Structure of common carotenoids in microalgae and their main mechanism of antioxidant action

Furthermore, some xanthophylls present exclusively in microorganisms, such as microalgae and cyanobacteria, exhibit a specified structural complexity as they may have allenic (C=C=C), acetylenic (C≡C), glycosylated (-O-CO-CH3), and keto (C=O) groups, such as fucoxanthin, crocoxanthin, myxoxanthophyll, canthaxanthin, and echinenone, respectively (Takaichi and Mochimaru 2007; Takaichi 2011).

The antioxidant effects of these isoprenoid compounds are closely related to their chemical structure, including the number of CDBs, the type of structural end-groups, and the oxygen-containing substituents (El-Agamey et al. 2004). According to Rodrigues et al. (2012), the number of CDBs that make up the chromophore is the most influential feature in the carotenoid's ability to scavenge ROS, while the addition of hydroxyl (OH) and ketone (C=O) in the terminal rings increases the antiradical capacity of compounds with the same chromophore.

The relationship between the extension of the CBD system and the bioactive potential can be explained by the preferential antioxidant mechanism of action of carotenoids involving the donation of polyenic chain electrons. In SET reaction, the antioxidant compound [CAR] donates electrons to the reactive species ($R^{\bullet}$), which is reduced and loses reactivity (R^{-}) while the antioxidant compound undergoes oxidation [$CAR^{\bullet+}$] (see Fig. 9.1). SET occurs only as long as electrons are available, so the longer the chromophore, the high the antioxidant potential (El Agamey et al. 2004). For this reason, the increasing interest in exploring microalgae extracts is

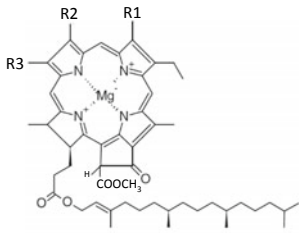
observed in the literature as they may present specific chromophore carotenoids with 12 and 13 CDBs, such as echinenone and canthaxanthin, respectively (Rodrigues et al. 2015; Patias et al. 2017; Nascimento et al. 2019).

In general, it seems that the antioxidant mechanism will be facilitated as the electromagnetic radiation energy of the molecule decreases, because the more linear, more CDBs and more symmetrical the structure, the smaller the energy charge, the more easily is the electron relocation, and therefore electron donation occurs more easily (Poliak et al. 2018). This observation makes sense when we look at the results of Rodrigues et al. (2012), where the opening of the β-ionone ring (lycopene—11 CDBs) and the increase of the chromophore extension (astaxanthin—13 CDBs) were the main factors that increased the peroxyl-scavenging capacity of carotenoids. Moreover, the addition of OH and CO groups in each terminal ring of carotenoid structures influenced the peroxyl-scavenging capacity of carotenoids depending on the number, type of functional groups and if it is part or not of the chromophore. For example, astaxanthin (13 CDBs, two OH, and two CO groups) was 2 times more efficient to scavenge peroxyl radicals than β-carotene (11 CDBs, 2 β-rings and none OH or CO groups), while β-cryptoxanthin (11 CDBs, 2 β-rings, and one OH group) exhibited the same scavenging capacity of β-carotene. The same authors also demonstrated that *cis* isomers of carotenoids have less antioxidant capacity than the corresponding *trans*-isomers. Additionally, a high concentration of extended chromophore compounds, such as canthaxanthin (12 CDBs), myxoxanthophyll (12 CDBs), and echinenone (11 CDBs), all of them found in *Chlorella vulgaris,* contributed to a higher antioxidant potential when compared to other microalgae.

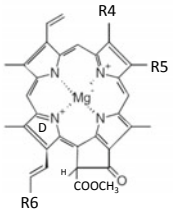
Another well-known antioxidant mechanism of tetraterpenoids is the quenching of singlet oxygen ($^1O_2$) and the triplet state of sensitizers, in which carotenoids physically interact with $^1O_2$ by absorbing excess energy (physical quenching) from the molecule in the chromophore region and releasing it into the environment as heat (energy transfer mechanism) (El Agamey et al., El-Agamey et al. 2004). In addition, whereas at a low rate, carotenoids are able to quench $^1O_2$ via chemical quenching, resulting in carotenoid oxidation products (Montenegro et al. 2004; Rios et al. 2007). Additionally, the antioxidant action of carotenoid may also occur through adduct formation and hydrogen atom transfer (HAT) (Poliak et al. 2018).

Chlorophyll, a green pigment, is a cyclic structure with a characteristic five-membered isocyclic ring, which may or may not have a central magnesium atom and a C17 phytol chain that confers hydrophobicity to the molecule (Roca et al. 2015). Figure 9.2 shows the main chlorophylls found in most microalgae, namely, chlorophylls *a*, *b*, *c*, *d*, and *f*, as well as their suggested antioxidant mechanism of action.

Chlorophylls *a* and *b* differ by the C7 (R1) carbon functional group, while chlorophyll *a* has a methyl group and chlorophyll *b* has an aldehyde (Fernandes et al. 2017). In contrast, chlorophyll *c* have a D-ring unsaturated and a characteristic propionic acid at C17 (R6) instead of a phytol group, which confers polarity to the molecule, and chlorophyll c_1 and c_2 differ from each other by the radical at C8 (R5) carbon, alkyl and vinyl, respectively, while c_3 is characterized by the presence of C7 (R4)



	R1	R2	R3
Chlorophyll *a*	CH_3	H_3-C=H_2	CH_3
Chlorophyll *b*	COH	H_3-C=H_2	CH_3
Chlorophyll *d*	CH_3	COH	CH_3
Chlorophyll *f*	CH_3	H_3-C=H_2	COH



	R4	R5	R6
Chlorophyll c_1	CH_3	CH_3-CH_3	CH_3CH_2COOH
Chlorophyll c_2	CH_3	H_3-C=H_2	CH_3CH_2COOH
Chlorophyll c_3	$COOCH_3$	H_3-C=H_2	CH_3CH_2COOH

MAIN ANTIOXIDANT MECHANISM

$$Single-Electron\ Transfer\ (SET)\text{: } [CHL] + R^{\bullet} \rightarrow [CHL^{\bullet+}] + R^{-}$$

Fig. 9.2 Structure of microalgae chlorophylls and their related antioxidant mechanism

methoxyl group. Chlorophylls *d* and *f* resemble chlorophyll *a*; however, chlorophyll *d* has an aldehyde in C3 (R2) while chlorophyll *f* in C2 (R3) (Zepka et al. 2019).

Due to their structural particularities, chlorophylls *a*, *b*, and *c* exhibit more intense absorption in the visible spectral region around 450 nm (Soret band) and relatively moderate around 620 nm (Q-band), while chlorophylls *d* and *f* have an extension in the chromophore that decreases the energetic need of the molecule and displaces the electromagnetic radiation absorption to the infrared region (above 700 nm) (Chen and Blankenship 2011).

According to Lanfer-Marquez et al. (2005), the antioxidant efficiency of the tetrapyrrole compounds varies considerably according to their chemical structures, which is influenced by the different substituents on the pyrrole rings, whether or not there is Mg in the center of the structure and the extent of the chromophore. Also, its structural characteristics have been shown to strongly influence the energy levels of the molecule that reflects both color and bioactive potential (Croce and Amerongen 2014).

Scientific evidence showed that chemical instability of chlorophyll *a* reduces its antioxidant capacity; in contrast, the absence of Mg in the center of the structure increases this property. Moreover, chlorophyll *b* derivatives have higher antioxidant capacity than chlorophyll a derivatives as a general tendency associated to the presence of aldehyde in the structures (Lanfer-Marquez et al. 2005), which increases the number of CDBs of chromophore. However, it is difficult to draw definitive conclusions since the literature presents conflicting data concerning the bioactive potential

for the same tetrapirolic structures, probably due to the physical structure of the test system, the nature of the oxidation substrate, and the analytical method employed (Becker et al. 2004).

The mechanism of antioxidant action of chlorophylls is not yet well understood; however, the conclusions about proportionality between the CBDs system and the ability to scavenge radicals established in the literature suggest that chlorophyll acts as an effective electron donor (see Fig. 9.2) (Kumar et al. 2001; Lanfer-Marquez et al. 2005; Fernandes et al. 2017). Despite being the most abundant structure in most studies, chlorophylls were not included in the trials (Lanfer-Marquez et al. 2005). As far as our knowledge is concerned, Rodrigues et al. (2015) and Fernandes et al. (2017) were the only ones to evaluate the antioxidant capacity of a microalgae chlorophyll extract. Rodrigues et al (2015) demonstrated that chlorophyll extract of *Phormidium autumnale* microalgae was almost 85 times more efficient as peroxyl radical scavenger than α-tocopherol in lipophilic media. Furthermore, in their findings, Fernandes et al. (2017) showed that commercial standards of chlorophylls *a* and *b* have higher ability to scavenge peroxyl radicals than *P. autumnale* extract with 11 characterized structures including chlorophyll *a*, *b*, and its derivatives.

In addition to these typical pigments, phycobiliproteins, which are open tetrapyrrole accessory pigments, they are also produced by aquatic microorganisms (Chen and Blankenship 2011). The main phycobiliproteins produced and marketed are phycoerythrin and phycocyanin from cyanobacteria and Rhodophyta (Viskari and Colyer 2003). In addition, these compounds are also found in a class of biflagellate unicellular eukaryotic algae (cryptomonads) (Román et al. 2002).

As shown in Fig. 9.3, phycobiliproteins are a group of proteins with a linear tetrapyrrole chromophore covalently attached to the protein backbone through covalent thioether bonds to cysteine residues. They are water-soluble, very stable at physiological pH, and highly fluorescent (Viskari and Colyer 2003; Spolaore et al. 2006).

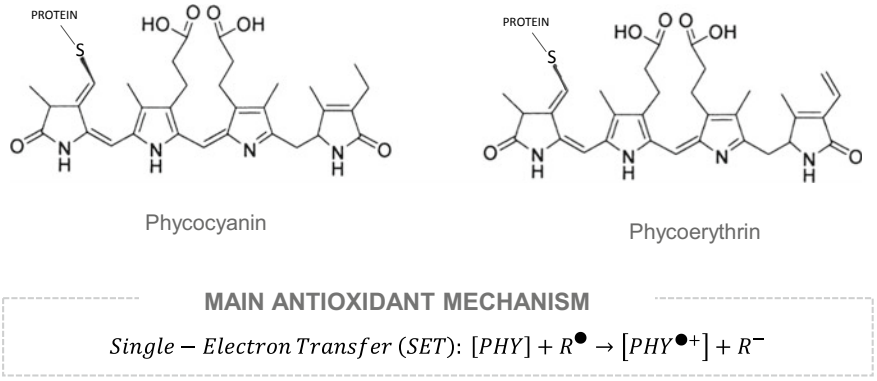


Fig. 9.3 Structure of phycobiliproteins and their main antioxidant mechanism

The phycocyanins are blue while the phycoerythrins are pink and they differ structurally from each other by replacing the CH_3–CH_2 group with $CH = CH_2$, being the absorption maximums at 540–570 nm and 610–620 nm for phycocyanin and phycoerythrin, respectively (Román et al. 2002; Spolaore et al. 2006).

The main application of these compounds is as a natural dye in the food industry, however, an increasing number of investigations have shown their health-promoting properties and wide range of pharmaceutical applications, mainly phycocyanin (Bhat and Madyastha 2000; Begum et al. 2015; Rodrigues et al. 2015; Jung et al. 2016; Sangeetha et al. 2017; Patel et al. 2018). According to Estrada et al. (2001), the increase in hydroxyl radical inhibition related to the increase of phycobilin content demonstrates the antioxidant capacity of these compounds. This biological activity has been linked to the structure's chromophore, which acts as a powerful scavenger of ROS suggesting SET as the primary mechanism involved in the same way as carotenoids and chlorophylls (see Fig. 9.3) (Bhat and Madyastha 2000).

Another type of bioactive compounds present in microalgae are the phenolic compounds. Although their determination is not frequent, phenolic compounds (including phenolic acids and flavonoids) are also present in microalgae biomass and have been explored about their antioxidant potential (Goiris et al. 2014; Pagnussatt et al. 2016; Scaglioni et al. 2018). Figure 9.4 presents the phenolic compounds already reported in microalgae, namely, gallic acid, chlorogenic acid, coumaric acid, caffeic acid, ferulic acid, quercetin, and apigenin. In addition, the main antioxidant mechanism of phenolic compounds is HAT and this fact is well known in the literature.

In terms of chemical structure, phenolic compounds are bioactive substances that occur as secondary metabolites and they are composed of aromatic rings attached to hydroxyl substituents (Mahmood et al. 2019). They can be classified by the number and arrangement of carbon atoms and are commonly linked to sugars and organic

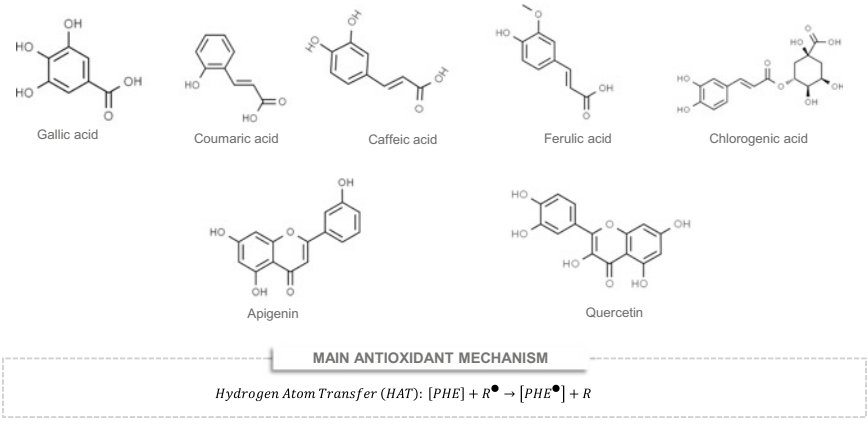


Fig. 9.4 Structure of phenolic compounds reported in microalgae and their main antioxidant mechanism

acids (Crozier et al. 2009). The phenolic acids (non-flavonoid compounds) are generally composed of an aromatic ring attached to a carboxylic acid and hydroxyl groups, they are divided into hydroxybenzoic acids (e.g., gallic acid) with the conformation of C6-C1 and hydroxycinnamic acids (e.g., coumaric, ferulic, and caffeic acids) with C6-C3 structures (Robbins 2003). Flavonoids, on the other hand, are characterized by the structure C6-C3-C6, (e.g., quercetin and apigenin), i.e., two aromatic rings linked by a central ring C3 (Panche et al. 2016). Their absorption spectra range from 280 to 370 nm for flavonoids and up to 320 nm for phenolic acids (Crozier et al. 2009).

The antioxidant mechanism of action involves a combined HAT from its hydroxyl groups to the radical, forming a transition state of an OH bond with an electron (see Fig. 9.4). Based on this, it is suggested that their ability to scavenge reactive species is proportional to the number and position of hydroxyls. However, this antioxidant capacity is significantly reduced when the reaction medium consists of a solvent prone to the formation of hydrogen bonds as alcohol (Santos-Sánchez et al. 2019).

Additionally, the chromophore present in the structure of some phenolic compounds, such as quercetin, may provide the ability to scavenge radicals via SET, but may also act as chelators of metal ions, such as iron and copper, due to the presence of 3', 4'-hydroxyl groups in B ring, inhibiting the oxidation of low-density lipoproteins (LDL) (Leopoldini et al. 2004).

9.3 Overview of Antioxidant Protocols

Natural extracts containing bioactive compounds are frequently evaluated for their effectiveness by different in vitro chemical methods in variable reaction media. Table 9.1 shows the relevant characteristics of the most cited protocols in the evaluation of microalgae antioxidants: DPPH, ABTS, FRAP, RC, ORAC-H, ORAC-L, and PRSC.

Among non-competitive assays, DPPH is a colorimetric method, in which the principle involves the scavenging of $DPPH^{\bullet}$ by antioxidants in a methanolic reaction medium at pH 6.0, and the loss of absorption at 515 nm is monitored by spectrophotometry (Brandt-Willians and Berset 1995). The main mechanism involved is SET, although HAT from the neutral antioxidant by DPPH was characterized as a marginal reaction path due to its very slow reaction rate in strong hydrogen-bond-accepting solvents, such as methanol and ethanol (Huang et al. 2005).

Unlike most reactive species, DPPH radical is characterized by its stability due to the displacement of the spare electron on the molecule as a whole, so the molecule does not dimerize (Alam et al. 2013). In its radical form, it has purple color resulting in an absorption band at 515 nm that fades after reduction by an antioxidant compound (assuming both radical and probe function) (see Fig. 9.5). Absorbance monitoring occurs for approximately 30 min (time required for the reaction kinetics of most compounds to reach steady state) (Santos-Sanchez et al. 2019). The whole reaction takes place at room temperature (25 °C), there is no need of prior radical preparation

Table 9.1 Characteristics of the most relevant in vitro chemical antioxidant capacity assays

Assay	Mechanism	Reaction medium	Radical generator/Reactive specie/Probe	T (°C)	Monitoring (λ)	Advantages	Limitation	References
DPPH	SET (main) and HAT (marginal reaction path)	Methanol	none/DPPH/DPPH	25 °C	515 nm	Rapid; Easy to execute; Evaluation of hydrophilic and lipophilic compounds	Non-competitive; Radical nonexistent in the organism; Reacts different from physiological ROS and RNS; Spectral interference with natural pigments that absorbs at the same monitoring wavelength	Brandt-Williams et al. (1995)
ABTS	SET	Ethanol or Phosphate buffer (pH ~ 7.4)	ABTS + Potassium persulphate/$ABTS^{\bullet+}$/$ABTS^{\bullet+}$	30 °C	750 or 734 nm	Rapid; Easy to execute; Evaluation of hydrophilic and lipophilic compounds; Low spectral interference	Non-competitive; Radical nonexistent in the organism; Reacts different from physiological ROS and RNS	Re et al. (1999)
FRAP	SET	Acetate buffer (pH ~ 3.4)	None (metal reduction)	37 °C	593 nm	Rapid; Easy to execute	Non-competitive; No reactive specie; pH outside physiological conditions (acid); Nonspecific reaction	Bezie and Strain (1996)
RC	SET	Aqueous solution (pH 11.0)	None (metal reduction)	25 °C	750 nm	Easy to execute; Many results available	Non-competitive; No reactive specie; pH outside physiological conditions (basic); Several compounds react with Folin-Ciocalteu reagent	Singlenton and Joseph (1965)

(continued)

Table 9.1 (continued)

Assay	Mechanism	Reaction medium	Radical generator/Reactive specie/Probe	T (°C)	Monitoring (λ)	Advantages	Limitation	References
ORAC-H	HAT	Phosphate buffer (pH 7.4)	AAPH/ROO•/Fluorescein	37 °C	Ex: 493 nm; Em: 515 nm	Competitive method; Conditions of the reaction similar to the physiological system; Applicable to hydrophilic samples	Long time of analysis; Not applicable to lipophilic extracts	Ou et al. (2001)
ORAC-L	HAT	Phosphate buffer (pH 7.4)—sample in acetone +RDMC	AAPH/ROO•/Fluorescein	37 °C	Ex: 493 nm; Em: 515 nm	Competitive method; Conditions of the reaction similar to the physiological system; Applicable to lipophilic samples	Long time of analysis; Not applicable to hydrophilic extracts	Huang et al. (2002)
PRSC	HAT	DMSO/MTBE	AIBN/ROO•/Bodipy$^{581/591}$	42 °C	Ex: 540 nm; Em: 600 nm	Competitive method; Applicable to lipophilic samples; Developed for carotenoids	Long time of analysis; No applicable to hydrophilic compounds	Rodrigues et al. (2012)

DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; FRAP: Ferric Reducing Antioxidant Power; RC: Reducing Capacity; ORAC-H: Oxygen Radical Absorbance Capacity for hydrophilic antioxidants; ORAC-L: Oxygen Radical Absorbance Capacity for lipophilic antioxidants; PRSC: Peroxyl Radical Scavenging Capacity; SET: Single-Electron Transfer; HAT: Hydrogen Atom Transfer; ROO•: Peroxyl radical; DMSO: Dimethyl sulfoxide; MTBE: Methyl *tert*-butyl ether; AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride; RDMC: Randomly methylated *β*-cyclodextrin; AIBN: 2,2′-Azobis(2-methylpropionitrile); ROS: Reactive oxygen species; RNS: Reactive nitrogen species; Ex: Excitation wavelength; Em: Emission wavelength.

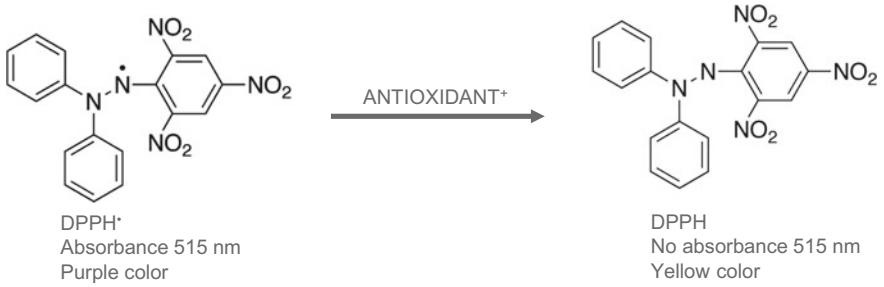


Fig. 9.5 Interaction between DPPH radical and antioxidant compound

once DPPH$^{\bullet}$ is preformed when DPPH is dissolved in methanol (Brandt-Willians and Berset 1995).

The scavenging efficiency of any antioxidant against DPPH radical can be defined as the amount of an antioxidant required to decrease the initial DPPH concentration by 50% (Efficient Concentration = EC_{50} or Inhibitory Concentration = IC_{50}). Therefore, most results are described in terms of EC_{50} or IC_{50} and the higher this value, the more efficient the antioxidant compound. Another way to look at antioxidant capacity in this method is to determine the amount of antioxidant needed to decrease the initial DPPH concentration by 100% (EC_{100}); however, the EC_{50} or IC_{50} is more frequent since some compounds would never react with more than 75% of the initial DPPH even after hours of reaction and at high concentrations (Brand-Williams et al. 1995).

Based on the above, the main advantages of this assay are its speed, ease of execution, and it is excellent for the evaluation of intermediate hydrophilic and lipophilic compounds since the reaction occurs in methanol. On the other hand, they have some limitations, such as being a non-competitive method, the DPPH radical being nonexistent in the human organism and reacting differently from ROS and RNS. Furthermore, it cannot be performed for light-absorbing compounds near 500 nm, such as anthocyanins and some carotenoids, due to spectrum-overlapping characteristic observed between DPPH solution and such compounds.

DPPH has been used extensively to evaluate the antioxidant efficacy of phenolic extracts of *Nostoc, Arthrospira, Anabean, Eucapsis, Porphyridium, Chlorella, Haematococcus, Oscillatória, Calothrix, Anabaena, Phormidium, Scenedesmus, Botryococcus, Nannochloropsis, Phaeodactylum* e *Tetraselmis* (Assunção et al. 2017; Babi et al. 2016; Banskota 2019; Blagojevi et al. 2018).

ABTS is another non-competitive assay, which has undergone some updates to eliminate interferences and facilitate radical generation (Miller et al. 1993). The most recent version was proposed by Re et al. (1999), the mechanism of action occurs by SET and its principle is based on the discoloration of ABTS$^{\bullet+}$ when an antioxidant is added to its blue-green solution (see Fig. 9.6). The reaction medium may occur in ethanol or saline phosphate buffer (pH ~ 7.4).

The assay is performed in two stages: in the first, ABTS is submitted to an oxidative reaction with potassium persulfate where it remains for 12 to 16 h to form ABTS

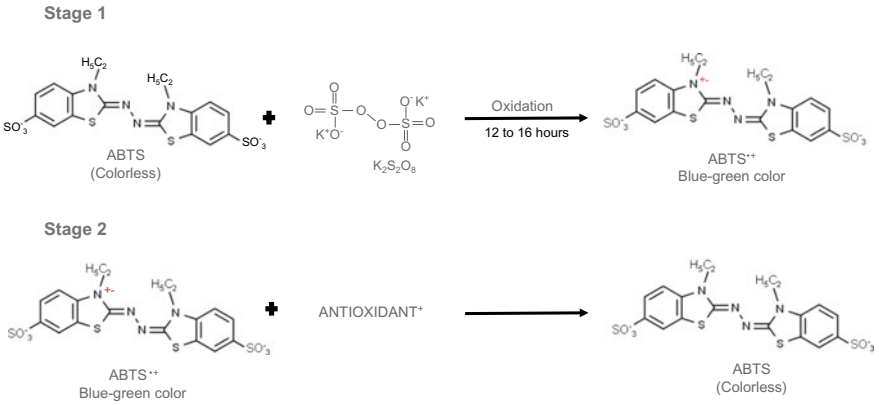


Fig. 9.6 $ABTS^{\bullet+}$ generation stage and reaction with antioxidant compounds

radical cation ($ABTS^{\bullet+}$). In the second stage, the radical is brought into contact with the antioxidant compound in a temperature at 30 °C, and monitored for absorbance loss at 750 nm (ethanol) or 734 nm (saline phosphate buffer) until reaction stabilization (usually 1 to 30 min) (Re et al. 1999). According to Shalaby and Shanab (2013), in determining the antioxidant activity of *Spirulina platensis*, the reaction occurred faster with saline phosphate buffer (approximately 30 min) than with alcohol. In this version of the assay, it is necessary to consider the influences of both antioxidant concentration and reaction stabilization on inhibition of radical absorption (Re et al. 1999).

Although some results are presented as a percentage, ABTS radical inhibition efficacy values can be calculated from the standard curve and expressed as equivalent (μmol Equivalent Standard per gram of sample extracted on dry basis = $\mu mol.SE.g^{-1}$) (Santos-Sanches et al. 2019). Trolox and ascorbic acid are the most recommended reference standards due to their high antioxidant capacities and solubility in the reaction medium.

The advantages of this assay are the simplicity of execution, low timing consuming (after radical generation), and the possibility of evaluation of hydrophilic and lipophilic compounds. Additionally, the spectral region of $ABTS^{\bullet+}$ solution (around 730 nm) has few spectral interferences with other natural pigments and can be successfully applied for evaluation of microalgae hydrophilic extracts, since the closest absorption occurs near 650 nm for phycobiliproteins. In contrast, they have the same limitations as DPPH in terms of the scheme's competitiveness, nature, and lack of similarity to those reactive species present in physiological environment.

The reducing capacity of a bioactive compound has also been considered as a significant indicator of its antioxidant capacity (Gülçin 2015). The reducing power by FRAP was originally developed to measure antioxidant capacity in blood plasma, but it has also been applied to other matrices (Benzie and Strain 1996). As shown in Fig. 9.7, the antioxidant capacity of any antioxidant is measured by

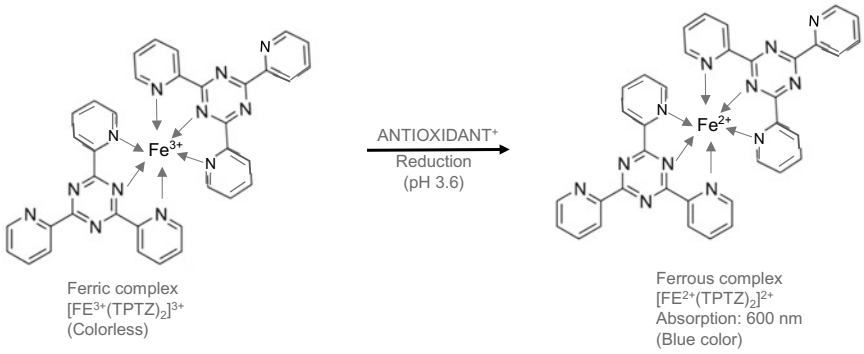


Fig. 9.7 Principle of Fe^{2+} reduction in FRAP assay

the ability of a compound to reduce a ferric complex 2,4,6 -tripyridyl-1,3,5-triazine ($[FE^{3+}(TPTZ)_2]^{3+}$) to a ferrous complex ($[FE^{2+}(TPTZ)_2]^{2+}$).

The ferric complex $[FE^{3+}(TPTZ)_2]^{3+}$ absorbs light in the UV region of the electromagnetic spectrum (colorless); however, when in the presence of an antioxidant, it receives an electron and $[FE^{3+}(TPTZ)_2]^{3+}$ is reduced to $[FE^{2+}(TPTZ)_2]^{2+}$,which has its absorption shifted to the visible region of the electromagnetic spectrum (~ 600 nm) (Benzie and Strain 1996). Then, the antioxidant capacity is estimated by measuring the absorbance increase in the visible region of the spectrum (Alam et al. 2013).

The reaction medium used is acetate buffer at 300 mM, the reaction is monitored at 593 nm, kept at 37 °C for approximately 30 min or until stabilization (Benzie and Strain 1996). In general, the reducing power of the samples is expressed in milligrams of ascorbic acid equivalents (AAE). An important detail of this protocol is the maintenance of the acid medium (pH 3.6), because at the physiological condition range (pH 7.4) the reaction does not occur due to iron precipitation (Santos-Sanches et al. 2019).

In one hand, FRAP is well regarded as a fast and easy assay to carry out. On the other hand, it has limitations since its reduction reaction is not a competitive scheme; it does not use any reactive species and it is performed at pH outside physiological conditions. Other limitations of the assay are the impossibility of evaluating lipophilic compounds and the reaction is nonspecific, where any compound with a redox potential leads to Fe^{3+} reduction (Benzie and Devaki 2017).

RC is another assay that measures the reducing capacity of samples through reduction between antioxidants with Folin-Ciocalteu reagent, which was initially designed to the analysis of proteins due to the reduction capacity of protein tyrosine that contains a phenol group residue in its structure (Huang et al. 2005). Later, the assay was extended to measure total phenolic compounds contents, whereas it is not specific for phenolics, i.e., any reducing agent can be accounted for this test (Singlenton and Rossi 1965). Therefore, it can be extended as an indication of reducing capacity.

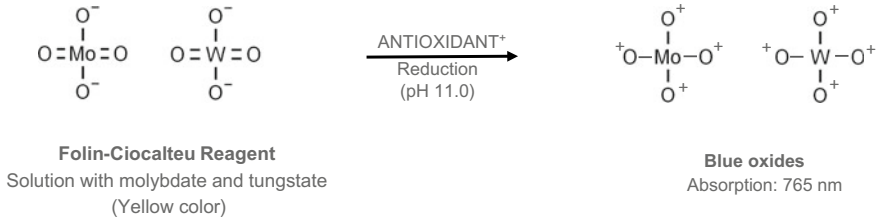


Fig. 9.8 Reaction of Folin-Ciocalteu reagent with antioxidant compound in reduction capacity assay

Figure 9.8 shows the principle of the technique: in basic aqueous solution (pH ~ 11.0), the Folin-Ciocalteu reagent (solution containing tungstate and molybdate) is reduced by reducing compounds via SET mechanism to generate a blue solution containing blue oxides (MoO^{4+} and WO^{4+}) (Amorati and Valgimigli 2015).

According to the original version of the method, the reaction occurs in approximately 2 h at room temperature (25 °C) by monitoring absorbance at 765 nm. The most frequent way to express the results is as gallic acid equivalent.

The advantages of RC refer to the ease of execution and a large number of results available in the literature, which facilitate comparisons. Thus, as FRAP test, RC assay limitations include the fact that it is not a competitive reaction, it does not involve reactive species, and the pH is outside physiological conditions. In addition, it is a slow reaction, and several compounds may react with Folin-Ciocalteu reagent, especially proteins that are extensively extracted by hydrophilic solutions (Amorati and Valgimigli 2015).

Unlike the assays described above, ORAC-H, ORAC-L, and PRSC are competitive inhibition mechanism. In this type of assay, the oxidant (reactive species) reacts with a probe leading to structural changes that cause changes in its fluorescence and the antioxidant compounds compete with the probe for the reactive species; thus, the higher the antioxidant capacity of the compound, the lower the oxidative conversion of the probe (Apak et al. 2016).

ORAC-H is an assay developed for the exclusive evaluation of samples with hydrophilic structural characteristics since the reaction system occurs in phosphate buffer (pH 7.4). Fluorescein (3',6'-dihydroxyspiro[2-benzofuran-3,9'-xanthene]-1-one) is the probe used while AAPH (2,2'-Azobis (2-amidinopropane)) is the free radical generator and the antioxidant mechanism involved is based on HAT (Arom et al. 2013). The free radical-probe-antioxidant compound interaction is briefly described in Fig. 9.9. AAPH is thermo-decomposed at 37 °C into peroxyl radicals ($ROO^{\bullet}$) and in the presence of an antioxidant compound and a fluorescent probe (fluorescein), the fluorescence loss due to the probe oxidation to non-fluorescent oxidation products of fluorescein is monitored by excitation wavelength at 493 nm and emission wavelength at 515 nm (Ou et al. 2001). Monitoring time varies with the decay in the fluorescence of fluorescein, generally 10 min for blank experiments and greater than 30 min for standard and samples.

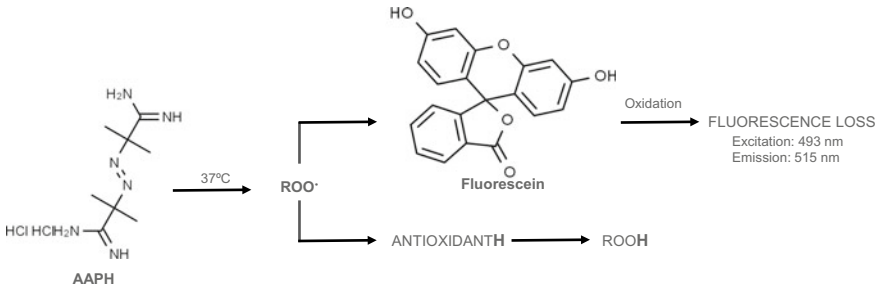


Fig. 9.9 The radical-probe-antioxidant interaction in the ORAC-H assay

The antioxidant efficiency by ORAC-H assay is calculated by evaluating the area under the curve (AUC) generated by the decay of fluorescence intensity as function of time, and the higher the AUC of the antioxidant compared to the blank experiment (in the absence of antioxidant compounds), the greater the antioxidant efficiency. Trolox is used as the standard and therefore, the result is expressed as Trolox equivalent (Ou et al. 2013).

The positive points of ORAC-H are the use of a competitive mechanism similar to physiological reactions, mainly in terms of pH and temperature, and a large number of results available in the literature. The extended operating time required is one of the weaknesses of the assay; another limitation is pointed out by López-Alarcón and Lissi (2006), regarding the probe used. According to the authors, the low reactivity of fluorescein with $ROO^{\bullet}$ may overestimate the result. Moreover, the impossibility of analyzing compounds of lipophilic nature is considered another negative point for the method.

To make the use of ORAC for nonpolar samples possible, Huang et al. (2002) inserted the use of a randomly methylated β-cyclodextrin (RDMC) as a solubility enhancer and validated ORAC-L as an applicable version for lipophilic compounds. In this version, the sample is solubilized in a solution containing 7% RDMC in acetone (50%). The other operating conditions remained the same as described by Ou et al. (2001). In 2012, the hydrophilic and lipophilic versions of the ORAC protocol were evaluated and recognized by the AOAC International (Official Methods of Analysis) (Ou et al. 2013).

Another assay based on a competitive scheme is the PRSC developed by Rodrigues et al. (2012) to evaluate the antioxidant potential of carotenoids. In PRSC, to meet compatibility requirements with the test sample, both the radical generator and the probe have lipophilic characteristics. The solvent considered the most suitable reaction medium was the mixture of dimethyl sulfoxide (DMSO) and methyl *tert*-butyl ether (MTBE) in a 10:1 (v/v) ratio.

Figure 9.10 shows the principle of the method, which measures the ability of nonpolar antioxidants to protect the fluorescent probe, C_{11}-Bodipy$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic

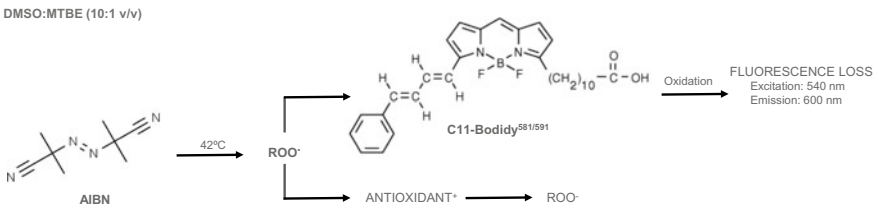


Fig. 9.10 PRSC principle

acid), from the oxidation induced by ROO• generated by AIBN (azobisisobutyronitrile) thermo-decomposition at 42 °C. The antioxidant mechanism involved is HAT. The probe monitoring occurs by excitation at 540 nm and emission at 600 nm for 180 min. The α -tocopherol is used as a reference standard, and the result is expressed in an admentional unit, i.e., it represents how many times the sample is more potent than the standard in scavenging ROO• (α-tocopherol relative) (Rodrigues et al. 2012).

In the same way that it is observed in ORAC-L and ORAC-H, the fluorescence loss of the probes is an indication of the extent of the oxidative damage mediated by ROO•; in other words, the longer the inhibition of probe fluorescence, the higher the antioxidant capacity of the antioxidant compound. The main advantage of this protocol is the structural compatibility of all lipophilic components involved, so that all remain dissolved. On the other hand, hydrophilic compounds cannot be analyzed, and the long analysis time and temperature outside physiological conditions are other limiting aspects.

All protocols described in this chapter are passible of miniaturization (e.g., adaptation to microanalyses in a microplate reader). In addition, they are not absolute and, therefore, can be optimized logically to achieve specific experimental purposes (Alam and Bristi 2013; Granato et al. 2018). However, to obtain reliable results, some important parameters, such as pH and temperature, must be highly controlled. Moreover, the use of adequate blanks, positive controls, testing of different antioxidant concentrations, and ensuring the solubility of tested compounds are fundamental for unequivocal measurement.

9.4 Antioxidant Protocols Applied in Microalgae Extracts

Finally, to facilitate the search for results for the determination of the antioxidant capacity of bioactive compounds from microalgae, a database was constructed and presented in Table 9.2. The database includes the target microalgae species, the extract fraction analyzed, the applied assay, and the literature reference where the original data can be found.

As can be seen in Table 9.2, in most studies, several in vitro chemical assays are used to evaluate the antioxidant capacity of the same sample. This fact may be

Table 9.2 Database of antioxidant capacity of microalgae extracts measured by different assays

Microalgae	Fraction evaluated	Assay	Antioxidant Capacity	References
Eucapsis alpina	soluble in ethanol	ABTS	15.31 mg AAE.100 g^{-1}	Assunção et al. (2017)
Eucapsis alpina	soluble in ethanol	ABTS	28.61 mg TE.100 g^{-1}	Assunção et al. (2017)
Porphyridium aerugineum	soluble in ethanol	ABTS	50.25 mg AAE.100 g^{-1}	Assunção et al. (2017)
Porphyridium aerugineum	soluble in ethanol	ABTS	67.95 mg TE.100 g^{-1}	Assunção et al. (2017)
Chlorella vulgaris	soluble in ethanol	ABTS	28.06 mg AAE.100 g^{-1}	Assunção et al. (2017)
Chlorella vulgaris	soluble in ethanol	ABTS	147.99 mg TE.100 g^{-1}	Assunção et al. (2017)
Haematococcus pluvialis	soluble in ethanol	ABTS	92.68 mg AAE.100 g^{-1}	Assunção et al. (2017)
Haematococcus pluvialis	soluble in ethanol	ABTS	89.77 mg TE.100 g^{-1}	Assunção et al. (2017)
Scenedesmus obliquus	soluble in methanol	DPPH	18%	Banscota et al. (2019)
Scenedesmus sp.	soluble in methanol	DPPH	28%	Banscota et al. (2019)
Botryococcus braunii	soluble in methanol	DPPH	28%	Banscota et al. (2019)
Chlorella sorokiniana	soluble in methanol	DPPH	20%	Banscota et al. (2019)
Nannochloropsis granulata	soluble in methanol	DPPH	25%	Banscota et al. (2019)
Neochloris oleoabundans	soluble in methanol	DPPH	12%	Banscota et al. (2019)
Phaeodactylum tricornutum	soluble in methanol	DPPH	25%	Banscota et al. (2019)
Porphyridium aerugineum	soluble in methanol	DPPH	35%	Banscota et al. (2019)
Tetraselmis chui	soluble in methanol	DPPH	45%	Banscota et al. (2019)
Nostoc 2S3B	phenolic content	DPPH	$IC_{50} = 9.47$ mg.mL^{-1}	Blogojevic et al. (2018)
Arthrospira S1	phenolic content	DPPH	$IC_{50} = 0.12$ mg.mL^{-1}	Blogojevic et al. (2018)
Anabeana C5d	phenolic content	DPPH	$IC_{50} = 0.12$ mg.mL^{-1}	Blogojevic et al. (2018)

(continued)

Table 9.2 (continued)

Microalgae	Fraction evaluated	Assay	Antioxidant Capacity	References
Eucapsis alpina	soluble in ethanol	DPPH	$IC_{50} = 380.29$ mg.mL^{-1}	Assunção et al. (2017)
Porphyridium aerugineum	soluble in ethanol	DPPH	$IC_{50} = 157.53$ mg.mL^{-1}	Assunção et al. (2017)
Chlorella vulgaris	soluble in ethanol	DPPH	$IC_{50} = 108.63$ mg.mL^{-1}	Assunção et al. (2017)
Haematococcus pluvialis	soluble in ethanol	DPPH	$IC_{50} = 528.88$ mg.mL^{-1}	Assunção et al. (2017)
Oscillatória sp	phenolic content + phycobiliprotein	DPPH	456.31 mg AAE.g^{-1}	Hossain et al. (2015)
Nostoc	phenolic content	DPPH	102.47 IC_{50} μg mL^{-1}	Babi et al. (2016)
Oscillatoria	phenolic content	DPPH	30.72 IC_{50} μg mL^{-1}	Babi et al. (2016)
Calothrix	phenolic content	DPPH	30.72 IC_{50} μg mL^{-1}	Babi et al. (2016)
Anabaena	phenolic content	DPPH	50.54 IC_{50} μg mL^{-1}	Babi et al. (2016)
Phormidium	phenolic content	DPPH	47.62 IC_{50} μg mL^{-1}	Babi et al. (2016)
Spirulina maxima	phycocyanin	DPPH	32.10%	Choi and Lee (2018)
Arthrospira platensis	phenolic content + phycobiliprotein	FRAP	482 μg TE.g^{-1}	Castro et al. (2019)
Arthrospira S1	phenolic content	FRAP	21.01 mg AAE.g^{-1}	Blogojevic et al. (2018)
Nostoc 2S3B	phenolic content	FRAP	13.69 mg AAE.g^{-1}	Blogojevic et al. (2018)
Anabeana C5d	phenolic content	FRAP	12.89 mg AAE.g^{-1}	Blogojevic et al. (2018)
Oscillatória sp	phenolic content + phycobiliprotein	FRAP	39.63 μM Fe[II].100 g^{-1}	Hossain et al. (2015)
Phormidium	phenolic content	FRAP	22.48 mg AAE.g^{-1}	Babi et al. (2016)
Nostoc	phenolic content	FRAP	21.50 mg AAE.g^{-1}	Babi et al. (2016)
Oscillatoria	phenolic content	FRAP	22.04 mg AAE.g^{-1}	Babi et al. (2016)
Calothrix	phenolic content	FRAP	16.88 mg AAE.g^{-1}	Babi et al. (2016)

(continued)

Table 9.2 (continued)

Microalgae	Fraction evaluated	Assay	Antioxidant Capacity	References
Anabaena	phenolic content	FRAP	21.93 mg AAE.g^{-1}	Babi et al. (2016)
Botryococcus braunii	phenolic content	ORAC-H	16.35 μmol TE.g^{-1}	Banscota et al. (2019)
Dunaliella salina	carotenoids	ORAC-H	577 μmol TE.g^{-1}	Ahmed et al. (2014)
Tetraselmis suecica	carotenoids	ORAC-H	300 μmol TE.g^{-1}	Ahmed et al. (2014)
Isochrysis galbana	carotenoids	ORAC-H	170 μmol TE.g^{-1}	Ahmed et al. (2014)
Pavlova salina	carotenoids	ORAC-H	275 μmol TE.g^{-1}	Ahmed et al. (2014)
Scenedesmus obliquus	phenolic content	ORAC-H	29.25 μmol TE.g^{-1}	Banscota et al. (2019)
Scenedesmus sp.	phenolic content	ORAC-H	24.49 μmol TE.g^{-1}	Banscota et al. (2019)
Chlorella sorokiniana	phenolic content	ORAC-H	14.36 μmol TE.g^{-1}	Banscota et al. (2019)
Nannochloropsis granulata	phenolic content	ORAC-H	16.5 μmol TE.g^{-1}	Banscota et al. (2019)
Neochloris oleoabundans	phenolic content	ORAC-H	30.97 μmol TE.g^{-1}	Banscota et al. (2019)
Phaeodactylum tricornutum	phenolic content	ORAC-H	16.85 μmol TE.g^{-1}	Banscota et al. (2019)
Porphyridium aerugineum	phenolic content	ORAC-H	16.71 μmol TE.g^{-1}	Banscota et al. (2019)
Tetraselmis chui	phenolic content	ORAC-H	14.52 μmol TE.g^{-1}	Banscota et al. (2019)
Phormidium Autumnale	phycocyanin	ORAC-H	237.4 μmol TE.g^{-1}	Rodrigues et al. (2015)
Scenedesmus obliquus	carotenoids	ORAC-L	14.81 μmol TE.g^{-1}	Banscota et al. (2019)
Scenedesmus sp.	carotenoids	ORAC-L	10.31 μmol TE.g^{-1}	Banscota et al. (2019)
Scenedesmus obliquus	carotenoids	ORAC-L	1779.9 μmol TE.g^{-1}	Nascimento et al. (2019)
Botryococcus braunii	carotenoids	ORAC-L	26.25 μmol TE.g^{-1}	Banscota et al. (2019)
Chlorella sorokiniana	carotenoids	ORAC-L	19.04 μmol TE.g^{-1}	Banscota et al. (2019)

(continued)

Table 9.2 (continued)

Microalgae	Fraction evaluated	Assay	Antioxidant Capacity	References
Nannochloropsis granulata	carotenoids	ORAC-L	52.98 μmol TE.g^{-1}	Banscota et al. (2019)
Nannochloropsis oleoabundans	carotenoids	ORAC-L	14.11 μmol TE.g^{-1}	Banscota et al. (2019)
Phaeodactylum tricornutum	carotenoids	ORAC-L	14.62 μmol TE.g^{-1}	Banscota et al. (2019)
Porphyridium aerugineum	carotenoids	ORAC-L	10.16 μmol TE.g^{-1}	Banscota et al. (2019)
Tetraselmis chui	carotenoids	ORAC-L	6.69 μmol TE.g^{-1}	Banscota et al. (2019)
Scenedesmus obliquus	phenolic content	RC	29.3 μmol GAE.g^{-1}	Banscota et al. (2019)
Scenedesmus sp.	phenolic content	RC	39.8 μmol GAE.g^{-1}	Banscota et al. (2019)
Dunaliella salina	phenolic content	RC	9.05 μmol GAE.g^{-1}	Ahmed et al. (2014)
Tetraselmis suecica	phenolic content	RC	4.52 μmol GAE.g^{-1}	Ahmed et al. (2014)
Isochrysis galbana	phenolic content	RC	1.38 μmol GAE.g^{-1}	Ahmed et al. (2014)
Pavlova salina	phenolic content	RC	1.32 μmol GAE.g^{-1}	Ahmed et al. (2014)
Botryococcus braunii	phenolic content	RC	52.9 μmol GAE.g^{-1}	Banscota et al. (2019)
Chlorella sorokiniana	phenolic content	RC	37.2 μmol GAE.g^{-1}	Banscota et al. (2019)
Nannochloropsis granulata	phenolic content	RC	43.6 μmol GAE.g^{-1}	Banscota et al. (2019)
Neochloris oleoabundans	phenolic content	RC	26.6 μmol GAE.g^{-1}	Banscota et al. (2019)
Phaeodactylum tricornutum	phenolic content	RC	44.7 μmol GAE.g^{-1}	Banscota et al. (2019)
Porphyridium aerugineum	phenolic content	RC	36.4 μmol GAE.g^{-1}	Banscota et al. (2019)
Tetraselmis chui	phenolic content	RC	57.5 μmol TE.g^{-1}	Banscota et al. (2019)
Phormidium autumnale	carotenoids	PRSC	28.1 (α-TR)	Rodrigues et al. (2015)
Chlorella vulgaris	carotenoids	PRSC	31.1 (α-TR)	Patias et al. (2017)
Aphanothece m. Nägeli	carotenoids	PRSC	7.3 (α-TR)	Patias et al. (2017)

(continued)

Table 9.2 (continued)

Microalgae	Fraction evaluated	Assay	Antioxidant Capacity	References
Scenedesmus obliquus	carotenoids	PRSC	14.0 (α-TR)	Patias et al. (2017)
Phormidium autumnale	chlorophyll	PRSC	200.0 (α-TR)	Fernandes et al. (2017)
Phormidium autumnale	chlorophyll	PRSC	84.9 (α-TR)	Rodrigues et al. (2015)

DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric Reducing Antioxidant Power; RC: Reducing Capacity; ORAC-H: Oxygen Radical Absorbance Capacity for hydrophilic antioxidants; ORAC-L: Oxygen Radical Absorbance Capacity for lipophilic antioxidants; PRSC: Peroxyl Radical Scavenging capacity; TE: Equivalent Trolox; AAE: Ascorbic acid equivalent; $IC_{50:}$ Inhibitory Concentration; GAE: Gallic acid equivalent; α-TR: α-tocoferol relative

explained because the same bioactive compounds can act by different mechanisms, although there is a preference, as can be seen in the previous sections. Thus, the scavenging capacity of bioactive compounds should not be completed based on a single assay, as this strategy cannot provide a total comprehensive prediction of antioxidant efficacy (Aruoma 2003). Considering, at least, two different antioxidant mechanisms of action is an excellent strategy to achieve greater relevance.

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Chapter 10
Analytical Protocols in the Measurement of Pigments' Bioavailability

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Abstract Microalgae have been recognized as the foundation of the food chain in aquatic ecosystems and one of the most prominent characteristics of algae is their color, which is determined by their pigments, hence, microalgal biomass is attracting worldwide attention. These pigments are colorful chemical substances that are part of the photosynthetic system of microalgae and are distinguished into three classes: carotenoids, chlorophylls, and phycobiliproteins. Besides the color, pigments have health-promoting properties and a broad range of potential industrial applications. Consumers are becoming increasingly aware of the correlation between diet, health, and disease prevention. Despite the beneficial properties of pigments provided by microalgae, their effectiveness at preventing a range of diseases depends on their bioaccessibility and bioavailability. The digestion process comprises several steps, which promotes an intense variation of the conditions that the pigments are exposed, and therefore, could in several ways compromises the health benefits caused by microalgae pigment consumption. Therefore, the present chapter aims to present the main methods used to assess the bioaccessibility and bioavailability of pigments from microalgae to better understand the processes involved. Consequently, providing information about the most accepted analytical protocols in measurement of bioavailability of pigments.

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10.1 Introduction

Microalgae have been recognized as the foundation of the food chain in aquatic ecosystems. In addition, they constitute a large and diversified group which includes primitive prokaryotic or eukaryotic photosynthetic microorganisms. Due to their simple constitution, they are able to grow quickly and live in difficult environments such as the ones that present extreme temperatures, absence of oxygen, high salinity, and exposure to ultraviolet radiation. One of the most prominent characteristics of algae is their color, which is determined by their pigments, hence, microalgal biomass is attracting worldwide attention. These pigments are colorful chemical substances that are part of the photosynthetic system of microalgae and are distinguished into three classes: carotenoids, chlorophylls, and phycobiliproteins. In addition to color, pigments have health-promoting properties and a broad range of potential industrial applications. Consumers are becoming increasingly aware of the correlation between diet, health, and disease prevention; in this context, dietary intake of microalgae has been shown to have the ability to protect organisms against various chronic disorders, such as cancer, diabetes, atherosclerosis, coronary disease, ischemic brain development, metabolic syndromes, gastrointestinal, and liver diseases, as well as neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Despite the beneficial properties of pigments provided by microalgae, their effectiveness at preventing or treating a range of diseases depends on their bioaccessibility and bioavailability. The digestion process comprises several steps, which promote an intense variation of the conditions to which pigments are exposed, and therefore, could in numerous ways compromise the health benefits of consuming microalgae pigments. Several methods to determine the bioaccessibility and bioavailability of pigments have been developed and studied in the last years, each one with its particularities in order to maximize and improve the results regarding this subject. Therefore, the present chapter aims to present the main methods used to assess the bioaccessibility and bioavailability of pigments from microalgae to better understand the processes involved, consequently providing information about the most accepted analytical protocols in measurement of pigments bioavailability.

10.2 Challenges to assess bioavailability of microalgae pigments

Microalgae pigments of major commercial interest are carotenoids, especially: 1- *Dunaliella salina* (β-carotene); 2- *Haematococcus pluvialis* (astaxanthin, canthaxanthin and lutein); 3- *Chlorella vulgaris* (canthaxanthin and astaxanthin); 4- *Coelastrella striolata* var. multistriata (canthaxanthin, astaxanthin and β-carotene); and 5- *Scenedesmus almeriensis* (lutein and β-carotene) (Guedes et al. 2011). Chlorophylls and phycobiliproteins are still of limited interest due to the insipient market.

Most studies on microalgae focus on the production and application of biomass and pigments extracted from biomass. Determination of the bioaccessibility and bioavailability of microalgal pigments has been the focus of very few researches in the last years. However, some aspects are more exploited than others. From the data shown in the literature, the most studied pigments from microalgae are carotenoids. Researches about the behavior of chlorophylls and phycobiliproteins are very rare. In addition, even for carotenoids, there is no unified protocol specific for microalgal sources, although, for food in general, a harmonized method called INFOGEST was developed and tested for the in vitro digestion method (Egger et al. 2016).

Just as the different pigments classes present distinct ways to be produced, they also behave differently in the gastrointestinal system and, hence, perform differently against bioavailability. So, it is mandatory to answer the main questions around the bioavailability of pigments evaluated by different protocols, regardless of the work applies in vitro models or in vivo (animal and/or human) protocols.

Several methods have been proposed to assess bioavailability, including in vitro, ex vivo, and in vivo protocols, and the found articles are summarized in Table 10.1. A discussion remains concerning the quality of the data produced from in vitro and/or in vivo methods. Some authors consider in vivo approaches more trustworthy since the reactions and components occur in the human body. However, several studies have shown a correlation between the results obtained for the same compound using in vivo and in vitro systems (Brown et al. 2014).

Bioavailability assessment is grounded on three main steps: digestibility and solubility of the component in the gastrointestinal tract; absorption of the component by the intestinal cells and transport into the circulation; and incorporation from the circulation to the functional entity or target (Mackie and Rigby 2015).

10.3 In Vitro Bioavailability Methods

All methods to assess the bioavailability of microalgal pigments applied and described in the articles follow almost the same steps and sequence of experiments as outlined in Fig. 10.1. Most of the studies about bioavailability of microalgal pigments used in vitro methods.

Particularly regarding in vitro methods, they range from simple enzyme reactions, such as hydrolysis of proteins by pepsin under rather non-physiological conditions in a beaker, to the complex sophistication of the TNO gastrointestinal model and other similar dynamic models (Mackie and Rigby 2015).

Static digestion models are widely used as the first step to determine bioavailability of pigments from microalgae. Gille and collaborators (2017) evaluated the in vitro digestion process for carotenoids' extraction from *Phaeodactylum tricornutum*. The authors performed in vitro digestion using porcine pepsin (40 mg/mL in 0.1 N HCl), then the pH was adjusted to a value between 2.2 and 2.4 and this step was followed by shaking at 37 °C in a water bath for 1 h. Subsequently, porcine bile extract (12 mg/mL in 0.1 M Na_2CO_3) and pancreatin (0.2 mg/ml in 0.1 M Na_2CO_3) were

Table 10.1 List of studies in the literature reporting the bioaccessibility and bioavailability of pigments from microalgae

In vitro				
Authors	Year	Pigments	Methods	
			In vitro digestion	In vitro bioavailability
Ferruzzi et al	(2002)	Sodium Copper Chlorophyllin	In vitro protocol using static digestion model	TC7 clone of the Caco-2
Gille et al	(2018)	Carotenoids	In vitro protocol using static digestion model	Not reported
Gille et al	(2017)	Carotenoids	In vitro protocol using static digestion model	Not reported
Gille et al	(2019)	Carotenoids	In vitro protocol using static digestion model	Caco-2 human cells
Minic et al	(2016)	C-phycocyanin	In vitro protocol using static digestion model	Not reported
Sugawara et al	(2002)	Carotenoids	Not reported	Caco-2 human cells
Wu et al	(2015)	R-phycoerythrin	In vitro protocol using static digestion model	Not reported
Ex vitro and In vivo				
Authors	Year	Pigments	Methods	
Gille et al	(2018)	Carotenoids	Feeding experiment with C57BL/6 J mice	
Hartmann et al	(2004)	Carotenoids	Plasma kinetics of synthetic zeaxanthin after repeated oral doses and assessment of the possible influence of other carotenoids on plasma zeaxanthin concentrations	
Ranga Rao et al	(2010)	Carotenoids	Feeding experiment with mice	
Rao et al	(2013)	Carotenoids	Feeding experiment with mice	
Sangeetha et al	(2009)	Carotenoids	Feeding experiment with mice	
Shibata and Hayakawa	(2009)	Carotenoids	Human Volunteers	
Sugawara et al	(2002)	Carotenoids	Feeding experiment with mice	

added, the pH set to a value between 7.2 and 7.6 and the samples were overlaid with nitrogen gas. This was followed by incubation for 2 h at 37 °C under dark in a shaking water bath. To separate micellized carotenoids, the remaining digestate was centrifuged at 13,000 × g for 10 min and the aqueous phase filtered (0.45 μm pore size) to remove any contaminating aggregates and oil droplets. After the in vitro digestion process, Caco-2 cells were used to assess cellular carotenoid uptake and transepithelial transport. The authors concluded that *P. tricornutum* represent a good source of carotenoids, particularly fucoxanthin. Thus, this diatom can contribute to the intake of bioaccessible carotenoids.

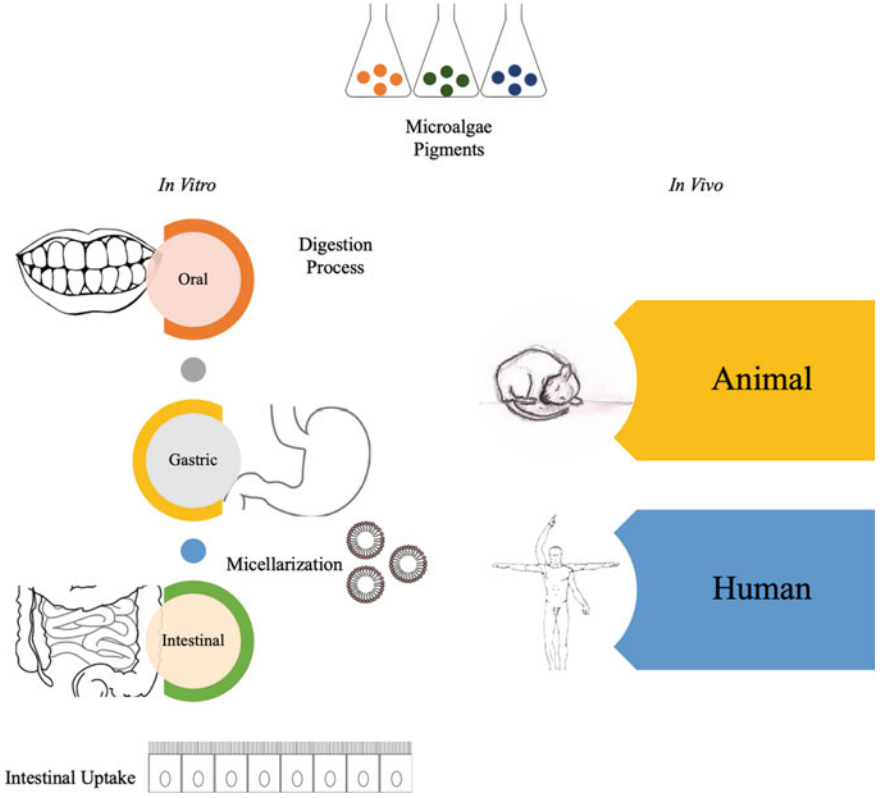

Fig. 10.1 Scheme representing methods applied in the determination of microalgal pigments bioavailability

The application of the microfluidization method was tested to increase bioaccessibility of carotenoids from *Chlorella ellipsoidea* during simulated digestion (Cha et al. 2012). The operating pressure of the microfluidizer was varied at three levels—5,000, 10,000, and 20,000 psi. The microfluidization products underwent three simulated digestion steps employing 200 mg of biomass. α-Amylase (3,000 units) was employed in the oral phase with pH adjusted to 6.5 and incubation at 37 °C for 5 min under 95 rpm agitation. Gastric digestion was performed with incubation for 1 h using porcine pepsin (0.04 g/mL HCl) at pH 2.0 and, finally, the intestinal phase was performed at pH 5.3 using bile salts and pancreatic lipase (40 mg in 1 mL bicarbonate) at 37 °C for 2 h under shaking. After cooling, the micelles were separated using ultracentrifugation. Very interesting results were obtained: for the antheraxanthin carotenoid, no micellarization was observed in any of the three conditions used for microfluidization; while for zeaxanthin and β-carotene, the bioaccessibility increased from 2.60% and 1.69% to 32.6% and 18.19%, respectively, when comparing the untreated and the microfluidized at 20,000 psi *C. ellipsoidea.* Probably, the microfluidization process promoted a disruption in the cell wall of *C.*

ellipsoidea, making the carotenoids more exposed and, consequently, more easily micellarized during the digestion process. Similar results have already been reported by Gille et al. (2016), who tested the sonication of the microalgae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* for 15 min using 5 cycles/min at a frequency of 20 kHz and observed an increase in bioaccessibility of ≥10% for lutein and ≥ 15% for β-carotene. However, the sonication process contributed to losses in carotenoid contents, probably due to temperature- and oxygen-mediated degradation reactions, with lutein and β-carotene contents being 21% and 35% lower when the sonication of *C. reinhardtii* was performed, respectively. Thus, the use of biomass pretreatment processes may contribute to increase bioaccessibility but may result in loss of carotenoid stability.

The use of cellulase as pretreatment to assist in the breakdown of microalgae cells was tested by Gille et al. (2019) in *Phaeodactylum tricornutum*, to increase carotenoid yield. One-hundred U of cellulase at pH 3.5—4.0 were added in 200 mg of biomass in HBSS medium under stirring for 4 h at 37 °C. After simulated digestion, the micellar fraction was applied in a Caco-2 cell model. Fucoxanthin, zeaxanthin, and β-carotene were the main carotenoids present in *P. Tricornutum*. Surprisingly, fucoxanthin showed 52% of bioaccessibility, a value considered high compared to other dietary sources. However, other studies carried out with macroalgae fucoxanthin showed a bioaccessibility of 70% for this carotenoid (Asai et al. 2008), probably due to the presence of high contents of unsaturated fatty acids, which are present both in microalgae and in higher algae. Regarding cell absorption in Caco-2 model, the authors observed that zeaxanthin and fucoxanthin were the most abundant carotenoids in the cell monolayer. In addition, fucoxanthin was metabolized to fucoxanthinol due to the enzymatic activity of the cells, while β-carotene was not detected in the basolateral fraction (Gille et al. 2019).

The incorporation of unsaturated fatty acids is a recurring alternative to ensure carotenoid micellarization during in vitro digestion processes. Granado-Lorencio et al. (2009) added olive oil to the biomass of *Scenedesmus almeriensis* and observed a significant increase of lutein and zeaxanthin in the micellar fraction.

Information on the digestion, absorption, and metabolism of chlorophylls and their derivatives is limited. Some studies focused on the isolation of chlorophyll derivatives in feces of humans and animals, assuming minor absorption. Since natural chlorophylls are adapted by heat and acid, they can be inclined to degradation when exposed to the severe digestive environment. Select chlorophyll derivatives have been shown to exhibit different properties in in vitro assays (Chernomorsky et al. 1999, Ferruzzi et al. 2001).

Sodium copper chlorophyllin (SCC), a mixture of water-soluble chlorophyll derivatives, is used as both a food colorant and as a common dietary supplement and, as observed for chlorophyll, limited information is available on its digestion and absorption by humans. Stability of SCC was studied during simulated gastric and small intestinal digestion by Ferruzzi et al. (2002). The authors prepared three test preparations from an aqueous stock solution of SCC. The first preparation simply contained aqueous SCC (W). The second preparation consisted of a homogenized mixture of SCC and 10% corn oil (wt/wt) in water (WCO). The third preparation

contained SCC homogenized in an applesauce matrix with 10% corn oil (AS). The final SCC concentrations ranged from 50 to 250 μg/mL for all experimental preparations. Aliquots of each preparation were exposed to a two-phase in vitro digestion protocol. The gastric phase included acidification of the sample to pH 2.0 with 100 mM HCl and exposure to porcine pepsin (3 mg/mL) with incubation at 37 °C for 1 h in a shaking water bath (95 rpm). The small intestinal phase was initiated by neutralizing the gastric phase with $NaHCO_3$, adding porcine pancreatin (0.4 mg/mL), lipase (0.2 mg/mL), and bile extract (2.4 mg/mL), and adjustment of the final pH value to 7.0 with 1 N NaOH prior to incubation at 37 °C in a shaking water bath (95 rpm). After 2 h, aliquots of the digestate were centrifuged at 167,000 g at 4 °C for 35 min to separate the aqueous micellar fraction from residual solids and oil. Isolated aqueous fractions were filtered (0.2-micron pore size) to remove contaminating aggregates. Caco-2 human intestinal cells were used to characterize the accumulation of SCC. The authors found that Cu(II)chlorin e_4, the major chlorin component of SCC, was relatively stable during simulated digestion. On the other hand, more than 90% of Cu(II) chlorin e_6 was degraded to undetermined products during digestion. Accumulation of SCC derivatives was also investigated by using differentiated cultures of the TC7 clone of the Caco-2 human intestinal cell line. Cellular accumulation in media containing 0.5 to 60 ppm SCC was linear with intracellular content ranging between 0.2 and 29.6 μg of total SCC per mg of cellular protein.

The literature data on bioavailability of phycobiliproteins is very scarce, showing the importance to investigate this field, especially in order to stablish protocols that allow the exchange and comparison between results obtained worldwide. Most studies that evaluate the bioavailability related to microalgae involve the availability and digestibility of some of the constituents, particularly the minerals. However, the steps to evaluate bioavailability are the same followed to evaluate the absorption of carotenoids and chlorophyll.

In recent years, there is a growing number of studies on protein hydrolysis as an effective method to produce bioactive peptides and several works have been pointing to the possibility that the biological effects that bring health benefits through the intake of microalgal pigments are related to these peptides. Bioactive peptides are obtained from enzymatic hydrolysis and exhibit excellent biological effects. Protein hydrolysis is generally performed by enzymes derived from microorganisms or plants, but digestion by enzymes of the gastrointestinal tract (GIT), such as pepsin or trypsin, present physiological relevance (Begum et al. 2016).

Ingestion of phycocyanin, resulting from the intake of *Spirulina* dietary supplements, is a very plausible way to evaluate the bioavailability for these pigments, since there is a potential susceptibility to GIT proteolysis related to the oral administration and consumption, to better understand the structure and bioactivities of the released chromopeptides. There are very few data on the literature about bioactivities of peptides obtained after C-phycocyanin digestion, as well as bioactivities of peptides with covalently bound bioactive chromophores (chromopeptides) in general.

Minic and others (2016) examined the digestibility of C-phycocyanin (C-PC) by pepsin in simulated gastric fluid. The authors used simulated in vitro digestion, followed by SDS polyacrylamide gel electrophoresis. The pepsin digest of C-PC was

separated using a semi-preparative C-18 column connected with a HPLC system. Further, each chromopeptide fraction (with absorbance at 615 nm) was collected and analyzed by mass spectrometry. For the bioactivity assays, the solvent was evaporated, and the resulting pellet was dissolved in 20% DMSO. Rechromatography of each fraction was performed under analytical conditions. Additionally, cytotoxicity of each chromopeptide fraction on Human cervical adenocarcinoma (HeLa) and human epithelial colonic carcinoma (Caco-2) cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay (Mosmann 1983). Data collected was expressed as percentage of viability with untreated cells taken as 100%. The authors showed that the released chromopeptides varied in size from 2 to13 amino acid residues and were identified in both subunits of C-phycocyanin. In addition, all five chromopeptide fractions obtained presented significant antioxidant and metal-chelating activities and showed cytotoxic effects on human cervical adenocarcinoma and epithelial colonic cancer cell lines. There was a positive correlation between the antioxidative potency and the other biological activities of chromopeptides. Finally, they concluded that the digestion by pepsin releases biologically active chromopeptides from C-phycocyanin, and the bioactivity is mostly related to the antioxidative potency provided by the chromophore.

In the same line of work, Wu et al. (2015) evaluated purified R-phycoerythrin (R-PE) after in vitro-simulated GI digestion to investigate the antioxidant activity of the resulting peptides. The results indicated that digestion-resistant antioxidant peptides of R-PE may be obtained by in vitro GI proteinases degradation.

Regardless of the protocol used to determine the behavior of microalgal pigments throughout the in vitro digestion process, the steps are very similar, but some differences related to enzyme concentrations, agitation speed, reagent concentrations, among others, are observed in the different studies.

Despite this diversity in the methods and applications, one can draw a few general conclusions about the design of gastrointestinal (GI) models. Firstly, the model should be as simple as possible, but not so simple that the results do not provide relevant information to the "real life" situation. Secondly, what has been done previously is not always the best or the most relevant approach. Finally, digestion is not a goal in itself and the way that digestate samples are collected is very dependent on the type of measurement to be made.

10.4 In Vivo Bioavailability Methods

Rats and mice models have been used to understand the microalgal carotenoid distribution on different organs and tissues, mainly in liver, plasma, and feces. Carotenoids were evaluated in rats after administration of microalgal biomass. The quantification of carotenoids was carried out by the absorbance of the microalgal extracts, previously obtained from *Spirulina platensis*, *Haematococcus pluvialis,* and *Botryococcus braunii*, measured at 450, 470, 645, and 661.5 nm to estimate the content of total carotenoids and chlorophyll using Lichtenthaler equations. Astaxanthin was

determined at 480 nm using an extinction coefficient of 2500 at the 1% level. To determine the bioavailability of carotenoids, a single dose (134, 33, 328 mg/rat, corresponding to 200 μM of β-carotene, astaxanthin, and lutein) of *S. platensis* or *H. pluvialis* or *B. braunii* biomass solubilized in olive oil was administered by intubations to the stomach of rats (n = 25). Each group was divided into five subgroups (n = 5/subgroup) to measure the time-course response of carotenoids in plasma and tissues for 9 h. The results showed peak levels in plasma, liver, and eyes at 2, 4, and 6 h, respectively, and the authors concluded that microalgae can be a good source of carotenoids of high bioavailability and nutraceutical value (Ranga Rao et al. 2010). Ranga Rao and contributors (2013) administered 200 μM equivalent of β-carotene, astaxanthin, and lutein from *Spirulina platensis*, *Haematococcus pluvialis,* and *Botryococcus braunii* biomass dispersed in olive oil to rats for a period of 15 days. The levels of these carotenoids in the plasma, liver, and eyes were examined by high performance liquid chromatography and also confirmed by mass spectroscopy. The maximum peak levels (nmol/g) of β-carotene (615.61 ± 85.54), astaxanthin (896.51 ± 101.76) and lutein (679.55 ± 74.08) were detected in the liver followed by the eyes and plasma. Astaxanthin accumulation in rats fed with *H. pluvialis* was higher when compared to *S. platensis* and *B. braunii* fed groups. In the *H. pluvialis* fed group, the levels of the antioxidant enzymes catalase, superoxide dismutase, peroxidase, and the lipid peroxidation levels were higher in the plasma and liver when compared to *S. platensis* and *B. braunii* fed groups. These results indicate that astaxanthin from the *H. pluvialis* group presents higher bioavailability and better antioxidant properties compared to other carotenoids.

Gille et al. (2018) performed a comparative study that evaluated the in vitro digestion method and a feeding experiment with C57BL/6 J mice. Female C57BL/6 J mice used in the study were 6–8 weeks old at the beginning of the feeding experiments. All mice had ad libitum food and water access. A total of 10 groups were formed, 8 animals to each group. Health scores were assessed daily and weight every three days. The experiment lasted 14 days. For carotenoid and retinoid determination, 30–50 mg of the liver and adipose tissue was weighed and homogenized briefly by sonication for 5 s in 250 μL HBSS. Afterwards, 400 μL THF containing 0.25 mg BHT/mL were added and the samples stored at -20 °C until used for carotenoid extraction. Accumulation of carotenoids in liver and adipose tissue was detected and ranged from 10 to 40%. The authors stated that data provided by these results can be used as the basis for the potential application of the microalgae species *C. vulgaris* and *P. tricornutum* in food products. The applied microalgae species might specially serve as good sources for the xanthophylls lutein and zeaxanthin due to their good bioaccessibility and accumulation in the liver of the mice. Moreover, it was stated that mice might not be the best model to assess carotenoid and retinoid bioavailability and metabolism. Therefore, further investigation is necessary especially in terms of bioactivity and metabolism of fucoxanthin in humans.

Shibata and Hayakawa (2009) assessed the effect of a single ingestion of *Chlorella* powder (CP) (3 or 6 g) in tablet form on serum lutein concentrations in humans (n = 21). The total experiment period was 11 days and subjects maintained their usual lifestyle (eating habits, exercise, sleep, and daily work) during the experiment;

however, they were instructed not to take vitamin supplement tablets, drink nutritional supplements, or drink vegetable juice during the study, because these products potentially contain large amounts of lutein. Before ingestion of the CP tablets, subjects (n = 21) were divided into three groups based on initial serum lutein levels, BMI, and age. A fasting blood sample was collected in the morning (08:00 to 09:00) at –7, 0, 1, 2, and 3 d. On the morning of 0 d, after the blood sampling, each subject took a single CP dose (0, 3, or 6 g) in tablet form with bread (40 g), olive oil (10 g), and water (280 mL). During the experiment, the subjects kept food diaries regarding their consumption of vegetables, fruits, and eggs to allow estimation of their dietary intake of lutein. The carotenoid content of the foods was estimated from data by the USDA National Nutrient Database for Standard Reference. The carotenoid content of the 3 and 6 g tablets was (respectively): 8.6 and 17.3 mg of lutein, 1.3 and 2.6 mg of α-carotene, and 2.1 and 4.2 mg of β-carotene. For the analysis of serum carotenoid concentrations, blood samples (5 to 10 mL) were drawn from a forearm vein, and serum was separated from the blood cells by centrifugation. At 1 d, serum lutein concentrations increased from baseline values by 34% (25.6 nM/mg-lutein intake) after 3 g CP and by 66% (21.4 nM/mg-lutein intake) after 6 g CP. These results suggest that lutein in CP is highly bioavailable.

10.5 Conclusion

To date, studies aimed at determining the bioaccessibility and bioavailability of pigments from microalgae are very restricted. It is necessary to apply more modern methods, such as the harmonized method by INFOGEST, in order to compare results obtained in different laboratories. On the other hand, it can already be seen that the microalgae cell wall is an important barrier for enzymatic digestion, resulting in poor bioaccessibility and consequently bioavailability of carotenoids. For applications in food products and food supplements, it is suggested to apply a technology to break down the cell wall and facilitate the digestion and utilization of these compounds.

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Chapter 11
Microalgae-Based Processes for Pigments Production

Mariana Manzoni Maroneze, Rosangela Rodrigues Dias, Ihana Aguiar Severo, and Maria Isabel Queiroz

Abstract The purpose of this chapter is to provide an overview of the microalgae-based processes for pigment production, especially astaxanthin, β-carotene, and C-phycocyanin. At first, chemistry and biochemistry will be described for a better understanding of pigment's production in the microalgae cells, focusing on the factors influencing the synthesis. Besides this, the upstream and downstream processing operations involved in the production process of the pigments in question will be reviewed in their main aspects. Finally, the main production systems (raceways, extensive unmixed lagoons, and tubular photobioreactors) used in the production of carotenoids and phycocyanin will be presented and discussed in the chapter.

Keywords Astaxanthin · B-carotene · C-phycocyanin · Cultivation systems

11.1 Introduction

Microalgae are prokaryotic and eukaryotic photosynthetic microorganisms adapted to live in very different environments and showing an enormous biochemical and genetic diversity (Riccio and Lauritano 2019). Thus, they represent an excellent source of new bioproducts with potential applications in several biotechnological sectors, being one of the most promising paths for the supply of next-generation foods, feeds, and biofuels. At the same time, it can be integrated with environmental management processes such as CO_2 capture and recycling of nutrients in wastewater (Barros et al. 2019).

Microalgal biomass has a wide range of products that can be explored due to its content of lipids, pigments, carbohydrates, minerals, enzymes, and vitamins.

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However, the high costs of producing microalgal biomass limit the commercialization of bioproducts to some niche markets. The most successful microalgae-based products available commercially belong to the two classes of pigments; carotenoids and phycobiliproteins, especially β-carotene, astaxanthin, and phycocyanin, from *Dunaliella salina*, *Haematococcus pluvialis*, and *Arthrospira platensis*, respectively (Holdmann et al. 2019; Jacob-Lopes et al. 2019).

The demand for natural pigments is growing, as consumers and regulatory agencies increasingly question the use of artificial colorants, which have been linked to health problems. Pigment production using microalgae offers advantages over its production from traditional sources like fruits and vegetables, as these microorganisms have faster growth rates and high photosynthesis efficiencies and also does not require arable land (García-López et al. 2020; Maroneze et al. 2019). Additionally, pigments produced from microalgae have been approved by several food administrations, as Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) and have been recommended to replace synthetic colorants in food, cosmetic, and nutritional markets (Rahman et al. 2016).

One of the main pillars needed to support the development of the microalgae industry is the efficient cultivation of microalgae on a large scale in an economical manner. The microalgae cultivation technology is related both to the design of the type and configuration of open or closed cultivation systems, and the operating conditions leading to the trade-off between optimal growth of the microalgae and the productivity of the target bioproduct (Yen et al. 2019).

This chapter discusses the chemistry, biochemistry, and synthesis of β-carotene, astaxanthin, and phycocyanin. The production processes of these pigments are presented, with emphasis on significant factors that influence their productivities. Moreover, the microalgae cultivation systems used to produce pigments on a commercial scale (raceway ponds, extensive unmixed ponds, and tubular photobioreactors) are presented and discussed.

11.2 Astaxanthin, β-Carotene, and Phycocyanin: Basic Aspects

Carotenoids and phycobiliproteins are accessory pigments that aid in photosynthesis and protect the photosynthetic apparatus against high radiation. The commercially consolidated microalgae-based pigment structures are shown in Fig. 11.1. As for functionality, both biocompounds present biological properties like antioxidants and anti-inflammatory (Siqueira et al. 2018).

Carotenoids are lipophilic substances composed mostly of forty carbon atoms, formed by eight isoprene units. The molecules are linear and symmetrical, and their basic structure can be chemically altered by reactions such as isomerization, cyclization, hydrogenation, rearrangement, and oxidation. These pigments are divided into

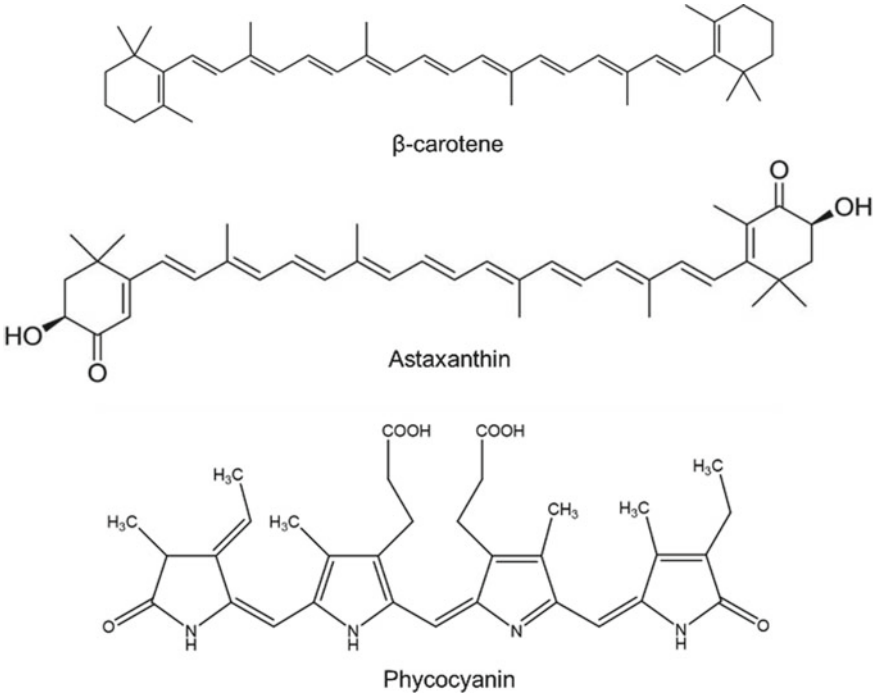

Fig. 11.1 Chemical structure of commercially consolidated microalgal pigments Adapted from Fernandes et al. (2018)

carotenes that are formed exclusively by carbon and hydrogen and xanthophylls that contain oxygen atoms in their molecule (Rodriguez-Concepcion et al. 2018).

Astaxanthin is a xanthophyll also known as 3,3′dihydroxyl-β,β′-carotene-4,4′-dione, widely utilized in food, aquaculture, cosmetic, nutraceutical, and pharmaceutical industries. In addition to its pigmentation, astaxanthin is quite attractive due to its high antioxidant capacity, which is responsible for its protective properties against cancer, cardiovascular diseases, diabetes, immune response, and inflammation (Yuan et al. 2011; Khoo et al. 2019). Astaxanthin from *Haematococcus* is recognized by the Food and Drug Administration (FDA) as Generally Regarded as Safe (GRAS) for use as an ingredient in several food categories (Jacob-Lopes et al. 2019).

The β-Carotene is a primary carotenoid, found in *Dunaliella* mostly in the form of stereoisomers all-*trans*-β,β-carotene and 9-*cis*-β,β-carotene, and a small fraction is composed of some other mono-*cis* and di-*cis* stereoisomers, (Ben-Amotz et al. 1982). The amount of β-carotene accumulated in the form *cis* or *trans* depends on light intensity and on the algal division time, which is determined by the cultivation conditions (Ben-Amotz 2009). This is different from what is found in synthetic β-carotene, composed only of *trans* isomers, which have lower liposolubility and lower antioxidant property than 9-*cis* isomer (Jayappriyan et al. 2013). As with astaxanthin, β-carotene obtained from *Dunaliella* also has GRAS status recognized by the FDA

and is frequently used for human and animal nutrition, food coloring, and cosmetics due to its provitamin A and antioxidant claims (Sui and Vlaeminck 2020).

The phycobiliproteins, in turn, are of a protein nature, hydrophilic, and highly fluorescent. These molecules are classified into phycoerythrin, phycoerythrocyanin, phycocyanin, and allophycocyanin according to their absorption spectra. Besides, based on their colors are classified into two major groups, to know: phycocyanin (blue) and phycoerythrin (red). Phycocyanin, especially C-phycocyanin, is found mainly in cyanobacteria and phycoerythrin in cyanobacteria, cryptophyte, and red microalgae (Pan-utai and Iamtham 2019). Phycocyanin is widely used as a natural dye for various purposes, in the food and cosmetic industry, due to its intense blue pigmentation and its excellent stability. Besides, interest in the use of phycocyanin in healthy foods and as a nutraceutical has grown thanks to its functional properties, such as antioxidant, anti-inflammatory, anti-viral, and anti-cancer (Vernès et al. 2015).

11.3 Biosynthesis of Carotenoids and Phycobiliproteins

The exploration of the microalgae photosynthetic machinery has led to the production of valuable biochemicals. In photoautotrophic metabolism, microalgae convert solar energy into chemical energy by fixing CO_2. From this metabolic pathway, biosynthetic precursors of carotenoid and phycobiliproteins are generated. Both accessory pigments are of commercial interest. The molecular basis of the microalgae pigments biosynthetic pathway is under investigation. The current understanding of metabolism and the regulatory mechanism of synthesis in microalgae is limited and inferred, mainly based on the knowledge obtained for plant cells (Sathasivam and Ki 2018). Figure 11.2 presents the biosynthetic pathways of carotenoids and phycobilins in microalgae.

The carotenoids are lipophilic isoprenoids biosynthesized from isopentenyl diphosphate (IPP) or its isomer dimethylallyl diphosphate (DMAPP) (Fernandes et al. 2017). There are two distinct routes for IPP biosynthesis: the mevalonic acid (MVA) route in the cytosol and the methylerythritol 4-phosphate (MEP) route in the chloroplast. Fungi and animals perform the MVA route. In microalgae, it is suggested that isoprenoid biosynthesis is derived from the MEP route (Paniagua-Michel et al. 2012).

The biosynthesis of IPP by the route MEP utilizes glyceraldehyde 3-phosphate and pyruvate as substrates to form deoxy-D-xylulose-5-phosphate (DXP); through the enzyme DXS (Huang et al. 2017). Subsequently, DXP is reduced by the enzyme DXR to yields to MEP. In the following, IPP and DMAPP are formed and pass a series of condensation and elongation reactions to produce geranylgeranyl diphosphate (GGPP, C20), the precursor of carotenoid biosynthesis. Head-to-head condensation of two GGPP molecules by the enzyme PSY gives origin to the C40 carotenoid, the phytoene (Gong and Bassi 2016). The phytoene is converted to lycopene and then cyclized by β-LCY, and ε-LCY followed by β-LCY to produce β-carotene and α-carotene, respectively.

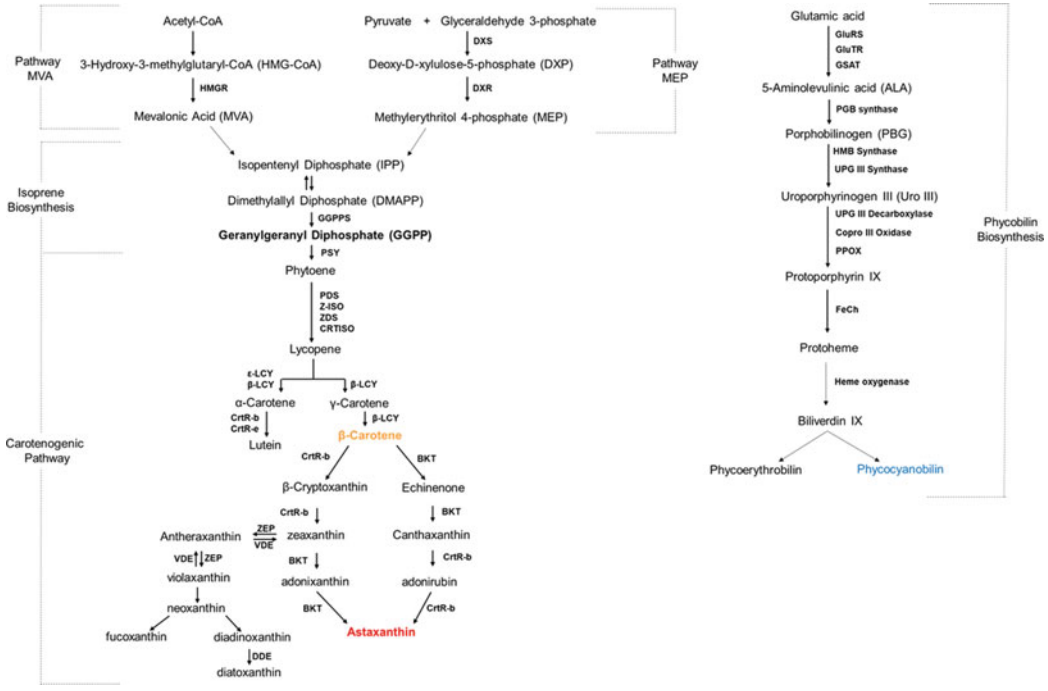

Fig. 11.2 Biosynthetic pathways of carotenoids and phycobilins in microalgae. Enzymes involved: HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; DXR, deoxy-D-xylulose-5-phosphate reductoisomerase; β-LCY, β-cyclase; CrtR-b, β-carotene hydroxylase; CRTISO, carotenoid isomerase; DXS, DXP synthase; ε-LCY, ε-cyclase; CrtR-e, ε-carotene hydroxylase; GGPPS, GGPP synthase; PDS, phytoene desaturase; DDE, diadinoxanthin de-epoxidase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; BKT, β-carotene ketolase; Z-ISO, ζ-carotene isomerase; ZEP, Zeaxanthin epoxidase; GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; GSAT, glutamate-1-semialdehyde aminotransferase; PGB synthase, porphobilinogen synthase; HMB synthase, hydroxymethylbilane synthase; UPG III synthase, uroporphyrinogen III synthase; UPG III Decarboxylase, uroporphyrinogen III decarboxylase; Copro III oxidase, coproporphyrinogen III oxidase; PPOX, protoporphyrinogen IX oxidase; FeChe, Fe chelatase; HOs, heme oxygenase Adapted from Paniagua-Michel et al. (2012), Raposo et al. (2015), Bertrand (2010), Gong and Bassi (2016) and Fernandes et al. (2017), Chakdar and Pabbi (2016)

The astaxanthin is formed from the β-carotene by the action of the enzymes BKT or CrtR-b. The intermediate compounds of catalytic activity are canthaxanthin and zeaxanthin, respectively (Rajesh et al. 2017). Both pathways are reported for microalgae *H. pluvialis*. However, the route where β-carotene is converted to canthaxanthin via echinenone and, after, converted to astaxanthin is the more proposed (Saini et al. 2019; Han et al. 2013; Henríquez et al. 2016).

The phycobilins, in its turn, are biosynthesized from heme by the action of the HOs enzyme (Mulders et al. 2014). The phycobilins biosynthesis starts from glutamic acid, which gives rise to ALA. The condensation of two ALA molecules forms porphobilinogen (PBG). The enzymes HMB synthase and UPG III synthase form from the PBG the UPG III. The UPG III leads to the formation of protoporphyrin IX (Chakdar and Pabbi 2016; Saini et al. 2018). The action of the enzyme FeCh catalyzes the formation of protoheme. Subsequently, the protoheme is converted to biliverdin IX by the action of the enzyme Hos and from the biliverdin IX phycocyanobilins and phycoerythrobilin are produced (Manirafasha et al. 2016; Stanic-Vucinic et al. 2018). For a more detailed panorama of the biosynthesis of carotenoids and phycobilins, see Fernandes et al. (2017), and Czarnecki and Grimm (2012).

11.4 Chemical Synthesis

In 1950 was described by Karrer and Eugster (1950), Inhoffen et al. (1950), and Milas et al. (1950), the chemical synthesis of pigments, starting with the synthesis of β-carotene. The scientific achievements of these three groups were the basis for the industrial production procedures. In 1954, Hoffmann-La Roche began commercial production of β-carotene. The method based on the Grignard reaction, which follows the synthesis principle C19 + C2 + C19, it presented a yield of 60%. In 1960, the BASF developed a higher yield method (85%) based on Wittig reaction (C20 + C20). The disadvantage of the Wittig reaction-based process is the undesirable formation of triphenylphosphine oxide, which needs of recycling due to its low biodegradability (Ribeiro et al. 2011).

Today, the two largest industrial producers are DSM and BASF, which produce the β-apo-8'-carotenal, β-carotene, canthaxanthin, astaxanthin, lutein, lycopene, zeaxanthin, and citranaxanthin pigments. Although chemical synthesis is a consolidated market, it became less desirable, given the awareness of the benefits associated with natural dyes. In this context, several companies are concentrated on developing methods for producing pigments from photoautotrophic cultures (Cardoso et al. 2017).

11.5 Microalgae-Based Pigments Processes and Particularities

The carotenoid and phycobiliproteins production from microalgae involve upstream (USP) and downstream (DSP) processing operations (Fig. 11.3). The USP includes the selection of microalgae species, carbon dioxide supply, nutrient availability, and light source.

Different approaches to the production system have been reported, such as photoautotrophic, heterotrophic, mixotrophic, two-stage, open, and closed systems. Between systems, raceway ponds and flat and tubular type photobioreactors are the most popular, and the two-stage systems approach has gained substantial space in industrial pigment production (Solovchenko and Chekanov 2014; Acién et al. 2017).

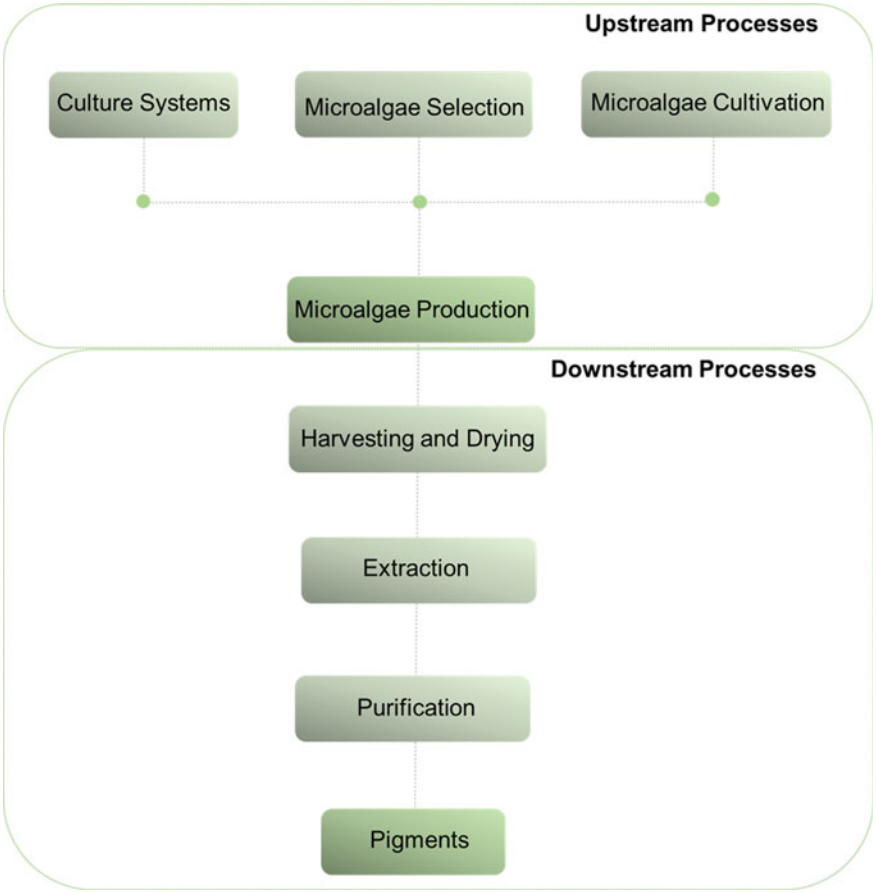

Fig. 11.3 Process flowchart upstream and downstream of microalgae-based pigment production

Once the USP is completed, the DSP begins. The microalgae biomass DSP comprises multiple unit processes involving harvesting, extraction, and purification (Deprá et al. 2018). Following described the technology used to produce carotenoids and phycobiliproteins from the microalgae *H. pluvialis* (astaxanthin), *D. salina* (β-carotene) and *A. platensis* (C-phycocyanin).

11.5.1 Astaxanthin Production Process

Currently, only two biological sources compete with synthetic astaxanthin, which presently dominates the market: the yeast *Phaffia rhodozyma* and the microalga *H. pluvialis*. *P. rhodozyma* is produced by fermentation and marketed in the form for salmonid feed powder. The average astaxanthin content in *P. rhodozyma* is about 8 mg/g, while for *H. pluvialis*, it is about 60 mg/g. Among natural sources, *H. pluvialis* is considered the primary producer of astaxanthin (AstaReal 2019).

The estimated market in 2019 was about 40 USD million for astaxanthin from *H. pluvialis*, based on a CAGR of 2.3%. The estimated cost of their production is about USD 552.0/kg, and the selling price is about USD 2,500/kg (Jacob-Lopes et al. 2019; Hu 2019). The microalgae *H. pluvialis* can be cultured photoautotrophically, heterotrophically, or mixotrophically. However, as the astaxanthin biosynthesis process requires light, a high cellular concentration of astaxanthin (up to ± 5% dw) is achieved only in photoautotrophic cultures (Kang et al. 2005).

Astaxanthin accumulates in cytosolic lipid bodies under environmental stress or adverse culture conditions, such as high light, nutrient depletion (especially N limitation), high temperature, and high salinity (Han et al. 2013). The metabolic stress conditions regulate the expression of carotenogenic genes. In *H. pluvialis*, the expression of caratonegonic genes that encode the enzymes PSY, PDS, lycopene cyclase (LCY), BKT, and CrtR-b are positively regulated. It is noteworthy that the strains differ from each other in the build-up of astaxanthin and the gene expression profile, which can be distinctly regulated in response to the culture conditions applied (Gao et al. 2015; Ma et al. 2018a; Córdova et al. 2018).

Haematococcus grows as motile bi-flagellated cells under optimal conditions and, under stress, turn into red cysts. Thus, a continuous culture process is not useful; instead, a two-stage process must be employed. During cultivation, the microalgae will undergo three cellular forms: (i) motile biflagellate; (ii) nonmoving palmella; and (iii) non-motile, haematocysts (Mobin and Alam 2017). In the first stage, flagellated macrozooids rapidly divide under favorable culture conditions, reaching a high cell density. Posteriorly, under unfavorable conditions, the macrozooids lose their flagella as they expand cell size and form the nonmoving palmella. When stress persists, the palmella become transform in haematocysts (aplanospore). At this stage, significant amounts of astaxanthin are accumulated, which brings red staining to the cells. Noteworthy, the *H. pluvialis* hematocysts can be parched and remain inactive by years and, after, return to life in the microzooid form when exposed to favorable conditions (Ma et al. 2018b).

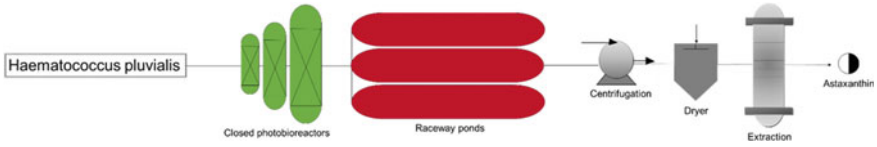

Fig. 11.4 Process flow diagram of the commercial production of astaxanthin from *H. pluvialis*

Most commercial *Haematococcus pluvialis* production facilities employ a combination of closed photobioreactors and open ponds. Traditionally, the process begins with the inoculating of the culture into small agitated flasks, where growth occurs by the supply of light, air, inorganic carbon, and nutrients. Posteriorly, they are transferred to external photobioreactors of small, medium, and large postage. The pH and temperature can be controlled automatically, and only air and sterile water enter the system. When a sufficient volume of cells is produced, the culture is stressed in open ponds under intense sunlight. The astaxanthin production is commonly induced by the high light, nutrient deprivation or by the addition of salt in cultivation. After the formation of hematocysts, they are harvested. The harvesting is typically realized by sedimentation, followed by centrifugation (Shah et al. 2016; Borowitzka 2018).

Subsequently, the hematocysts are dried and pass by a cracking mill. The extraction of astaxanthin-rich oleoresin from dry biomass is realized using supercritical carbon dioxide. The quality of pre-harvest crops, dry biomass, and end product are monitored (Cyanotech 2019). Details of the exact techniques used in downstream processing are of property of the companies. Therefore, they may vary from company to company. Figure 11.4 feature a general flow diagram of astaxanthin production from *H. pluvialis*.

In addition, production processes in enclosed environments, under fully controlled conditions, using multi-tanks also are being used to produce astaxanthin. The AstaReal owns a large-scale internal system that allows a more effective, clean, and safe procedure. Microalgae are cultivated in tanks, transferred by piping to tanks each time bigger. The process occurs under artificial light without any exposure to the atmosphere. In the final phase, microalgae are stressed by increased light intensity and nutrient deprivation. The downstream processing is similar to that done in external systems. The biomass is harvested, dried, and then goes for the grinding process to break the cell wall, preparing them for extraction. The extraction is done using supercritical carbon dioxide, and the resulting product is an astaxanthin-rich oleoresin. The products deriving from the upstream and downstream processing of *H. pluvialis* are biomass in powder containing a high astaxanthin content, which can be refined to obtain a more top purity ingredient (Schultz 2016).

11.5.2 β-Carotene Production Process

β-Carotene was the first high-value product commercially produced from a microalgae (Borowitzka 2013). The most important source for the natural production of β-carotene is the halophilic green microalgae *D. salina*, which can contain up to 14% of dw as β-carotene (Borowitzka 2018). The estimated market in 2019 was about 200 USD million for β-carotene from *D. salina*, based on a CAGR of 3.3%. The estimated cost of its production is about USD 105.0/kg, and the selling price is about USD 790/kg (Jacob-Lopes et al. 2019; Hu 2019).

In *D. salina*, adverse environmental conditions like nitrogen limitation, high salinity, low temperature, and high luminosity favor the β-carotene biosynthesis. As the carotenogenic pathway of microalgae appears to be similar to that of plants, it is believed that the generation of reactive oxygen species (ROS) triggers the accumulation of β-carotene. The ROS induction by applied stress conditions acts as messenger molecules for biosynthesis (Ye et al. 2008; Von Alvensleben and Heimann 2018). Although the β-carotene inductors conditions in *D. salina* have become clear, via that leading to this response still has not been adequately elucidated (Paliwal et al. 2017). To date, the known genes involved in the β-carotene biosynthetic pathway are PSY, PDS, ZDS, and β-LCY (Ramos et al. 2011).

Recent researches are focused on the coordinated expression of multiple enzymes and the regulation of specific enzymes that control the flow to the desired product (Varela et al. 2016). An example of this approach would be the positive regulation of β-LCY accompanied by the down-regulation of ε-LCY is desired to promote β-carotene accumulation, so that the conversion of lycopene to α-carotene does not occur (Li et al. 2019). In this sense, considerable efforts are being devoted to the massive accumulation of carotenoids in *D. salina* (Galarza et al. 2018).

Currently, at a commercial scale, the cultivation of *D. salina* is based on autotrophic growth in saline media containing inorganic nutrients, with CO_2 as the carbon source. Generally, these production plants are located in semidesert regions (i.e., Hutt Lagoon, Western Australia, Whyalla, Israel), where solar irradiance is maximal, cloudiness is minimal, the climate is warm, and hypersaline water is available (Del Campo et al. 2007).

The optimum salinity for growth lies between 18 and 22% NaCl, while a higher salinity at or around 27% of NaCl is ideal for carotenogenesis (Borowitzka et al. 1984). As β-carotene is an intracellular product, which accumulates in oil globules in the chloroplasts, it is necessary to find a balance between the production of biomass and the production of carotenoids. The maximum β-carotene yield occurs at an intermediate salinity, but it is advisable to use salinity above the ideal level to avoid protozoan predators and non-carotenogenic microalgal competitors, as *Dunaliella viridis* (Raja et al. 2007). *D. salina* also has a high-temperature tolerance, with optimal growth in temperatures around 28 °C (Borowitzka 2018).

Due to the adverse cultivation conditions employed, open ponds are the most used commercial systems. Among the different open system projects, extensive unmixed ponds and raceway ponds are the most employed. These cultivation systems are also

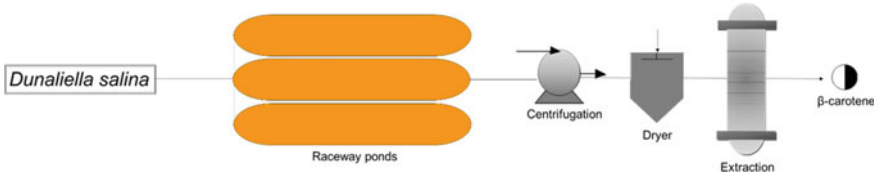

Fig. 11.5 Process flow diagram of the commercial production of β-carotene from *D.salina*

referred to as extensive and intensive approaches, respectively, and will be discussed later in this chapter.

In the intensive approach, a two-stage technology, similar to that used for astaxanthin, can be adopted. Considering that the use of stressors slows microalgae growth, but favors the synthesis of β-carotene, the two-stage strategy aims to improve overall β-carotene yield (Ben-Amotz 1995). In the first stage, cultivation is performed in a nutrients-rich medium with ~ 18% NaCl to maximize biomass production. Then, the culture is transferred to a depleted-medium containing 27% NaCl in stage two, where the carotenogenesis will be induced (Saha and Murray 2018).

The choice of intensive or extensive cultivation mode may depend on location, i.e., climatic conditions in the region, availability of a salt source, and land costs. For example, if a free brine source is available near the installation, and the cost of land is low, extensive cultivation may be more appropriate. However, regardless of this, higher productivity of β-carotene is achieved in the intensive cultivation (Raja et al. 2007).

Regarding the downstream process, the harvesting of carotene-rich biomass in extensive production is generally done using flocculation and cell surface adsorption, while in intensive production, centrifugation is employed. After harvesting, the cells generally are spray dried and then is extracted to produce a mixed carotenoid extract consisting mainly of β-carotene. Alternatively, after the drying, the whole biomass can be stabilized to produce *Dunaliella* powder that is used mainly as an animal feed, especially for aquaculture (Borowitzka 2018). Figure 11.5 feature a general flow diagram of β-carotene production from *D. salina.*

11.5.3 Phycocyanin Production Process

After the screening of potential cyanobacteria for phycocyanin production, it has been found that *A. platensis* (*Spirulina*) is the microorganism with the highest production of this pigment. Thanks to the high content, well as large availability, the phycocyanin marketed on the world market are extracted, exclusively, from *Spirulina* (Vernès et al. 2015; Ouada and Ammar 2017). The estimated market for 2022 is 114.8 USD million for C-phycocyanin from *A. platensis*, based on a CAGR of 4.7%. The estimated cost of its production is about USD 46.0/kg, and the selling price is about USD 548/kg (Jacob-Lopes et al. 2019; Hu 2019).

Regardless of the desired final product, almost all commercial production of *A. platensis* is conducted in open raceway ponds. As with other commercial microalgae, some factors regulate the yield of C-phycocyanin in *A. platensis*. Abiotic signals, as light, temperature, and nutrient availability, are closely linked to C-phycocyanin biosynthesis. Temperatures around 25 to 35 °C, a pH of 8 to 10, a NaCl concentration of 0.02 to 0.2 M, and a light intensity around 2500 lx are indicated for biosynthesis this pigment as from *A. platensis* (Pagels et al. 2019).

This microorganism grows naturally in tropical and subtropical alkaline lakes, which is why its performance is better at high pH and high temperatures (35–38 °C) and require minimum temperatures of 15–20 °C. In regions where the weather conditions are less than optimal, the production system can raceway ponds under plastic greenhouses, common facilities in the northern and central regions of China. In some more extreme cases, such as at Earthrise Farms, located in California, USA, the company only operates seven months a year because of the low temperatures recorded in winter (Borowitzka 2018).

The seawater is a source of trace elements and is commonly used for cultivation in the commercial when the plants are located near to this resource. The cultivation medium may employ a mixture of seawater, freshwater, and sodium bicarbonate. For *A. platensis* cultivation, pH control is critically important. This parameter is adjusted by supplying CO_2 gas to the medium, which besides providing pH control, also is the source of carbon for photosynthesis (Beley 2013).

The extreme cultivation conditions make the use of open systems suitable for these microorganisms. However, studies showed that C-phycocyanin productivity is increased when cultivated in closed photobioreactors since these systems provide better control of the cultivation parameters, and the culture can be kept axenic. However, currently, the operation of large-scale *A. platensis* culture in closed systems does not feature a competitive cost (Eriksen et al. 2008; Soni et al. 2017).

The harvesting, as in other production processes, is performed by centrifugation or filtration. The filtration is done through inclined or vibrating screens, or a combination of both, with up to 95% biomass removal efficiency. The water can be recycled and return to the ponds for the next cultivation cycle. The final harvest paste is typically dried by spray dryer or drying drum. Some companies employ exclusive processes to eliminate the oxidation that occurs in this operation (Cyanotech 2019). The cell disruption is conducted at pH 6 to 7 and then centrifuged, and phycocyanin extract is obtained (Mehar et al. 2019). Figure 11.6 feature a general flow diagram of phycocyanin production from *A. platensis*.

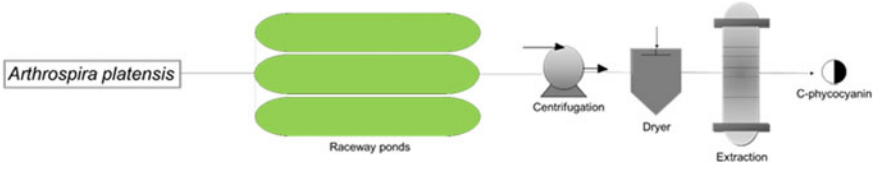

Fig. 11.6 Process flow diagram of the commercial production of phycocyanin from *A. platensis*

11.6 Commercial Microalgae Culture Systems for Pigments Production

The industrialization of any microalgae-based product requires large-scale cultivation, which can be done in open (ponds and their variations) or in closed systems (photobioreactors and heterotrophic/mixotrophic bioreactors). Concerning commercial microalgae biomass cultivation for pigment production, the most extended types of culture systems are open raceways, shallow ponds, and tubular photobioreactors, as can be seen in Table 11.1, where the main companies producing astaxanthin, β-carotene, and c-phycocyanin were listed.

At present, open systems, especially raceway ponds, are the predominant types, being used in over 90% of commercial cultivation around the world. This is justified by the fact that open systems are much cheaper and easier to operate than closed systems, and yet the cost of construction is about one order of magnitude lower than that of closed systems. On the downside, open systems have some operational problems as the dependence on climate conditions and geographic position, contamination, evaporation, and extensive land requirements. Due to the limited control of cultivation conditions and contamination, the use of open systems is limited to robust microalgae species (e.g., *Chlorella*, *Scenedesmus,* and *Nannochloropsis*), that grow rapidly or under very selective conditions (e.g., *D. salina* and *Arthrospira*) (Solovchenko and Chekanov 2014; Acién et al. 2017).

Table 11.1 Commercial producers of astaxanthin, β-carotene, and c-phycocyanin from microalgae

Pigment	Company	Location	Culture system
Astaxanthin	Cyanotech	USA	Closed tubular PBRs + Raceway ponds
	Mera Pharmaceuticals	USA	Closed tubular PBRs + Raceway ponds
	AstaReal	Sweden	Vertical (indoor, mixotrophic)
	Algatechnologies	Israel	Closed tubular PBRs
	Beijing Gingko Group	China	Closed tubular PBRs
β-carotene	Betatene (BASF)	Australia	Extensive unmixed ponds
	Western Biotechnology	Australia	Extensive unmixed ponds
	AquaCarotene	Australia	Extensive unmixed ponds
	Cyanotech	USA	Raceway ponds
	Nature Beta Technologies	Israel	Raceway ponds
	Tianjin Lantai Biotechnologies	China	Raceway ponds
C-phycocyanin	Cyanotech	USA	Raceway ponds
	Zhejiang Binmei Biotechnology	China	Raceway ponds
	Earthrise Farms	USA	Raceway ponds
	Parry Nutraceuticals	India	Raceway ponds

To eliminate these limitations, closed photobioreactors (PBRs) were developed, that support the growth of a much wider selection of strains, under precisely controlled conditions, with a low risk of contamination. These systems are especially important when working with sensitive species such as *H. pluvialis*. The major drawbacks of PBRs are related to high construction and operation cost, difficulty in scaling up, and high shear stress. Due to the high productivity and the high operational control provided by the PBRs, a considerable effort is being invested into the development of a wide range of configurations to reduce these limitations and thus increase the technical and economic viability of microalgae-based processes and products. However, until now, for large-scale commercial use, tubular photobioreactors are almost exclusively used (Table 11.1) (Borowitzka 2018). Another type of closed system that deserves special mention is the vertical bioreactors used by AstaReal to induce astaxanthin production in *H. pluvialis* mixotrophic cultivations (AstaReal 2019).

It is essential to consider that all culture systems have advantages and limitations, and there is no perfect system suitable for all applications and microalgae species. Therefore, the choice of culture system and culture mode for commercial production depends on several factors, including (i) reliability of the culture; (ii) type and quality of the desired product; and (iii) operating and capital costs (Chang et al. 2017; Maroneze and Queiroz 2018).

The first criterion to be considered is the reliability of the culture, which means a guaranteed supply of biomass. To achieve reliability, microalgal strains must meet specific requirements, such as (i) rapid growth in growing conditions (exceptionally light and temperature); (ii) broad temperature and irradiance tolerance; (iii) high shear and oxygen tolerance; and (iv) growth in a selective environment (e.g., high salinity or high pH) to reduce the likelihood of contamination (Borowitzka 2018).

The type and quality of the desired product will define the price of the final product and the required purity and is, therefore, a deciding factor in choosing the cultivation system. Here the final biomass must contain a high content of the target product; for this, often, the reaction system must allow the use of stress conditions that induce the synthesis of these compounds, such as irradiance, pH, nutrient content, or salinity. As already mentioned, to associate high biomass productivity with the high intracellular contents of the product of interest, two-stage systems are often used.

Finally, influenced by the parameters mentioned above, capital and operating costs will determine the economic viability of commercializing microalgae-based products. Consideration should be given to the costs of land (open pond systems require a much larger land area than closed photobioreactors), construction, installation, maintenance, labor, energy and nutrients requirements, and labor. Besides this, should be considered the climate conditions and geographic position, that will reflect in potential costs for cooling or heating the cultures.

11.6.1 Open Systems

11.6.1.1 Raceways Ponds

Raceway ponds have so far been the most widely used method for commercially producing microalgae biomass owing to their flexibility and ease of scaling up. It is also known as high-rate lagoons or Oswald lagoons, a tribute to W.J. Oswald, who described the technology for the first time, which was initially devised for wastewater treatment (Oswald and Golueke 1968; Acién et al. 2017). Currently, the industrial outdoor culture of microalgae and cyanobacteria in raceways is well established, especially for *Arthrospira* and *Dunaliella* since these species require growth media with high alkalinity and salinity, respectively, making it unsuitable for other competing species (Zitelli et al. 2013).

In raceway ponds, as the name suggests, the microalgae suspension is recirculated around a racetrack loop (Suparmaniam et al. 2019). At large-scale, the surface area of the pond could range between 1000 and 5000 m^2. Although pond configurations with multiple channels and bends are available, raceways with a minimum number of bends are more common because they imply less pressure drop and, consequently, less energy expenditure. The length of the channels should be proportional to the width, where proportions between 10 and 20 are generally adequate (Chisti 2012; Zhou et al. 2020).

Light is the driver of the photosynthesis reaction; thus, it is the primary factor that most influences the microalgal growth rate. In this sense, a key variable of the raceways is the depth of the channels, which should be shallow, in the range of 0.25 to 0.30 m, to allow the penetration of light inside the culture. As both area and depth are limited, larger facilities are implemented by multiplying the number of ponds (Acién et al. 2017).

In commercial microalgae production, these ponds generally are constructed in compacted soil covered with a PVC membrane. This type of construction carries a higher risk of contamination, so in some cases, ponds made of concrete with a plastic liner can be used, which are more expensive but guarantee a lower risk of contamination (Maroneze and Queiroz 2018).

To provide the energy required for the circulation and mixes the nutrients and cells along the reactor are utilized paddlewheel. It consists of a wheel with ten to twelve paddles, with a total diameter of four times the water depth, working at velocities between 10 and 20 rpm. In addition to what has already been mentioned, mixing is responsible for keeping cells into suspension, removal of photosynthetically generated oxygen, and to provide the periodic exposure of cells to sunlight, which is important to avoid photolimitation and photoinhibition and enhances the light utilization efficiency. However, excessive energy application in the mixing should be avoided, mainly to minimize the operational cost since this operation represents up to 69% of the total costs of microalgal cultures in raceways (Kumar et al. 2015).

In addition to light, the temperature is another determining parameter in biomass productivity. The optimal temperature for growth of microalgae varies widely with

species, but most raceway-grown species are mesophilic, which have ideal growth temperatures in the range of 24–40 °C. Since cultivations in these systems are usually operated continuously, temperature control can be done by cooling or heating the feed water or by circulating the culture in external heat exchangers. However, these are unlikely to be affordable options, so control is rarely done. Because of this, the process performance of open systems is dependent on the prevailing weather conditions in a particular locality. Therefore, to achieve a viable process, the selection of suitable locations as a function of its climatic conditions is critically important (Brusca et al. 2017).

In terms of cost, one of the great appeals of this type of system is the relatively low investment value that varies from 0.13 to 0.37 M€/ha at 100 ha scale (Norsker et al. 2011; Chisti 2012). The data from raceway ponds are evidently highly variable depending on the species, culture medium, climate, geographical position, in addition to other variables. Although high biomass productivities, like 37 g/m^2/d for *D. salina* has been reported (Moheimani and Borowitzka 2006), in average much lower productivities are usually described, such as 9–13 g/m^2/d for *Spirulina* sp. (Olguin et al. 2003), 1.6–3.5 g/m^2/d when producing *D. salina* (Garcia-González et al. 2003), and 4–10 for *Spirulina* sp. (Delrue et al. 2017). This variation will also reflect on the cost of biomass production, which, according to Acién et al. (2019), is 4.5 €/kg. Delrue et al. (2017) estimated that the cost to produce *Spirulina* in raceway ponds ranges from 3.8 to 9.5 €/kg depending on the system's productivity.

11.6.1.2 Extensive Unmixed Ponds

The two largest producers of β-carotene from *D. salina* in the world are Western Biotechnology Ltd. (Perth, Western Australia) and Betatene Ltd. (BASF) (Melbourne, Victoria) in Australia. These and other Australian companies grow microalgae in extensive and shallow ponds built on the bed of a hypersaline coastal lagoon or formed by the artificial expansion of a lagoon (Borowitzka 2013). The extensive unmixed ponds or shallow ponds, Unmixed extensive lagoons or shallow lagoons are so-called because they are generally less than 0.5 m deep, up to 250 ha in area, and do not have a mixing system, except for wind and convection (Kumar 2015).

Unmixed ponds can represent the most economical and least technical of all commercial culture methods when suitable climatic conditions allow almost year-round cultivation, as is the case in some regions of Australia. In Western Australia and South Australia, where are located the two largest commercial microalgae production plants in the world, there is very high annual irradiance, warm weather, and low rainfall, ideal conditions for growth in natural ponds (Trediti 2004).

The main advantages of this type of approach are linked to the economic appeal as they operate without CO_2 addition with minimal control, plus in Australia, the cost of land is low, and water is free except for pumping costs (del Campo 2007). On the other hand, such ponds are mainly limited to growing microalgae which are capable of surviving in poor conditions or have a competitive advantage that allows them to

outgrow contaminants such as unwanted microalgae species, bacteria, viruses, and protozoa (Kotzen et al. 2019). Besides, biomass productivity hardly exceeds 1 g/m^2/d, and, given the very low concentration of cells, a very efficient system should be used to harvest the biomass (Trediti 2004).

The cultivation process in these systems consists of pumping seawater from the adjacent ocean to the pond, where nutrients are added to this saline water. When the system has reached the appropriate β-carotene content, the culture is pumped to a harvesting plant. The rate of harvesting and the growth period varies with changing climatic conditions throughout the year. After harvesting, the remaining medium is returned to the unmixed pond, where salinity and nutrient content is adjusted as needed (Borowitzka 1990).

11.6.2 Closed Systems

Due to the high operational control and high productivity provided by photobioreactors, researchers and companies have invested heavily in the development of a wide variety of configurations over the last decades. However, for large-scale commercial use, tubular photobioreactors are almost exclusively used, mainly because they are easy to scale up, have a large illumination surface area, are suitable for outdoor cultures, present good biomass productivities, and are economically reasonable. Although used by only a portion of microalgae-based companies, bioreactors for heterotrophic and mixotrophic cultures have also gained prominence (Borowitzka 2018). Other systems such as flat-plate and vertical photobioreactors have exploration potential but are still in their early stages where some significant limitations need to be overcome, which are mainly associated with scale-up difficulty and high cost (Ugwu et al. 2008).

11.6.2.1 Tubular Photobioreactors

Tubular photobioreactors were first described in 1953 (Tamiya et al. 1953), but were not consolidated until the 1990s, after being gradually optimized (Gudin and Chaumont 1983; Pirt et al. 1983; Chaumont et al. 1988; Chaumont 1993; Richmond et al. 1993). Only from this point is it possible to commercially produce astaxanthin from microalgae, as far as we know *Haematococcus* single-phase cultivation in large-scale raceway systems has proved unsatisfactory (Bubrick 1991; Margalith 1999; Olaizola 2000).

Unlike open systems, tubular photobioreactors allow greater control of cultivation conditions, the possibility of contamination is lower, and the high availability of solar radiation, which results in higher yields. It this allows using these reactors to produce sensible strains such as *H. pluvialis*. Due to the lower water depth (tube diameter), which ranges from 0.03 to 0.12 m, the biomass concentration can reach 3.0 g/L.

The company Algatechnologies Ltd. (Ketura, Israel) established tubular photobioreactors in two stages for astaxanthin production from *Haematococcus pluvialis* (Ayalon 2014), and similar systems are widely employed in other companies around the world, as was showed in Table 11.1 (Maeda et al. 2018).

Tubular photobioreactors consist of an array of straight transparent tubes that are usually made of plastic or glass and have a diameter of 0.1 m or less, in which the culture is circulated by pumps or air streams (airlift) (Acién et al. 2017; Maroneze and Queiroz 2018). These transparent tubes can be arranged in different patterns, including horizontal/serpentine (Chaumont et al. 1988; Molina Grima et al. 2001), vertical (Chen et al. 2016), near-horizontal (Tredici and Zittelli 1998), conical (Watanabe and Hall 1996), and inclined (Ugwu et al. 2002, 2008).

This distribution of the tubes in the solar collector, together with the tube diameter, will determine the amount of solar radiation that the culture will be able to capture, which will directly reflect on the productivity in biomass and photosynthetic bioproducts. Besides, the choice of the diameter of the tube will affect the temperature of the culture, the oxygen concentration in the culture, and the length of the tubes. It is already well established that smaller diameters favor the performance of the system since it will reflect in a higher surface-volume ratio (S/V) (Torzillo and Zitelli 2015). Generally, tubes lower than 0.1 m required, but it must be considered that although it can increase the kinetic performance of the process, the use of very small diameters leads to a significant increase in energy consumption; therefore, minimum values of 0.03 m are recommended (Molina Grima et al. 2001).

As for the length, it must be dimensioned so that there is no accumulation of excess oxygen in the PBR loop. Photosynthesis is a well-established biochemical reaction, where to produce one ton of microalgal biomass, it generates 1.9 tons of oxygen. Which needs to be efficiently removed as its excess presence has a toxic effect on cells. In this sense, it is recommended that the maximum length is in the range of 100 to 400 m, depending on the configuration (Torzillo and Zitelli 2015).

The investment cost of tubular PBRs is a limiting factor in the use of these systems, as it can exceed twice the amount reported for raceways. According to Nosker et al. (2011), this value varies around 0.51 M€/ha at 100 ha scale. On the other hand, high productivity is achieved in these systems. It has been reported biomass productivities of 10–40 g/m^2/d for *Spirulina* (Delrue et al. 2017), 13 g/m^2/d for *Haematococcus pluvialis* (Olaizola 2000). Regarding the cost of biomass production, the values are quite variable, as they depend on the species used, productivity, and the stages of the production process. Delrue et al. (2017) reported costs ranging from 18 to 74 €/kg, Molina Grima (2009) found a cost of 25 €/kg, and Nosker et al. (2011) 4.15 €/kg. Despite the innumerable advantages of closed cultivation systems, the high cost of both investment and production is still the most significant limitation in the establishment of processes and products based on microalgae.

11.7 Concluding Remarks

Pigments derived from microalgae are steadily gaining ground in the most diverse branches of industry. The successful marketing of β-carotene, astaxanthin, and phycocyanin derived from these microorganisms, reflects the presence and importance of niche markets in which consumers are willing to pay more for products that contain natural ingredients with bioactive properties.

However, to expand this market, they must be produced at a competitive price, because synthetic pigments or extracted from conventional sources can generally be produced more economically. In this sense, researchers and companies around the world must develop strategies to improve the performance of microalgae-based processes, reduce production costs, and consequently increase viability.

The main challenges to consolidate the microalgae industry are related to the development of cultivation systems that allow higher productivity at a lower cost, development of integration strategies and intensification of processes that will enable the complete commercial exploitation of biomass, as well as the improvement of downstream processes, especially harvesting, and cell disruption.

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Chapter 12
Industrial Extraction of Microalgal Pigments

Ronald Halim

Abstract Pigments are valuable commodity products that have found many applications in pharmaceutical, food and cosmetic industries. Under different light and nutrient regimes, microalgal cells can be cultivated to accumulate a broad range of valuable pigments throughout their life cycle. The effective recovery of these pigments is critical to the development of a commercially viable and sustainable microalgal bio-industry. In this chapter, the recovery of pigments from microalgal biomass is examined. The chapter starts by evaluating the range of technological options that are currently available for rupturing microalgal cell walls to release intracellular pigments (high-pressure homogenisation, bead milling, ultrasonication, pulse electric field, osmotic shock, microwave, enzymatic treatments and chemical treatments). It then reviews a number of extraction technologies that can be used to extract the released pigments from ruptured biomass (organic solvent extraction, pressurised solvent extraction, ionic liquid extraction and supercritical carbon dioxide extraction) and examines the different biomass processing pathways that have been recently studied for the recoveries of industrially valuable pigments from algal sources (astaxanthin, β-carotene, phycoerythrin, phycocyanin). The last part of the chapter evaluates the industrial scalability of pigment extraction in the context of a biorefinery system that enables energy-efficient and cost-effective co-production of lipids, proteins and pigments from microalgal biomass.

Keywords Microalgae pigment · Pigment extraction · Biomass pretreatment · Astaxanthin · B-carotene · Phycobiliprotein

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12.1 Overview of Microalgal Pigments, Pigment Extraction and Cell Walls

12.1.1 Pigment Class and Solubility

Microalgal pigments can be classified into 3 different groups: chlorophylls, carotenoids and phycobiliproteins (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Chlorophylls are green photosynthetic pigments found universally in all microalgal and cyanobacterial species. Chlorophyll molecules have a porphyrin ring coordinated to a central magnesium ion as their basic structure. Chlorophylls are soluble in organic solvents (such as methanol, ethanol, acetone) and supercritical carbon dioxide.

Carotenoids are a group of accessory pigments made of linear hydrocarbon polyenes (some of which have oxygenated derivatives) that are able to absorb light between 400 and 550 nm (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Carotenoids are divided into separate classes based on their molecular structure and functionality. In terms of molecular structure, carotenoids composed of a pure hydrocarbon chain are classified as carotenes (e.g. β-carotene), while those with molecules that include oxygenated derivatives (such as–OH and–CO substitutes) are classified as xanthophylls (e.g. astaxanthin). In terms of functionality, carotenoids can be grouped as either primary or secondary carotenoids (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Primary carotenoids (such as β-carotene, lutein and violaxanthin) transfer absorbed light energy to chlorophylls and thus act as energy harvesters that expand the light-absorbing spectrum of the cells. On the other hand, secondary carotenoids (such as astaxanthin and canthaxanthin) serve to protect chlorophylls from photo-damage by forming a protective layer over the microalgal cells upon exposure to extreme light intensities (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). The synthesis of secondary carotenoids in the cells generally intensifies under environmental stresses. All carotenoids demonstrate strong antioxidant properties and are able to scavenge reactive oxygen species (ROS) to protect the cells from free radical attacks and oxidation under environmental stresses (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Even though a microalgal species may have a number of different carotenoids, it normally has a dominant carotenoid (between 70—80wt% of its total carotenoid content) (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Research works on the species are thus often focused on the recovery of this primary carotenoid (β-carotene from *D.salina*, astaxanthin from *H. pluvialis*, lutein from *Scenedesmus* sp.). Similar to chlorophylls, carotenoids are soluble in organic solvents (such as methanol, ethanol, acetone) and supercritical carbon dioxide.

Phycobiliproteins are coloured antennae-protein pigments bound to supramolecular complexes (known as phycobillisomes) on the outer surface of the thylakoid membranes. The pigments absorb energy in the visible light spectrum (450—650 nm) and function as accessory pigments that enhance cellular light collection for photosynthesis (Cuellar-Bermudez et al. 2015; Khanra et al. 2018; Rammuni et al. 2019).

Phycobiliproteins are found in prokaryotic cyanobacteria and eukaryotic red algae. They are divided into four main classes according to their molecular structure: allophycocyanin (bluish-green in colour), phycocyanin (blue in colour), phycoerythrin (red in colour), phycoerythrocyanin (orange in colour) (Cuellar-Bermudez et al. 2015; Khanra et al. 2018). Phycoerythrin (PE), a red phycobiliprotein with an absorption peak at 565 nm, is primarily found in the chloroplast of cyanobacteria (such as *Synechococcus* sp., *Leptolyngbya sp.*) and red algae (such as *Porphyridium cruentum*), while phycocyanin (PC), a blue phycobiliprotein with an absorption peak at 610—620 nm, is almost exclusively found in cyanobacteria (such as *Spirulina platensis*) (Cuellar-Bermudez et al. 2015; Khanra et al. 2018; Rammuni et al. 2019). Unlike chlorophylls and carotenoids, phycobiliproteins are soluble in water and aqueous buffers (e.g. phosphate buffer) and can thus be recovered from microalgal biomass without the use of organic solvents (Cuellar-Bermudez et al. 2015; Khanra et al. 2018; Rammuni et al. 2019).

12.1.1.1 Astaxanthin and H.Pluvialis

H.pluvialis has received significant industrial attention because of its ability to accumulate astaxanthin during the encystment stage. Astaxanthin is a red-coloured alipathic carotenoid ($C_{40}H_{52}O_4$) with powerful antioxidant, anti-inflammatory and immunoprotective properties (Cuellar-Bermudez et al. 2015; Denery et al. 2004; Molino et al. 2018; Rammuni et al. 2019). It is currently used as an oral tablet for treating Alzheimer's disease, Parkinson's disease, stroke, high cholesterol, liver disease and age-related macular degeneration, a feed additive in aquaculture for fish growth as well as a food colorant to provide salmon, trouts and crustaceans their characteristic pink colours. With astaxanthin content up to 7 wt% of its dry biomass (or 90 wt% total carotenoid), red-phase *H.pluvialis* cells accumulate significantly more astaxanthin than any other known natural sources, such as yeast (*Phaffia rhodozyma)* or other algal cells (e.g. *B braunii*, *Chlorella* sp. *Chlorococcum* sp. *Dunaliella* sp. and *Scenedesmus* sp.) (Cuellar-Bermudez et al. 2015; Denery et al. 2004; Molino et al. 2018; Rammuni et al. 2019).

H.pluvialis cells only accumulate astaxanthin in their cytoplasm during the encystment stage. The onset of the stage is triggered by unfavourable growth conditions, such as nitrogen and phosphorous starvation, high solar intensities, salt stress and elevated temperature. The encystment process is, however, accompanied by the formation of a new thick, physically resistant and multi-layered cell wall (Sect. 12.1) which limits the accessibility of the accumulated intracellular astaxanthin. Because of the presence of hydroxyl (–OH) and keto (–C = O) bonds in its molecule, astaxanthin is easily esterified with fatty acids and thus typically accumulated in its ester forms (Cuellar-Bermudez et al. 2015; Denery et al. 2004; Molino et al. 2018; Rammuni et al. 2019). In terms of distribution, up to 95% of astaxanthin in the cells can exist in its ester forms (70% monoester and 25% diester) and only 5% in its free form. Despite having a much stronger antioxidant property than the corresponding ester forms, astaxanthin in its free form is unstable and highly susceptible to oxidation,

(Cuellar-Bermudez et al. 2015; Denery et al. 2004; Molino et al. 2018; Rammuni et al. 2019).

12.1.1.2 Beta Carotene and D. Salina

β-carotene ($C_{40}H_{56}$) is a yellow-coloured highly unsaturated hydrocarbon (Marchal et al. 2013; Rammuni et al. 2019). The 9-cis and all-trans stereoisomers are considered to be the most important because of their physiological roles as pro-vitamin A and radical quenchers. The 9-cis-β-carotene can only be produced from natural sources (such as vegetables and microalgae) and has a stronger antioxidant capacity than the all-trans isomer (Marchal et al. 2013; Rammuni et al. 2019). Even though β-carotene is present in various microalgal and cyanobacterial species (such as *Chlorella zofingiensis, Spirulina platensis* and *Caulerpa taxifolia*), only *D.salina* has been reported to be able to accumulate the pigment up to 13 wt% of its cellular biomass (Marchal et al. 2013; Rammuni et al. 2019). In terms of composition, β-carotene extracted from *D.salina* typically consists of ~ 40% all-trans isomer, ~ 40% 9-cis isomer, ~ 10% 15-cis isomer and ~ 5% other isomers (Marchal et al. 2013; Rammuni et al. 2019). *D. salina* cells accumulate β-carotene as droplets in their chloroplast stroma when exposed to high temperature, high light intensity, high salinity and nitrogen starvation. *D. salina* is a highly promising source of β-carotene not only because of its ability to accumulate 9-cis isomer of the pigment but also because of the fact that the vegetative form of the cells lacks cell walls and can thus easily be ruptured to release the intracellular pigments (Marchal et al. 2013; Rammuni et al. 2019).

12.1.2 An Overview of Microalgal Pigment Extraction

Microalgal pigments are intracellular in nature and can generally only be recovered from the biomass after they have been liberated from the encapsulation of the cell wall. Microalgal cell walls, however, are composed of tough interlinking biopolymers (Sect. 1.3) that confer the cells with structural strength and formidable defense. The first step of pigment recovery, therefore, generally consists of a biomass pretreatment step where the cells are subjected to one or more externally applied mechanical, chemical or enzymatic treatment in order to facilitate disruption, free intracellular products and improve pigment accessibility to a subsequent extraction step. The structural strength of microalgal cells is dependent on the thickness, composition and stratification of their cell walls though these relationships are yet to be fully understood. Some species have thick and highly rigid cell walls (e.g. *Haematococcus pluvialis* and *Nannochloropsis* sp.) which require energy-intensive mechanical treatments (such as high-pressure homogenization and bead milling) or chemical hydrolyses to rupture, while other species have no cell wall (e.g. *Dunaliella salina*) or have less robust cell walls that can be ruptured by simple freeze-thawing or changes in osmotic pressure (e.g. *Rhodomonas salina*).

Once the cells are ruptured, they are then subjected to an extraction step in order to recover the released intracellular pigments. The extraction solvent used for the recovery step will depend on the solubility of the targeted pigment(s). Chlorophylls and carotenoids are not soluble in water and therefore require the addition of organic solvents (such as acetone, ethanol, methanol, ethyl acetate) or supercritical carbon dioxide for their recoveries. The extraction step is often pressurised or coupled with an ancillary rupture process (such as microwave or ultrasonication) in order to enhance mass transfer and thus pigment recovery. Phycobiliproteins, on the other hand, are soluble in water and do not require the use of organic solvents or supercritical carbon dioxide for their extraction. Aqueous buffer with a stable pH (such as phosphate buffer) is often used instead of water in order to prevent potential pigment degradation.

Table 12.1 provides a summary of the different biomass pretreatments and extraction solvents that have been used in previous research to recover pigments from microalgal biomass. Despite our best efforts to ensure its completeness, we note that the list is by no means comprehensive and some technological options might have been overlooked. Cyanobacteria (such as *Spirulina platensis*) and their pigment recoveries have been included in Table 12.1 and various discussions throughout this review for their high phycobiliprotein contents and processing similarities to microalgal biomass. Even though the chapter reviews a wide spectrum of principles and technologies associated with biomass pretreatment and pigment recovery, it focuses its discussion on the recoveries of four industrially valuable pigments from their algal biomass sources (e.g. astaxanthin from *H. pluvialis*, β-carotene from *D. salina*, phycoerythrin from *Porphyridium cruentum* and phycocyanin from *Spirulina platensis*).

Section 12.2 of the chapter evaluates the range of technological options that can be used to rupture microalgal cell walls and release intracellular pigments, while Sect. 12.3 examines the different extraction technologies that are currently available for extracting the released pigments from ruptured biomass. Section 12.4 reviews different biomass processing pathways that have been recently studied for the recoveries of industrially valuable pigments from algal sources (astaxanthin, β-carotene, phycoerythrin, phycocyanin) and summarises key findings from these studies. Section 12.5 outlines the array of technological options available for the purification of pigments after they have been extracted from the microalgal biomass. Finally, Sect. 12.6 evaluates the industrial scalability of pigment extraction in the wider context of a microalgal biorefinery system and provides a recommendation on the future research direction of microalgal pigment extraction.

12.1.3 Microalgal Cell Wall: Structure and Composition

Microalgal cell walls are complex, extremely diverse and poorly understood. The ultrastructure and biochemical composition of a microalgal cell wall are generally determined by the strain's phylogenetic classification, with different species grouped

Table 12.1 Outline of the different pretreatments, cell rupture technologies and extraction solvents that have previously been used to recover astaxanthin, β-carotene, lutein, phycoerythrin and phycocyanin from algal biomass. m: microalgae, cya; cyanobacteria, mac: macroalgae

Pigment	Pigment group	Microalgal/cyanobacterial/macroalgal species with abundance of the pigment	Pretreatment and cell rupture	Extraction solvent
Astaxanthin	Carotenoid	*Haematococcus pluvialis* (m), *Monoraphidium* sp. (m), *Botryococcus braunii* (m), *Chlorella* sp. (m), *Chlorococcum* sp (m)., *Chloromonas nivalis* (m), *Dunaliella* sp. (m), *Scenedesmus* sp. (m), *S. obliquus* (m)	• acid treatment (HCl) • bead milling • freeze drying • ionic liquid permeabilisation (imidazolium, ammonium and phosphonium) •microwave • physical grinding with pestle and mortar • ultrasonication	• organic solvent or pressurized organic solvent: acetone, chloroform/methanol, DMSO, dodecane, ethanol, ethyl acetate, hexane, methanol, methylene chloride, vegetable oil or a mixture of the above solvents (such as chloroform/methanol, hexane/IPA, ethanol/ethyl acetate, acetone/ethanol, acetone/methanol, methylene chloride/methanol) • supercritical carbon dioxide • supercritical carbon dioxide with an organic co-solvent (such as ethanol)
β-carotene	Carotenoid	*Dunaliella salina* (m), *Chlorella zofingiensis* (m), *Spirulina platensis* (cy), *Caulerpa taxifolia* (mac)	• bead milling • freeze drying • spray drying	• organic solvent or pressurized organic solvent: acetone, dichloromomethane, ethanol, ethyl oleate, hexane • supercritical carbon dioxide

(continued)

Table 12.1 (continued)

Pigment	Pigment group	Microalgal/cyanobacterial/macroalgal species with abundance of the pigment	Pretreatment and cell rupture	Extraction solvent
Lutein	Carotenoid	*Chlorella vulgaris* (m), *Chlorella sorokiniana* (m), *Chlorella pyrenoidosa* (m), *Chlorella* sp. (m), *Haematococcus pluvialis* (m), *Scenedesmus obliquus* (m), *Scenedesmus* sp. (m)	• alkali treatment (KOH) • autoclave • bead milling • freeze drying • French press • pulsed electric field • physical grinding with pestle and mortar • ultrasonication	• organic solvent or pressurized organic solvent: acetone, chloroform, dichloromethane, diethyl ether, ethanol, hexane, methanol, petroleum ether, tetrahydrofuran (THF) • supercritical carbon dioxide with an organic co-solvent (such as acetone and ethanol)
Phycoeryhtrin	Phycobiliprotein	*Porphyridium cruentum* (m), *Rhodomonas salina* (m), *Synechococcus* sp. (cy), *Phormidium* sp. (cy), *Leptolyngbya* sp. (cy)	• freeze drying • freeze thawing • grinding with mortar and pestle • high-pressure homogenisation • nitrogen-gas cavitation • pressure filtration • ultrasonication	• culture medium • phosphate buffer • DI water • polyethylene glycol (PEG) • Chaps and asolectin solution in DI water
Phycocyanin	Phycobiliprotein	*Spirulina platensis* (cy), *Galdieria sulphuraria* (cy), *Limnotrhix* sp. (cy), *Synechococcus bacillaris* (cy).	• bead milling • freeze thawing • grinding with mortar and pestle • ultrasonication	• phosphate buffer • DI water • protic ionic liquid: 2-hydroxy ethylammonium acetate (2-HEAA), 2-hydroxy ethylammonium formate (2-HEAF) or a mxiture of 2-HEAA and 2-HEAF

under the same taxon generally sharing common cell wall features and structures (Baudelet et al. 2017; Gerken et al. 2013).

The cell walls of green microalgae are known to have rigid wall components embedded within a plastic polymer-based matrix (Baudelet et al. 2017; Gerken et al. 2013). The rigid fraction is resistant to hydrolysis by either sodium hydroxide or trifluoroacetic acid (TFA) and is composed of glucosamine-derived polymer (such as chitin), glucose-mannose polysaccharides or other biopolymers (such as algaenan) (Baudelet et al. 2017; Gerken et al. 2013). The plastic matrix is hydrolysable by sodium hydroxide or TFA and is generally composed of uronic acids and neutral sugars, such as rhamnose, arabinose, fucose, xylose, mannose, galactose or glucose (Baudelet et al. 2017; Gerken et al. 2013).

Chitin is a crystalline polymer commonly found in shellfish, the exoskeletons of crustaceans, fish scales and the cell walls of fungi. Algaenan is a non-hydrolyzable biopolymer that is commonly found in the rigid fraction of microalgal cell walls across different taxonomical groups (Baudelet et al. 2017; Gerken et al. 2013). It normally forms part of the trilaminar structure (TLS) that is located in the thin outer layer of the cell wall (10–20 nm). The biopolymer is able to withstand harsh acid/alkali hydrolyses and energy-intensive mechanical processes. The presence of algaenan in microalgal cell wall has often been postulated to be the reason for the wall's recalcitrance to cell rupture and resistance to bacterial degradation. Even though its composition is yet to be fully elucidated, algaenan has been shown to comprise long, straight-chain, saturated aliphatic compounds (~ C30) that are joined together with ether cross linkages to form a structure closely resembling that of cutan in vascular plants (Baudelet et al. 2017; Gerken et al. 2013).

12.1.3.1 Cell Wall of Haematococcus Pluvialis

Under nutrient deprivation, *H.pluvialis* cells enter encystment stage and begin to accumulate starch, lipid droplets and astaxanthin. During this stage, the cells increase their volumes and transform themselves from an ellipsoidal flagellate to a spherical red cyst (also known as aplanospore) (Baudelet et al. 2017; Desai et al. 2016).

The cells also synthesise a robust and physically resilient multi-layered cell wall (Fig. 12.1a) as part of the encystment process. This new cell wall (up to 16 wt% of the biomass) consists of an outer trilaminar sheath (TLS) layer and a thick inner secondary wall. The trilaminar sheath is composed of algaenan, while the secondary wall is made of non-fibrillar mannan polymers (Baudelet et al. 2017; Desai et al. 2016). As mentioned above, algaenan is a long-chain alipathic hydrocarbon that is able to withstand harsh oxidative treatments and resists acid/alkali hydrolyses. It is this algaenan that confers *H.pluvialis* cell walls with rigidity and recalcitrance to breakage (Baudelet et al. 2017; Desai et al. 2016). In terms of mass composition, carbohydrate accounts for 70 wt% of the cyst cell wall, while protein and acetolysis-resistant materials (algaenan) make up 6 and 3 wt% of the cell wall respectively. Mannose is the principal monosaccharide of the cell wall (89 wt% of carbohydrates). Glucose, arabinose and xylose are also present in smaller quantities in the cell wall

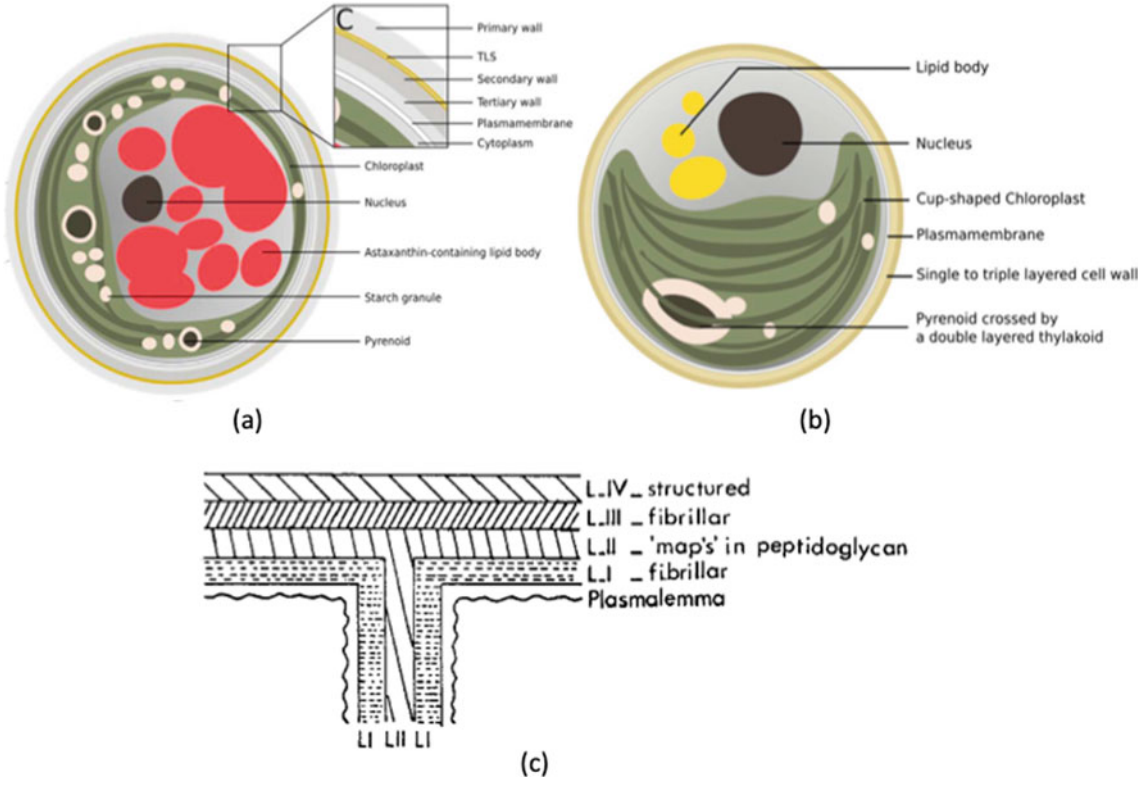

Fig. 12.1 **a** The structure of *Haematococccus* sp. cell and cell wall during red-cyst stage [extracted from Baudelet et al. 2017]. **b** The structure of *Chlorella* sp. cell and cell wall [extracted from Baudelet et al. 2017]. **c** The structure of *Spirulina platensis* cell wall [extracted from Van Eykelenburg 1977]

(respectively at 6, 1.6 and 1.3 wt% of carbohydrates) (Baudelet et al. 2017; Desai et al. 2016).

12.1.3.2 Cell Wall of Chlorella Sp

Since there are dramatic interspecies (between species) and intraspecies (the same species but grown under different conditions) variations in *Chlorella* cell walls, it is difficult to describe a representative *Chlorella* cell wall. Nevertheless, three distinct classes of *Chlorella* wall structures have been identified (Fig. 12.1b): type 1 is bilayered with a microfibrillar inner layer and a TLS outer layer, type 2 is bilayered without the TLS layer and type 3 is a single microfibrillar layer (Baudelet et al. 2017). The cell wall of *C vulgaris* UTEX 395 is a type-2 configuration with long hair-like fibres protruding out of the outer layer. In terms of composition, *Chlorella* cell wall generally comprises 22–25 wt% hemicellulose and 60–66 wt% rigid non-alkali soluble wall fraction. The main neutral sugar monosaccharides found in the rigid fraction are glucosamine, galactose and rhamnose (Baudelet et al. 2017).

12.1.3.3 Cell Wall of Dunaliella Salina

D.salina lacks a cell wall. Instead of a rigid cell wall, the cell's plasmalemma is covered by a thick electron-dense mucilaginous coating composed of lysozyme-sensitive glycoproteins. The genus *Dunaliella* is known to secrete large amounts of exopolysaccharides (EPS) under certain environmental conditions (e.g. salt stress) (Baudelet et al. 2017).

12.1.3.4 Cell Wall of Spirulina Platensis

The cell wall of *Spirulina platensis* consists of four longitudinal layers with recurrent ingrowths of a three-layered septum (Fig. 12.1c) (Van Eykelenburg 1977). L1 and L3 are fibrillar in nature and composed of a glucose polysaccharide known as β-1,2,-glucan. L2 contains peptidoglycan that confers the cells with structural strength and the ability to counteract osmotic pressure, while L4 has a structure analogous to the cell wall of gram-negative bacteria. The thickness of each layer ranges from 10 to 15 nm and the whole wall is roughly 60 nm in thickness.

12.2 Biomass Pretreatment

The purpose of biomass pretreatment or cell disruption is to rupture cell walls in order to liberate intracellular products and improve their accessibilities to extracting solvents. The release of intracellular products from the biomass enhances their mass

transfers during subsequent extraction step and ultimately increases the extraction efficiencies/yields of the products (Dong et al. 2016). An ideal biomass pretreatment needs to be able to avoid/minimise emulsion formation and be energetically efficient and scalable. Biomass can be subjected to more than one pretreatment prior to extraction, though the energy and operational costs of performing multiple pretreatment steps should be taken into consideration. Cell disruption can be classified as either mechanical, chemical or enzymatic treatment. Mechanical treatments include high-pressure homogenisation, bead milling, ultrasonication, pulsed-electric field treatment, microwaving and osmotic shock treatment.

12.2.1 High-Pressure Homogenisation (HPH)

HPH is a mechanical process that pumps cell culture under high pressure to achieve disruption. During HPH operation, microalgal cellular suspension is pumped radially across a narrow valve seat before colliding with an impact ring and subsequently released into a low-pressure chamber. Cell disruption is attributed to several phenomena: the impingement of the cells on the valve seat and impact ring, the pressure drop that the cells experience as they pass from the valve to the chamber and the cavitation caused by the rapid release of gas bubbles from within the cells under sudden pressure drop (Dong et al. 2016; Halim et al. 2013; Martin 2016). HPH is currently used in the biotechnological industry for the recovery of high-value protein. HPH has frequently been shown to be an effective means to rupture microalgal cells for lipid and pigment recoveries (Halim et al. 2012b, 2013, 2019; Jubeau et al. 2013; Yap et al. 2015). In Halim et al. (2012), HPH was found to be more effective in rupturing *Chlorococcum* cells than ultrasonication, bead milling and sulphuric acid treatment. Martin (2016) recently demonstrated that HPH could be an energetically scalable approach for microalgal cell disruption provided that a species with a weak cell wall and high lipid content is used. Dong et al. (2016), however, noted that the efficiency of HPH varies remarkably across different species and growth conditions due to differences in cell wall rigidity. They also noted that the use of HPH could lead to the formation of severe emulsion which hinders solvent recovery and reduces extraction yield.

12.2.2 Bead Milling

Bead milling achieves cell disruption by violently grinding the biomass against solid bead surfaces (Dong et al. 2016; Doucha and Lívanský 2008). The technology has been routinely applied on microalgal biomass for both lipid and pigment extraction (Doucha and Lívanský 2008; Halim et al. 2012b). The use of bead milling, however, can often lead to a dramatic temperature rise which denatures pigments as well as the formation of micelles which hinders solvent recovery and reduces extract purity.

12.2.3 Ultrasonication

During ultrasonication, sonic waves are transmitted to the suspending medium to create a continuous cycle of microbubble formation (rarefaction phase) and implosion (compression phase). The collapse of microbubbles releases shock waves which bombard microalgal cells and shear their cell walls (Dong et al. 2016; Halim et al. 2013). The technology has successfully been used to enhance the yield of both water-insoluble (e.g. astaxanthin) and water-insoluble (e.g. phycocyanin and phycoerythrin) pigments from various microalgal biomass (Benavides and Rito-Palomares 2006; Dong et al. 2014; Furuki et al. 2003; Lawrenz et al. 2011; Rodrigues et al. 2018; Zou et al. 2013). Ultrasonication, however, has been shown to have a slower disruption rate than HPH and a relatively high energy requirement (Halim et al. 2013).

12.2.4 Pulse Electric Field (PEF)

PEF treatment subjects cells to pulses of high-intensity electric field. At sufficiently high transmembrane voltage (0.5–1 V), the field causes cell membrane to become irreversibly permeable (a phenomenon known as 'electropermeabilisation') (Dong et al. 2016; Eing et al. 2013; Luengo et al. 2015; Sheng et al. 2011). Unlike the other mechanical methods, PEF generally does not result in cell rupture and can be particularly useful for the selective extraction of small intracellular components that can pass through the permeabilised cell membrane/wall complex, such as water-soluble enzymes and ionic compounds (Dong et al. 2016; Eing et al. 2013; Luengo et al. 2015; Sheng et al. 2011). Since PEF treatment requires an electrically non-conductive medium for its operation, saltwater algal species will have to be dewatered and washed with a large amount of fresh water prior to being subjected to the treatment. In Luengo et al. (2015), PEF treatment was shown to be able to substantially increase (by a factor of 4) the yield of lutein extraction from *Chlorella vulgaris* biomass.

12.2.5 Osmotic Shock

Sudden change in the solute concentration of culture medium creates pressure difference between the cell cytoplasm and the medium. This can potentially result in cell rupture if the species has a relatively weak cell wall (Dong et al. 2016). For freshwater species (such as *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp.), osmotic shock is carried out at hypertonic shift, triggered by adding solutes (such as NaCl and sorbitol) to the culture medium. For saltwater species (such as *Dunaliella viridis*), osmotic shock is performed at hypotonic shift, induced by transferring biomass to a

medium with lower salt concentration or fresh water (Dong et al. 2016). The effectiveness of osmotic shock appears to be highly species dependent and, as such, the treatment may not be the most reliable method for cell rupture.

12.2.6 Microwaves

The transmission of microwave energy into cell suspension triggers rapid vibration of the liquid particles and leads to rapid heating of the cell matrix. The increase in intracellular kinetic energy exerts pressure on the cell wall and leads to cell rupture (Balasubramanian et al. 2011; Dong et al. 2016; Rammuni et al. 2019). A number of previous studies (Balasubramanian et al. 2011; Lee et al. 2010; Zhao et al. 2009) have applied microwave treatment on different microalgal species and demonstrated its ability to increase the yield of both lipid and pigment extractions (total lipid from *Scenedesmus obliquus*, *Botryococcus* sp., *C. vulgaris*, *and Scenedesmus* sp., astaxanthin from *H.pluvialis*) (Table 12.3). Similar to HPH, the use of microwave treatment can potentially lead to severe emulsion formation that impedes solvent recovery.

12.2.7 Enzymatic Treatment

Various studies have used enzymatic treatments as means to hydrolyse algal cell walls for carbohydrate solubilisation, lipid extraction and pigment extraction (Demuez et al. 2015; Dong et al. 2016; Gerken et al. 2013; Grossman et al. 2011; Kobayashi et al. 1997; Zuorro et al. 2016). The hydrolysis step normally requires a cocktail of different enzymes, the formulation of which will depend on the composition of the cell wall to be hydrolysed. Since cell wall configuration varies considerably between species, the composition of the enzyme cocktail needs to be tailored and optimised for each specific biomass. Enzymes are expensive and need to be recovered and reused for the treatment to be economically scalable. Because enzymatic hydrolysis is normally carried out at a relatively low temperature, it has the potential to be used in the recovery of thermally sensitive/labile products (Demuez et al. 2015; Dong et al. 2016; Gerken et al. 2013; Grossman et al. 2011; Kobayashi et al. 1997; Zuorro et al. 2016). *Chlorella* has previously been shown to be vulnerable to enzymes that attack sugar polymers containing N-acetylglucosamine, such as chitinases and lysozymes. *Nannochloropsis*, on the other hand, was found to be susceptible to enzymes that degrade glucose polymers, such as cellulose and hemicellulose (Demuez et al. 2015; Dong et al. 2016; Gerken et al. 2013; Grossman et al. 2011; Kobayashi et al. 1997; Zuorro et al. 2016). Kobayahi et al. (1997) found the extractability of astaxanthin from *H. pluvialis* to be significantly enhanced after the biomass was treated with one of the following enzymes: β-1,3-glucanase, cellulase, β-1,4-glucanase or β-glucuronidase.

12.2.8 Chemical Hydrolysis (Alkali and Acid)

Acid and alkali treatments have been shown to be effective means of hydrolysing microalgal cell wall. Unlike mechanical treatments which generally use a form of shear force in order to rupture the cell walls, chemical treatments work by attacking certain linkages in the wall, hydrolysing these bonds and ultimately dissolving the entire wall or the wall layer away (Dong et al. 2016; Laurens et al. 2015, 2017). Chemical treatments generally have low energy requirements and can often suppress emulsion formation as they hydrolyse long chains of surfactant molecules (e.g. polysaccharides, protein and phospholipids) into constituent units (Dong et al. 2016; Laurens et al. 2015, 2017). The main drawback of chemical treatments will be their relatively slow kinetics in comparison to mechanical treatments. Laurens et al. (2015) showed that dilute acid pretreatment can assist lipid extraction from microalgal biomass by degrading cell wall and enabling the release of oil droplets (lipid extraction from acid-treated biomass can recover up to 97% of fatty acids). Dilute acid pretreatment (1-10 N HCl at 70 °C) has also been applied to astaxanthin recovery from *H. pluvialis* biomass and was shown to be able to improve both the astaxanthin yield and the antioxidant activity of the lipid extract. (Dong et al. 2014; Sarada et al. 2006).

12.3 Pigment Extraction

After cell rupture, released intracellular pigments are recovered from the debris using an extraction solvent. For water-soluble pigments (such as phybiliproteins), the extraction solvent can be water, the culture medium itself or an aqueous buffer. For water-insoluble pigments (such chlorophylls and carotenoids), one of the following extraction systems is used: organic solvent extraction, pressurised solvent extraction, ionic liquid extraction or supercritical carbon dioxide extraction.

12.3.1 Organic Solvent Extraction

Pigment extraction is based on the principle of 'like dissolves like'. Chlorophylls and carotenoids have high partition coefficients in organic solvents and will migrate out of the biomass (cell debris or intact cells) into the solvent during the extraction process (Halim et al. 2012a; Rammuni et al. 2019). Various organic solvents have previously been used for the extraction of chlorophylls and carotenoids from microalgal biomass (Table 12.1): acetone, chloroform/methanol, DMSO, dodecane, ethanol, ethyl acetate, hexane, methanol, methylene chloride, vegetable oil and a mixture of one or more of the above solvents. After the extraction step, a solid/liquid separation step is used in order to separate solvent from the cell debris. The solvent phase is then subjected to a distillation step in order to obtain crude lipid extract containing

the targeted pigment and to recycle the organic solvent. In order to reduce the energy requirement of distillation, the selected solvent for industrial-scale pigment extraction should be relatively volatile and have a low boiling point. Since the extracted pigments will likely be used for nutraceutical or food applications, it is also important to use green solvents or solvents of low toxicity for the extraction (e.g. acetone and ethanol instead of chloroform and methanol).

12.3.2 Pressurised Liquid Extraction (PLE)

Pressurised liquid extraction (PLE), also known as pressurised solvent extraction or accelerated solvent extraction, is an organic solvent extraction that is performed at elevated temperature and pressure. (Halim et al. 2012a; Herrero et al. 2006; Jaime et al. 2010). Solvent is maintained in its liquid state throughout the operation. The temperature and pressure elevation help to disintegrate cellular structures and accelerate mass transfer kinetics. As a result, PLE uses less solvent and can complete the extraction process in a shorter timeframe compared to a conventional organic solvent extraction (Halim et al. 2012a; Herrero et al. 2006; Jaime et al. 2010). However, PLE has a higher energy requirement than conventional solvent extraction due to its use of elevated temperature and pressure. High temperature (up to 200 °C) may also lead to the degradation of thermally sensitive proteins and pigments (Halim et al. 2012a; Herrero et al. 2006; Jaime et al. 2010). In their study investigating the use of PLE (with either hexane or ethanol) to obtain astaxanthin from red-cyst *H. pluvialis*, Jaime et al. (2010) found that temperature increase during PLE improved extraction yield but had a negative effect on the structural integrity and thus the antioxidant activity of the extract (Table 12.3). PLE (with hexane, ethanol, water or acetone) has also been applied for the extraction of β-carotene from *D. salina* (Denery et al. 2004; Herrero et al. 2006) (Table 12.4).

12.3.3 Ionic Liquid Extraction

Ionic liquids are salts of loosely held anions and cations that remain in liquid state over a wide range of temperature (melting point < 100 °C) (Desai et al. 2016; Praveenkumar et al. 2015; Rodrigues et al. 2018). Many of their physical properties, such as polarity, hydrophobicity and viscosity, are adjustable and can be controlled by the exchange or combination of ions. Such physical versatility means that their solvating power can be specifically tailored to the target compound in order to enhance solvent-solute interaction and increase extraction efficiency. This makes ionic liquids a potent extracting solvent and an attractive alternative to organic solvents for various extraction systems, including pigments from microalgae (Desai et al. 2016; Praveenkumar et al. 2015; Rodrigues et al. 2018). Most ionic liquids,

however, are expensive and have low biodegradability. The high temperature requirement for their operation (> 100 °C) can potentially be detrimental to the integrity of targeted pigments and other valuable components in the microalgal biomass (e.g. protein) (Desai et al. 2016; Praveenkumar et al. 2015; Rodrigues et al. 2018). Ionic liquids are classified as either aprotic (which includes imidazolium and pyridine cations) or protic (which includes ethylammonium cations) (Desai et al. 2016; Praveenkumar et al. 2015; Rodrigues et al. 2018). Rodrigues et al. (2018) found protic ionic liquids to be more effective than standard sodium phosphate buffer in extracting phycobiliprotein from *Spirulina platensis* (Table 12.5). They attributed the high extraction efficiency to the liquid's high diffusional power and strong interaction with the pigment molecules. Desai et al. (2016) used ionic liquids as an agent to permeabilise *Haematoccous pluvialis* cell wall before subjecting the biomass to ethyl acetate extraction for astaxanthin recovery (Table 12.2).

12.3.4 Supercritical Carbon Dioxide ($SCCO_2$) Extraction

When carbon dioxide is subjected to temperature and pressure beyond their critical values (T_c at 31.1 °C and P_c at 72.9 atm), it is transformed into a supercritical fluid that exhibits physical properties intermediate between a liquid and a gas (Cuellar-Bermudez et al. 2015; Halim et al. 2011, 2012a; Rammuni et al. 2019; Reyes et al. 2014; Soh and Zimmerman 2011). Supercritical carbon dioxide or $SCCO_2$ is a highly effective extraction solvent because of its transitional properties, such as high diffusivity and adjustable solvating power.

$SCCO_2$ has a high diffusivity which enables it to rapidly penetrate cellular matrix and complete extraction within a shorter timeframe. The fluid's solvating power is a direct function of its density and can thus be tailored to the targeted pigment through pressure and temperature adjustments (Cuellar-Bermudez et al. 2015; Halim et al. 2011, 2012a; Rammuni et al. 2019; Reyes et al. 2014; Soh and Zimmerman 2011). In addition to being a powerful extraction solvent, $SCCO_2$ is non-toxic, produces a solvent-free pigment extract (no additional solvent recovery step is needed—refer to operational description below) and operates at a moderate temperature range which minimises degradation of thermally sensitive pigments (Cuellar-Bermudez et al. 2015; Halim et al. 2011, 2012a; Rammuni et al. 2019; Reyes et al. 2014; Soh and Zimmerman 2011).

The main disadvantages of $SCCO_2$ extraction are the high capital cost of its infrastructure and the high energy cost associated with biomass drying step prior to the extraction (Cuellar-Bermudez et al. 2015; Halim et al. 2011, 2012a; Rammuni et al. 2019; Reyes et al. 2014; Soh and Zimmerman 2011). Since $SCCO_2$ is generally assumed to have a limited capacity to interact with solutes in wet biomass, lipid extraction using the fluid has almost always been carried out on dried (or freeze dried) biomass. This introduces the need for an energy-intensive dehydration step before the extraction and significantly inflates the operational energy requirement of the extraction process. In their study investigating $SCCO_2$ extraction from wet

Table 12.2 Summary (1st) of previous studies investigating astaxanthin extraction from microalgal biomass

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Molino et al. (2018)	*Haematococcus pluvialis*	Red cyst, freeze-dried	Mixed with diatomaceous earth, bead milled	Pressurised solvent extraction with acetone, ethanol or chloroform/methanol (1:1), hexane	87 wt% of available astaxanthin with acetone at 100 bar & 40 °C	Mechanical pretreatment improved astaxanthin yield by 3 orders of magnitude. Astaxanthin was degraded at high temperature beyond 40 °C for acetone and 67 °C for ethanol
Desai et al. (2016)	*H. pluvialis*	spray-dried	Ionic liquid permeabilisation at 25, 45, 55 or 65 °C. Seven ionic liquids from three different classes (imidazolium, ammonium and phosphonium) were trialled	Two stages of ethyl acetate extraction	>70 wt% of available astaxanthin with biomass pretreatment using 1-ethyl-3-methylimidazolium dibutylphosphate (EMIM DBP) at 45 °C and 40% w/w in water	Ionic liquids did not disrupt the cell wall; instead, the liquids dissolved part of the mannan polysaccharide in the cell wall, perforating/permeabilising the walls to form tiny passages that enabled subsequent organic solvents to penetrate the cells and extract astaxanthin

(continued)

Table 12.2 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Dong et al. (2014)	*H. pluvialis*	Freeze-dried	n/a	Four different extraction methods: 1. 70 °C HCl (4 M) biomass treatment followed by ultrasound-assisted acetone extraction, 2. ultrasound-assisted hexane/IPA extraction, 3. sequential ultrasound-assisted methanol and acetone extractions, 4. vegetable oil extraction	19.8 mg astaxanthin/g biomass with HCl-acetone extraction (method 1)	The highest lipid extraction yield (33.3 wt% of biomass) and antioxidant activity were also obtained with HCl-acetone extraction (method 1) Antioxidant activity of the extract can be directly correlated to its astaxanthin content
Reyes et al. (2014)	*H. pluvialis*	Powder	n/a	Supercritical carbon dioxide extraction with ethanol as a co-solvent	82.29 wt% of available astaxanthin at 55 °C, 20.0 MPa, 13 wt% ethanol in $SCCO_2$	Ethanol content had more impact on the yield, astaxanthin content and antioxidant activity of the extract than pressure and temperature

(continued)

Table 12.2 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Zou et al. (2013)	*H. pluvialis*	Powder	n/a	Ultrasound-assisted solvent extraction with a mixture of ethanol and ethyl acetate. ultrasound power = 200 W, frequenzy = 40 kHz	27.58 mg astaxanthin/g biomass at 48% ethanol in ethyl acetate, solvent-to-biomass ratio of 20:1, 16 min and 41.1 °C	The yield of ultrasound-assisted extraction was almost double that obtained from conventional extraction. Astaxanthin yield from ultrasound-assisted extraction increased from 20 to 40 °C and decreased from 40 to 70 °C due to thermal degradation
Fujii (2012)	*Monoraphidium* sp. GK12	Freeze-dried	n/a	Supercritical carbon dioxide extraction with ethanol as a co-solvent. Centrifugation to separate biomass from ethanol. Ethanol phase was mixed with acids (sulphuric, hydrochloroic, acetic or phosphoric acid) to remove chlorophyll from the extract	2.46 mg astaxanthin/g biomass at 60 °C, 20 MPa, 60 min and ethanol addition (20 ml/g biomass)	The addition of ethanol as a co-solvent improved astaxanthin yield (from 83% to 87–100% of available astaxanthin) The addition of sulphuric acid (0.01 N) or hydrochloric acid (0.01 N) to the extract was able to precipitate out ~ 80 wt% of the co-extracted chlorophyll without affecting the astaxanthin yield

Scenedesmus dimorphus, Zimmerman and Soh (2011), however, found that extraction from wet slurry was able to achieve similar lipid and fatty-acid methyl ester yields to that from freeze-dried biomass. These findings are in conflict with current consensus in the field and allude to the fact that further research may be needed in order to examine the ability of $SCCO_2$ to extract bio-compounds from wet biomass.

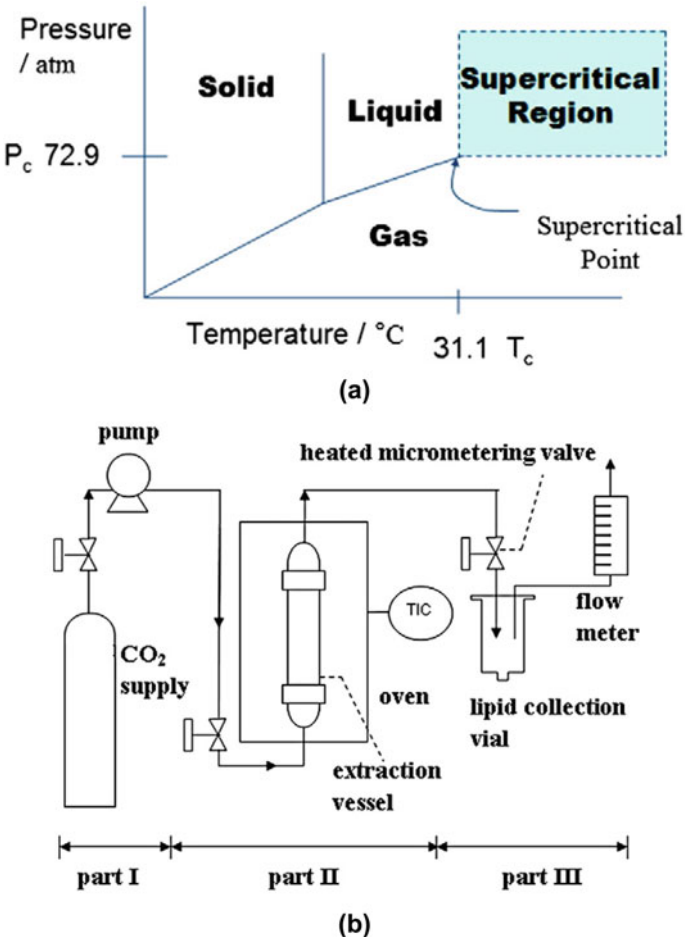

Fig. 12.2 **a** P-T Phase diagram for CO_2, showing the supercritical region [extracted from Halim et al. 2012]. **b** Schematics of a $SCCO_2$ extraction unit [extracted from Halim et al. 2011]. Part I consists of a feed pump which compresses and delivers liquid CO_2 to the extraction vessel. Part II comprises a stainless steel extraction vessel installed inside an oven module. Once the oven is heated, the compressed CO_2 enters the vessel in a supercritical state and performs extraction on the microalgal biomass. Part III consists of a heated micrometering valve to depressurise incoming $SCCO_2$. Once completely decompressed, CO_2 evaporates as gas to the ambient, forcing the extracted lipid/pigment to precipitate out in the lipid/pigment collection glass vial

An $SCCO_2$ extraction setup can be divided into 3 separate parts (Fig. 12.2b). Part I consists of a feed pump which compresses and delivers liquid CO_2 to the extraction vessel. Part II comprises an extraction vessel where the microalgal biomass is placed and an oven module that heats incoming CO_2 and converts it into supercritical fluid in the extraction vessel. Part III consists of a heated micrometering valve that depressurises the outgoing $SCCO_2$. Once completely decompressed, CO_2 evaporates back to the ambient as a gas and the recovered pigment is forced to precipitate out as a solvent-free liquid extract.

The use of $SCCO_2$ extraction for the recoveries of astaxanthin from *Haematococcus pluvialis* (Fujii 2012; Machmudah et al. 2006; Reyes et al. 2014) and β-carotene from *Dunaliella salina* has been demonstrated (Pour Hosseini et al. 2017) (Tables 12.2–12.4). Because of its non-polar nature, $SCCO_2$ is unable to effectively interact with polar pigment molecules such as astaxanthin. A polar modifier (also known as co-solvent or entrainer), such as ethanol, is therefore often added to the solvent during the extraction process in order to enhance its affinity towards the targeted pigment and increase extraction yield.

12.4 Processing Pathways for Pigment Recovery from Microalgal Biomass

This section examines the different biomass processing pathways used for extracting pigments from microalgae. We focus our discussion on the extraction of four industrially relevant and highly valuable microalgal pigments: astaxanthin, β-carotene, phycoerythrin and phycocyanin.

12.4.1 Astaxanthin Recovery

Tables 12.2 and 12.3 summarise previous studies that have investigated astaxanthin recovery from microalgal biomass (Denery et al. 2004; Desai et al. 2016; Dong et al. 2014; Jaime et al. 2010; Kang and Sim 2007; Kobayashi et al. 1997; Molino et al. 2018; Sarada et al. 2006; Zhao et al. 2009; Zou et al. 2013). The tables report the biomass pretreatment (cell rupture) and the extraction method used in each study as well as key findings obtained from each investigation. As can be seen from the tables, *Haematococcus pluvialis* was the species used in majority of the studies. *H.pluvialis* cells can accumulate significant amounts of astaxanthin (up to 7 wt%) in their cytoplasm during the encystment phase of their life cycles. The cells, however, are protected by thick, robust and exceedingly tough bilayered cell walls that consist of an outer trilaminar sheath (made of algaenan) and an inner secondary polysaccharide wall (made of mannan). Astaxanthin recovery from *H.pluvialis* generally subsribes to a two-step cell rupture and extraction procedure. During the cell rupture step, the biomass is subjected to a mechanical pretreatment (bead miling/ultrasonication/microwave), a chemical pretreatment (HCl hydrolysis/ionic

Table 12.3 Summary (2nd) of previous studies investigating astaxanthin extraction from microalgal biomass

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Jaime et al. (2010)	*H. pluvialis*	Green phase and red cyst, freeze-dried	Freezed-dried biomass was mashed with liquid nitrogen in a mortar	Accelerated (pressurised) solvent extraction with hexane or ethanol	37.1 g extract/100 g biomass for green cells with ethanol at 10.34 MPa, 200 °C, 20 min. 29.1 g extract/100 g biomass for red cells with ethanol at 10.34 Mpa, 200 °C, 20 min	Ethanol was a more effective extraction solvent than hexane Mono- and diesters of astaxanthin displayed a lower antioxidant activity than free astaxanthin. Enzymatic hydrolysis of these esters resulted in the formation of free astaxathin and significantly increased antioxidant activity of the extract

(continued)

Table 12.3 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Zhao et al. (2009)	*H. pluvialis*	Powder	n/a	Microwave-assisted solvent extraction with a mixture of ethanol and ethyl acetate (2:1 v/v)	594 μg astaxanthin/100 mg biomass at 141 W, 83 s, 9.8 ml/200 mg biomass and 4 extraction cycles	Microwave-assisted extraction increased astaxanthin yield and dramatically reduced extraction time The extracts obtained from microwave-assisted extraction exhibited strong antioxidant activities, strong radical scavenging properties against DPPH and strong reducing power

(continued)

Table 12.3 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Kang and Sim (2007)	*H. pluvialis*	Red cyst, direct culture from cultivation (without dewatering)	n/a	Two-stage dodecane and methanol extraction	Over 85 wt% of total astaxanthin with the developed procedure	Up to 95 wt% of available astaxanthin could be extracted from the intact cyst cells into the dodecane phase without the need for dewatering and cell rupture. The addition of hydroxyl group (NaOH) in methanol was able to saponify/hydrolyse astaxanthin ester to yield free astaxanthin and carboxylate salt. The free astaxanthin preferentially partitioned in the methanol phase (98 wt%) instead of dodecane (2 wt%)

(continued)

Table 12.3 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Machmudah et al. (2006)	*H. pluvialis*	Powder	n/a	Supercritical carbon dioxide extraction with ethanol as a co-solvent	77.9 wt% of available astaxanthin at 55 MPa, 343 K and 3 ml/min CO_2	The amount of total extract, the astaxanthin recovery and the astaxanthin content in the extract increased with increasing temperature and pressure
Sarada et al. (2006)	*H. pluvialis*	Red cyst, freeze-dried	In some experiments, biomass was treated for chlorophyll removal: 5% methanolic KOH at 70 °C	Three different extraction methods: 1. biomass homogenisation with pestle and mortar followed by acetone extraction. 2. direct extraction with acetone, methanol and DMSO. 3. biomass treatment with HCl (1–10 N) and other acids at 70 °C followed by acetone extraction	86.40 wt% of available astaxanthin with method 3	During HCl-acetone extraction, HCl acted as a disruption agent which cleaved vital bonds in the cell wall to facilitate solvent access into the cells

(continued)

Table 12.3 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Denery et al. (2004)	*H. pluvialis*	Red cyst, freeze-dried	n/a	Pressurised solvent extraction with acetone, ethanol, acetone:ethanol (7:3 v/v), acetone:methanol (7:3 v/v) or methylene chloride:methanol (1:3 v/v). pressure = 1500 psi. temperature = 40 °C	10.9 mg astaxanthin/g biomass with methylene chloride:methanol (1:3 v/v)	Acetone extraction obtained the highest total pigment yield (47.7 mg pigment/g biomass). Increasing extraction temperature resulted in pigment degradation and decreased total pigment yield

liquid permeabilisation) or a combination of two or more pretreatments (HCl hydrolysis followed by ultrasonication) in order to disrupt the thick cell walls of *H. pluvialis* cysts. This was then followed by a subsequent extraction step where organic solvent, pressurised solvent or supercritical carbon dioxide is used to extract the released pigment from the cell debris. For organic solvent extraction or pressurised solvent extraction, acetone, ethanol, ethyl acetate, hexane and methylene chloride are frequently used. The cell rupture step (ultrasonication, microwave or HCl treatment) and the extraction step are often combined and can be carried out simultaneously as a single step (Dong et al. 2014; Zhao et al. 2009; Zou et al. 2013).

Across the different studies, the integration of a cell rupture step prior to or during astaxanthin extraction consistently led to an improved extraction efficiency and overall increase in the pigment yield. In Molino et al. (2018), subjecting *H.pluvialis* red cysts to bead milling prior to the extraction step was able to triple the amount of extracted astaxanthin. Similarly, the use of ultrasonication in Zou et al. (2013) doubled the astaxanthin yield obtained by both ethyl acetate and ethanol extraction. In Sarada et al. (2006), a higher astaxanthin yield was obtained from acetone extraction of HCl-treated biomass compared to that from untreated biomass.

For $SCCO_2$ extraction, the addition of ethanol to the extraction process was shown to be able to consistently improve astaxanthin yield (Fujii 2012; Jaime et al. 2010; Machmudah et al. 2006; Reyes et al. 2014). $SCCO_2$ is a non-polar solvent and the addition of a polar entrainer such as ethanol to the solvent was able to improve its affinity (and thus interaction) with astaxanthin molecules.

Free astaxanthin has a stronger antioxidant property and thus a higher market value than its corresponding mono- or diester forms. Since up to 95% of astaxanthin in *H.pluvialis* cells can exist in its ester forms, astaxanthin extract from the biomass is often subjected to a post-recovery hydrolysis step in order to saponify any ester in the crude extract to free astaxanthin (Jaime et al. 2010). The hydrolysis step typically involves reaction of the extracted astaxanthin with a base (such as NaOH or KOH) or cholesterol esters (Denery et al. 2004; Jacobs et al. 1982; Jaime et al. 2010; Kang and Sim 2007).

We note that the majority of previous studies on astaxanthin recovery have used freeze-dried *H. pluvialis* powder as the starting biomass for their extraction (Tables 12.2 and 12.3). Since biomass drying or dehydration (whether it is thermal drying or freeze drying) forcefully removes water molecules from cell cytoplasm, it inadvertently disrupts the cell walls and can be considered as an extra cell rupture or pretreatment step. Biomass drying, however, requires a tremendous amount of energy and is unnecessary if the biomass is later to be subjected to another cell rupture step (such as high-pressure homogenisation or ultrasonication). We have not included drying in our discussion in Sect. 12.2 as we do not consider it as a scalable pretreatment. Recent life-cycle analyses on microalgal biorefineries for both food and fuel applications have established that biomass drying on a commercial scale is energetically prohibitive and thus has to be avoided in order for the systems to achieve a positive energy balance (Dong et al. 2016; Martin 2016). The economic prospects of biomass dehydration will be further discussed in Sect. 12.6.

12.4.2 β-Carotene Recovery

Table 12.4 provides a summary of previous studies that have investigated β-carotene recovery from microalgal biomass (Abu-Rezq et al. 2010; Herrero et al. 2006; Marchal et al. 2013; Pour Hosseini et al. 2017; Rammuni et al. 2019; Thoisen et al. 2017; Viskari and Colyer 2003). The table reports the biomass pretreatment (cell rupture) and extraction method used in the studies as well as the key findings obtained from them. As can be seen in the table, *Dunaliella salina* was the species used in all of the studies. *D. salina* cells are able to accumulate significant amounts of β-carotene (up to 13 wt% of biomass) as droplets in their chloroplast stroma under certain environmental stresses (e.g. high temperature, high light intensity, high salinity and nitrogen starvation astaxanthin). The cells do not have rigid cell walls. Instead, each cell is encapsulated by a thick mucilaginous coating that is composed primarily of glycoproteins.

Similar to astaxanthin recovery from *H. pluvialis*, β-carotene recovery from *D.salina* also follows a two-step cell rupture and extraction formula. During the cell rupture step, the biomass is subjected to a mechanical pretreatment (such as bead milling) in order to rupture the cells. This was then followed by a subsequent extraction step where pressurised solvent (such as acetone, ethanol, or hexane) or supercritical carbon dioxide is used to extract pigments from the biomass. However, since *D. salina* lacks cell walls, their cells tend to rupture more easily than other microalgal cells with rigid cell walls (e.g. *H. pluvialis*). For this reason, a dedicated biomass pretreatment (or cell rupture) step is often not needed as the cells are readily ruptured to release their intracellular pigments upon solvent contact during the extraction step. This automatic cell rupture simplifies β-carotene recovery from *D.salina* into a single-step extraction procedure. As can be seen in Table 12.4, the pretreatment or cell rupture step was omitted in the majority of studies that have previously attempted to optimise β-carotene recovery from *D. salina.*

12.4.3 Phycobiliprotein Recovery

Since phycobiliproteins are water-soluble pigments, they do not require the use of organic solvent or supercritical carbon dioxide for their recoveries (Furuki et al. 2003; Lawrenz et al. 2011; Thoisen et al. 2017). The recovery process for phycobiliproteins therefore entails a combined cell rupture and extraction process, whereby the biomass is first suspended in the aqueous extraction solvent (such as water, phosphate buffer, culture medium or ionic liquid) before being subjected to a mechanical cell rupture step (such as ultrasonication, high-pressure homogenisation, bead milling, grinding and nitrogen cavitation) to liberate the phycobiliproteins to the aqueous solvent. Tables 12.5 and 12.6 provide a summary of previous studies examining phycobiliprotein recovery (both phycoertyhrin and phycocyanin) from microalgal and cyanobacterial biomass. The tables report the cell rupture and the extraction methods used

Table 12.4 Summary of previous studies investigating β-carotene extraction from microalgal biomass

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Pour et al. (2017)	*Dunaliella salina*	Spray dried	Spray-dried biomass was homogenised with glass bead	Supercritical carbon dioxide extraction	115.43 μg β-carotene/g biomass at 400 bar and 55 °C	Pressure had more impact on the extraction yield than temperature. At optimum condition, the carotenoid recovery of $SCCO_2$ extraction was half that of solvent extraction
Marchal et al. (2013)	*D. salina*	Direct culture from cultivation	n/a	Centrifugal partition chromatography (CPC) with a mixture of ethyl oleate and dichloromethane or a mixture of decane and dichloromethane. The organic solvent acts as the stationary phase and the culture acts as the mobile phase	65% of available β-carotene with ethyl oleate/dichloromethane mixture (95: 5 v/v)	For CPC, it is critical that the solvent is biocompatible (non-toxic) with the cells, exhibits maximum β-carotene solubility and is immiscible with water Post-extracted culture can be re-cultivated as only 35% of the cells lost their viability (was damaged) during the extraction process

(continued)

Table 12.4 (continued)

Study	Species	Biomass state	Pretreatment and cell rupture	Extraction solvents and conditions	Maximum yield/recovery at optimum conditions	Key findings
Herrero et al. (2006)	*D. salina*	Freeze-dried	n/a	Pressurised solvent extraction with hexane, ethanol or water	34.6 wt% of available β-carotene for ethanol at 160 °C, 17.65 wt% of available β-carotene for hexane at 160 °C, 9.2 wt% of available β-carotene for water at 160 °C.	Hexane extract had a higher antioxidant activity than ethanol extract. Water was not a very effective extraction solvent
Denery et al. (2004)	*D. salina*	Freeze-dried	n/a	Pressurised solvent extraction with acetone. pressure = 1500 psi	2.7 mg β-carotene/g biomass with acetone at 1500 psi	Pigment extracts consisted of lutein (32 wt%), chlorophyll *b* (18 wt%), chlorophyll *a* (13 wt%) and β-carotene (13 wt%)

Table 12.5 Summary (1st) of previous studies investigating phycobiliprotein recovery from microalgal and cyanobacterial biomass

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Rodrigues et al. (2018)	*Spirulina platensis*	Phycobiliprotein with a focus on allophycocyanin (A-PC), phycocyanin (PC) and phycoerythrin (PE)	Oven-dried (60 °C for 24 h) and ground powder	Ultrasound assisted protic ionic liquid extraction: ultrasound frequency = 25 kHz, temperature = 25 °C. Solvents: 2-hydroxy ethylammonium acetate (2-HEAA), 2-hydroxy ethylammonium formate (2-HEAF), equimolar mixture of 2-HEAA and 2-HEAF, 1-butyl-3-methylimidazolium chloride [Bmim][Cl] or 0.1 M sodium phosphate buffer	6.34 mg A-PC/g biomass, 5.95 mg PC/g biomass and 2.62 mg PE/g biomass with 2-HEAA and 2-HEAF mixture at pH = 6.50, solvent:biomass ratio = 7.93 ml/g	Protic ionic liquids were generally more effective than sodium phosphate buffer and commercial ionic liquid [Bmim] [Cl] in extracting all 3 phycobiliproteins from the cyanobacterial cells due to their greater diffusional power and interaction with the pigments
Thoisen et al. (2017)	*Rhodomonas salina*	Phycoerythrin (PE)	Cells were filtered onto 0.2 μm filter under 34 kPa	Freezing and phosphate buffer extraction: 0.05 M K_2HPO_4, and 0.05 M KH_2PO_4 (pH 6.7)	8.04 ρg PE/cell	Treating the biomass with ultrasonication was found to be unnecessay for cryptophytes as they do not possess a cell wall

(continued)

Table 12.5 (continued)

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Jubeau et al. (2013)	*Porphyridium cruentum*	B-phycoerythrin (B-PE)	direct culture from cultivationn (exponential phase)	2-stage high-pressure homogenisation (HPH): low-pressure HPH (27–70 MPa) in culture medium to extract protein, centriugation, resuspension in distilled water, then high-pressure HPH at 270 MPa to recover B-PE	almost 100 wt% of available B-PE (3.6 mg B-PE/g biomass) with a purity ratio of 0.79	Intracellular proteins are extracted more effectively extracted at low HPH pressure than B-PE. Hence, with a two-step HPH procedure, it is possible to selectively remove protein in the first low-pressure HPH step and then recover enriched B-PE with the second high-pressure HPH step
Sorensen et al. (2013)	*Galdieria sulphuraria*	C-phycocyanin (C-PC)	Centrifuged concentrate	Ball milling followed by phosphate buffer extraction: three cycles in 50 mmol/L potassium phospate buffer (pH 7.2) followed by cell rupture in a ball mill (3,500 rpm). The supernatant (or crude extract) was collected and purified	25–30 mg C-PC/g biomass	Without further purification steps, the crude extract had a low purity

(continued)

Table 12.5 (continued)

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Gantar et al. (2012)	*Limnotrhix* sp	C-phycocyanin (C-PC)	Dewatered concentrate	Repeated (3x) freeze-thawing in distilled water. The lysate was filtered and subjected to a sequence of purification steps: mixing with activated carbon (1% w/v) and chitosan (0.01 g/l), ammonium sulphate precipitation (20–60%) and concentration with tangential flow filtration (30 kDa)	180 mg C-PC/g biomass with a purity ratio (A_{620}/A_{680}) of 2.0 without purification, 80 mg C-PC/g biomass with a purity ratio 4.3 with the use of all 3 purification steps	Subjecting C-PC extract to purification steps increased its purity but reduced its overall yield or recovery

Table 12.6 Summary (2nd) of previous studies investigating phycobiliprotein recovery from microalgal and cyanobacterial biomass

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Lawrenz et al. (2011)	*R. salina* and *Synechococcus bacillaris*	Phycoerythrin (PE) from *rhodomonas salina* and phycocyanin (PC) from *Synechococcus bacillaris*	The culture was either vacuum filtered onto a 0.7 μm glass fiber filter and frozen at –80 °C or centrifuged	Various methods involving phosphate buffer extraction (0.1 M, pH 6), freeze-thawing (−20 °C), lyophilisation, physical grinding and ultrasonication (8 W)	100 wt% of available PE from *rhodomonas salina* with freeze-thawing and centrifugation. > 98 wt% of available PC from *Synechococcus bacillaris* with freeze-thawing, ultrasonication and centrifugation	Extraction performed on concentrate harvested by filtration had a lower efficiency than that performed on centrifuged concentrate (32 vs 54–98 wt% of available phycobiliprotein) Ultrasonication was more effective in rupturing cells and resulted in higher extraction efficiencies than grinding

(continued)

Table 12.6 (continued)

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Benavides and Rito-Palomares (2006)	*P. cruentum*	B-phycoerythrin (B-PE)	Centrifuged concentrate	Ultrasonication followed by aqueous two-phase systems (ATPS) partition: The sonicated concentrate was gently mixed with a stock solution of polyethylene glycol (PEG) and potassium phosphate to form the ATPS system. The ATPS mixture was placed in a batch settler to enable gravity-driven biphasic separation. B-PE was recovered in the top PEG-rich phase	90% of available B-PE with a purity ratio of 3.2. The final ATPS system comprised of 29% (w/w) PEG at 1000 g/gmol, 9% (w/w) potassium phosphate, 40% (w/w) crude extract, pH 7.0, and volume ratio of top phase: bottom phase = 4.5	The dual method simplified B-PE recovery as it avoided a precipitation stage and combined a debris removal step with a B-PE recovery step Sonication was able to release 5 × more B-PE than manual maceration
Furuki et al. (2003)	*S. platensis*	Phycocyanin (PC)	Dried powder	Ultrasonication: biomass powder was re-suspended in 0.1 M phosphate buffer (pH = 6.8) at the concentration of 4.8 w/v% and subjected to sonication at 80 W, 20 kHz and 10 °C	Almost 100 wt% of available PC (almost 60 mg PC/g biomass) with a purity of 80%	PC release during ultrasonic disruption followed first-order kinetics with respect to irradiation time Other biological compounds such as chlorophyll were also released during the ultrasonic disruption

(continued)

Table 12.6 (continued)

Study	Species	Pigment	Biomass state	Cell rupture and extraction conditions	Maximum yield/recovery at optimum conditions	Key findings
Viskari and Colyer (2003)	*Synechococcus CCMP 833*	Phycobiliprotein with a focus on phycoerthyrin (PE)	Culture was vacuum filtered through a 0.22 μm filter	buffer extraction coupled with nitrogen cavitation. Extraction buffer consisted of 3% (w/v) Chaps 0.3% (w/v) asolectin solution in distilled water. Nitrogen cavitation vessel was charged with nitrogen gas at 1500 psi	>85 wt% of available phycobillin	With nitrogen cavitation, physical and chemical stresses on subcellular components are held to the minimum during cell rupture, thus ensuring the preservation of delicate enzymes and proteins

in the studies as well as specific key findings from each investigation. The extraction efficiency of phycobiliproteins is almost entirely dependent on the degree of cell disruption as the water-soluble pigments are readily transferred to the aqueous solvent after being liberated from the biomass. This is in contrast to the extraction efficiency of water-insoluble pigments (such as chlorophylls and carotenoids), which is determined by both the degree of cell disruption during biomass pretreatment and the affinity of the incoming extraction solvent (organic solvent, pressurised organic solvent or supercritical carbon dioxide) to the pigment molecules.

12.4.4 Pycoerythrin

Phycoerythrin (PE) is a red phycobiliprotein that is primarily found in the chloroplasts of cyanobacteria (such as *Synechococcus* sp., *Phormidium* sp., *Leptolyngbya sp.*), red algae (such as *Porphyridium cruentum*) and cryptophytes (*Rhodomonas salina*) (Benavides and Rito-Palomares 2006; Cuellar-Bermudez et al. 2015; Jubeau et al. 2013; Thoisen et al. 2017; Viskari and Colyer 2003). Most studies on PE recovery have used either filtered or concentrated wet biomass as their starting material (Tables 12.5 and 12.6). The most suitable cell rupture method for PE extraction will depend on the cell wall structure and is therefore species specific. Instead of a cell wall, each cryptophyte cell possesses a fragile periplast underneath the plasma membrane that can easily be ruptured through freeze-drying action (freezing the biomass at −20 °C and then thawing it to room temperature). Freeze-thawing *Rhodomonas salina* cells suspended in a phosphate buffer has been shown to be able to achieve almost complete PE recovery from the biomass (Lawrenz et al. 2011; Thoisen et al. 2017). On the other hand, cyanobacterial species have tougher cell walls made of peptidoglycan that can only be disrupted with a mechanical pretreatment step, such as ultrasonication (*Porphyridium cruentum)* and nitrogen cavitation (*Synechococcus* CCMP 833) (Benavides and Rito-Palomares 2006; Viskari and Colyer 2003). In their study investigating PE recovery from red algae (*Porphyridium cruentum)*, Jubeau et al. (2013) were able to extract almost 100 wt% of available PE by using a 2-stage high-pressure homogenisation which permeabilised the cell membrane in its first step (< 100 MPa) to recover low molecular-weight protein and then ruptured the cell wall in its second step (> 100 MPa) to selectively recover PE. Microalgal or cyanobacterial PE has to undergo a number of post-recovery purification steps (refer to Sect. 12.5) in order to meet the strict standards of pharmaceutical or molecular biology field. The A585/A280 value indicates the molar ratio of PE to other contaminating proteins in the solution and is used to determine PE purity (Cuellar-Bermudez et al. 2015). Diagnostic and pharmaceutical-grade PE must have an A585/A280 value greater than 4.

12.4.5 Phycocyanin

Phycocyanin (PC) is a blue phycobiliprotein that is almost exclusively found in cyanobacteria, such as *Spirulina platensis, Galdieria sulphuraria*, *Limnotrhix* sp. and *Synechococcus bacillaris* (Cuellar-Bermudez et al. 2015; Furuki et al. 2003; Gantar et al. 2012; Lawrenz et al. 2011; Papadaki et al. 2017; Rodrigues et al. 2018; Sørensen et al. 2013). Studies on cyanobacterial PE recovery have used filtered wet biomass, concentrated wet biomass or dried biomass as their starting material. *Spirulina platensis* has a relatively thick and robust cell wall that consists of four longitudinal layers composed of a glucose polysaccharide known as β-1,2,-glucan and peptidoglycan. A mechanical pretreatment (such as ultrasonication, ball milling and manual grinding) is generally required to rupture cyanobacterial cell walls (Furuki et al. 2003; Lawrenz et al. 2011; Rodrigues et al. 2018; Sørensen et al. 2013). In their study examining PC extraction from *Synechococcus bacillaris,* Lawrenz et al. (2011) found ultrasonication to be more effective in rupturing the cyanobacterial cells and liberating PC compared to manual grinding. Phosphate buffers (pH range $= 6 - 7.2$), ionic liquids (2-hydroxy ethylammonium acetate or 2-HEAA and 2-hydroxy ethylammonium formate or 2-HEAF) and distilled water have previously been demonstrated as effective extraction solvents for PC (Tables 12.1, 12.5 and 12.6). In Rodrigues et al. (2018), the protic ionic liquid, 2-HEAA/2-HEAF (an equimolar mixture of 2-HEAA and 2-HEAF), was found to be a more effective PC extraction solvent than sodium phosphate buffer because of its high diffusional power and ability to penetrate *S. platensis* cellular matrix. PC extract has a limited stability and can be degraded by light, temperature and other microorganisms. It is therefore critical to ensure that selected recovery procedure does not have any adverse impact on the pigment's structure and antioxidant functionality. PC has been shown to be stable under ultrasonic irradiation (Furuki et al. 2003). The purity of phycocyanin extract is determined by its A620/A280 value, a ratio that measures the proportion of PE to other contaminating proteins in the solution (Cuellar-Bermudez et al. 2015). Extracted PC has to undergo a number of purification steps (refer to Sect. 12.5) in order to meet the required food-grade (A620/A280 > 0.7) or analytical-grade (A620/A280 > 4.0) standards of its applications.

12.5 Pigment Purification and Separation

Pigments extracted from microalgal or cyanobacterial biomass generally contain other impurities that may interfere with their applications and functionalities. These impurities, e.g. chlorophylls and lipids in carotenoid extracts and proteins in phycobiliprotein extracts, can be separated from the desired pigments with a number of different chemical and/or chromatographic techniques. Calcium hydroxide precipitation, acid precipitation and column chromatography have previously been used to remove chlorophylls from astaxanthin and β-carotene extracts (Rammuni et al. 2019).

When reacted with calcium hydroxide or acid (such as sulphuric acid, hydrochloric acid, acetic acid and phosphoric acid), chlorophylls are converted to water-soluble salts which precipitate out of the organic solvent or pigment mixture and can thus be removed using a solid-liquid separation technique (such as centrifugation) (Rammuni et al. 2019). Sulphuric acid (0.01 N) and hydrochloric acid (0.01 N) were able to successfully precipitate out ~ 80 wt% of the chlorophyll that was co-extracted with astaxanthin from *Monoraphidium* sp. GK12 (Fujii 2012).

Phycobiliprotein purification generally involves subjecting the crude pigment extract to one or more of the following steps: ammonium sulphate precipitation, activated carbon and chitosan precipitation, aqueous two-phase extraction with polyethylene glycol, concentration with ultrafiltration or tangential flow ultrafiltration (30-50 kDa), anion exchange chromatography with Q-Sepharose column, gel permeation chromatography with Sephadex G-150 column and anionic chromatography with diethylaminoethanol cellulose (Benavides and Rito-Palomares 2006; Bermejo Román et al. 2002; Gantar et al. 2012; Parmar et al. 2011; Sørensen et al. 2013). In their study purifying PC extracts from *Galderis sulphuraria,* Sørensen et al. (2013) found the combination of ammonium sulphate precipitation with aqueous two-phase extraction and ultrafiltration to result in both the highest PC yield (42 wt% of PC in the crude extract) and the highest product purity (A620/A280 = 4.5). Gantar et al. (2012) noted that the increased purities obtained by subjecting pigment extracts to fractionation steps generally come in expense of their final yields/recoveries. Further research is needed in order to establish the technical and economic scalability of pigment purification steps.

12.6 Industrial Scalability

From an industrial perspective, the scalability of a biomass processing pathway is primarily determined by the intended use of the generated microalgal products. Even though it is difficult to make this determination without performing a comprehensive techno-economic analysis of the operational units in each pathway, certain generalisations can be stipulated. Microalgal biomass processing for applications related to human consumptions (such as pigment recovery for food colorants, nutraceutical and pharmaceutical products, protein recovery for human diets or food supplements and cell debris isolation for animal/aquaculture feed) must satisfy the safety and purity requirements associated with food production. On the other hand, biomass processing for fuel applications (such as lipid recovery for biodiesel conversion) must achieve a positive energy balance by minimising the energy requirement of individual unit operation, in particular that associated with biomass dehydration during pretreatment and evaporative solvent recovery after the extraction step. For this reason, the use of less toxic solvents or solvents that are generally accepted as safe by regulatory food agencies (such as ethanol or acetone) is ideal for pigment extraction, while the use of volatile solvents with relatively low boiling points and low enthalpies of vapourisation (such as hexane and ethyl acetate) is preferred for

biofuel lipid extraction as it facilitates facile solvent recovery. For biofuel application, since large-scale biomass dehydration is energetically prohibitive, the lipid recovery process needs to be able to effectively extract lipids from wet microalgal biomass (e.g. slurry or paste). Recent studies have asserted hexane extraction from previously ruptured wet microalgal concentrate as a biofuel production pathway that is able to achieve large-scale energy scalability (Dong et al. 2016; Martin 2016). Hexane is a water-immiscible solvent that readily forms a biphase with wet microalgal concentrate and thus does not require energy-intensive distillation for its separation (and recycling) from the post-extracted concentrate.

A biorefinery production strategy takes advantage of microalgal biomass compositional complexity and exploits both its nutritional and calorific fuel values by fractionating the biomass into an array of food and fuel products. The strategy reduces the cost of producing a single product through a shared processing scheme, maximises total possible revenue from the biomass and thus has a higher chance of achieving commercial viability than a linear strategy that focuses on the sole production of a single food or a single fuel product. In this context, microalgal pigment extraction will likely form part of a larger food-and-fuel biorefinery system that includes lipid recovery steps for biofuel production as well as steps to co-generate other product streams (such as the recovery of biomass sugar for ethanolic fermentation and the isolation of processed cell debris for animal or aquaculture feed). The biomass processing pathway deployed for pigment recovery will therefore have to comply with the requirements of both food and fuel production, i.e. using solvent that is safe for human consumption while at the same time using processing steps and solvents that facilitate a positive net energy balance. Even though we are unable to provide specific recommendations on the perfect solvent at this juncture in time, we postulate that the selected solvent will likely be one that is able to achieve a compromise between the two requirements. Hexane, for example, is a volatile solvent that requires minimal amount of energy for its evaporation and recovery (boiling point at 69 °C and enthalpy of vapourisation at 365 kJ/kg), while at the same time can still be considered safe for human consumption as long as it is present below its mandatory residual limit in the final food product. The European Commission has imposed the maximum residual limit for hexane in food products to be between 1 and 30 mg/kg depending on the type of food product and the extraction process used (Council 2009); we note that there is currently no safety guideline for solvent content in algal-derived food products.

To avoid energetically prohibitive large-scale biomass dehydration, the lipid or pigment recovery steps in a microalgal biorefinery system will have to be performed on wet microalgal biomass (slurry or paste). As shown in Tables 12.2–12.6, the majority of studies previously investigating microalgal pigment recovery, however, have used freezed/spray- or thermal dried biomass as their raw materials. Future studies must stop this practice and direct their efforts into developing scalable pigment recovery pathways from wet microalgal concentrate instead.

Equally importantly, more research efforts should also be directed into the development of a scalable lipid/pigment purification system. The solvent used for the lipid or pigment extraction step is unlikely to be selective only to the targeted pigment or

lipid. As an example, acetone extraction aimed at astaxanthin recovery from *H. pluvialis* cyst cells will likely extract astaxanthin as well as chlorophyll, other pigments and fatty-acid lipids from the biomass. Crude hexane extract from nitrogen-deprived *Nannochloropsis* biomass will contain not only the targeted biodiesel-convertible non-polar triglycerides but also meaningful amounts of ω3-rich polar lipids, chlorophyll *a* and a number of different carotenoids that could potentially be separated from the primary lipid product to form valuable products. For this reason, it is of critical importance to be able to refine crude extract obtained from a lipid or pigment extraction step into individual product streams that can add to the overall commercial value of the biorefinery system. Recent studies on microalgal pigment purification using various precipitation, chromatographic and esterification methods have generally focused on lab-scale application and have not examined the industrial scalability of their proposed processes (Benavides and Rito-Palomares 2006; Fujii 2012; Gantar et al. 2012; Kang and Sim 2007; Sørensen et al. 2013).

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Chapter 13
Process Intensification Aspects of Extraction of Pigments from Microalgae

Parag R. Gogate and Saurabh M. Joshi

Abstract Currently, microalgae are in significant focus as a potential source for value added products like pigments, lipids and carbohydrates. Microalgae can be grown quickly without competition to food chain on non-arable land with lesser requirement of nutrients and beneficial utilisation of greenhouse gas (GHG). Among the different value added products from algae, pigment offer immense potential. Chlorophylls, carotenoids and phycobiliproteins are the classes of pigments found in varied proportions in different microalgae strains. Zeaxanthin, astaxanthin, fucoxanthin, lutein, chlorophyll a and carotenes are some of the specific commercially important pigments possessing higher value in food and pharmaceutical industry market. It is important to note that the conventional pigment extraction processes such as bead-beating, homogenisers, etc. have not been very successful at commercial level due to high costs. Thus, evaluating process intensification approaches for increased process efficiency are necessary. Process intensification plays a crucial role in minimising process cost with significant reduction in quantum of chemicals required and processing time. The current chapter focuses on presenting overview of novel intensified separations with consideration of the governing mechanisms, set of best operating conditions and also detailed literature review to highlight the obtained benefits. It is demonstrated that significant intensification benefits are obtained based on the application of newer approaches and scaling it up to commercial scale will indeed lead to favourable economics for the recovery of pigments from microalgae.

Keywords Microalgae · Process intensification · Pigments · Carotenoids · Operating parameters

13.1 Introduction

Microalgae are unicellular microscopic organisms with a potential source of high-value products such as proteins, lipids and pigments. Microalgae are grown in closed

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or open systems and can produce many high value chemicals with the help of photosynthesis. The ability to grow on wastewater and absorb carbon dioxide helps in curbing environmental pollution along-with offering value addition. These aspects make microalgae a value added feedstock shifting major focus of researchers on commercialising their production with favourable economics (D'Alessandro and Antoniosi Filho 2016) and also optimising the processes for recovery of value added products. The overall processing of microalgae consists of different steps like cultivation in controlled conditions, harvesting and further processing with operations like coagulation, filtration, centrifugation and foam fractionation depending on the desired end product. Each product requires a specific pretreatment for the harvested microalgae and extraction process further increasing the complexity of process. Pigments from microalgae find application in food, cosmetic and pharmaceutical industry. They are typically used as colouring agents, food supplements and possess anti-inflammatory, anti-ageing, antioxidant and anticancer properties making them a strong constituent in key medicines (Khanra et al. 2018). Recovery of pigments consists of steps like cell disruption followed by selective extraction to obtain specific pigments with exclusion of other intra cellular components. Pigments obtained from microalgae are generally of three types, namely carotenoids, chlorophylls and phycobiliproteins. Astaxanthin, β-carotene and chlorophyll are the pigments which reportedly possess higher commercial markets. Following are brief details on different classes of pigments available and recovered using extractions.

13.1.1 Carotenoids

Carotenoids are the most diverse and wide spread pigment, lipophilic in nature and yellow, orange or red in colour. Carotenoids are mainly made up of C40 backbone of isoprene units and are divided into two groups, namely carotenes and xanthophylls. Carotenoids are antioxidants and are sensitive to light, heat and oxygen making them difficult to store and handle. Carotenoids function to absorb excess light in visible spectrum and also play a role in energy transfer and cell protective mechanisms (Gong and Bassi 2016). Carotenoids are mainly used as feed additives, food colorants and important ingredients in cosmetics and also recently there is reported application in human health care. The major carotenoids available in markets are astaxanthin, β-carotene, lycopene, canthaxanthin and lutein with astaxanthin and β-carotene accounting generally half the market. Astaxanthin comes with a potential to enhance antibody production, as well as anti-aging and sun-proofing characteristics and also exhibits stronger antioxidant activity than Vitamin E and β-carotene. Microalgae, especially those strains belonging to chlorophyta, such as *Chlorella vulgaris, Chlorella zofingiensis, Dunaliella salina*, *Chlorella pyrenoidosa* and *Haematococcus pluvialis* are mainly employed in production of astaxanthin, β-carotene, lutein, canthaxanthin and other carotenoids.

13.1.2 Chlorophylls

Chlorophylls are primary photosynthetic pigments widely found in plants and are lipid soluble with low polarity. They play a critical role of light harvesting in photosynthesis, selective absorption of lights from blue and red regions and emission of green colour. There are mainly two types of chlorophyll i.e. chlorophyll a and chlorophyll b with major occurrence of chlorophyll a in most microalgae. Replacement of magnesium ion with copper ion in the extracted material results in stable chlorophyll, namely chlorophyllin which is further used in commercial applications like body and fecal odour controlling in geriatric patients and as dietary supplements. They are also reported to have anti-mutagenic and anticancer properties (Halim et al. 2010).

13.1.3 Phycobiliproteins

Phycobiliproteins are water soluble pigments comprising a major portion of total cell protein and act as a light collecting accessory during photosynthesis. They are also used as natural dyes in food products and pharmaceutical industry. They are commonly used as fluorescent markers in immunoassay and dyes for microscopy. They also exhibit anticancer, antioxidant, antiviral, anti-inflammatory, neuro protective and anti-allergic properties. Red algae, namely *Glaucophytas* and *Rhodophyta* are reported to produce 4 types of Phycobiliproteins i.e. phycocyanin, allophycocyanin, phycoerythrocyanin and phycoerythrin (D'Alessandro and Antoniosi Filho 2016).

We will now discuss the overall process of pigment production from microalgae followed by brief on the conventional extraction process and subsequently details of the intensified extraction processes. Processes like supercritical and subcritical extraction, pulsed electric field (PEF) assisted extraction, pressurised liquid extraction, microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), high pressure homogenisation (HPH), etc. will be discussed with respect to their application in pigment extraction process.

13.2 Process of Pigment Production

Process of pigment production starts from an initial phase of cultivating microalgae with supply of specific nutrients and offering conditions required by different species of microalgae for maximising the specific pigment content. Microalgae are generally grown in open pond (raceway ponds, natural ponds, circular ponds and inclined systems) or closed systems (PBR-photobioreactor) (Joshi and Gogate 2018). Among the different steps depicted in Fig. 13.1, harvesting is the next step following

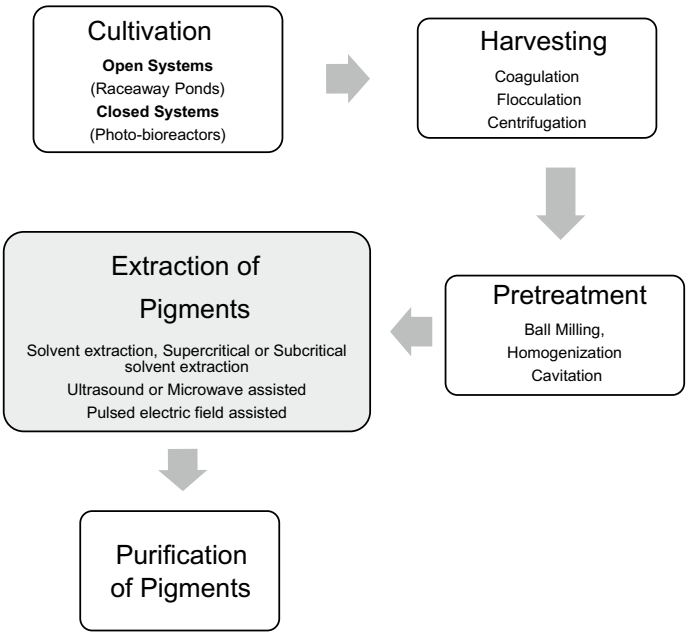

Fig. 13.1 Overview of pigment production from microalgae

cultivation and is a necessary step as it separates the algal cells from cultivation medium. Type of harvesting technique is selected based on cell density, cell size and market value of product. Harvesting of algae involves use of chemical, biological, mechanical and electrical methods wherein the mechanical methods are reported to be the most common. Coagulation, flocculation and floatation, filtration, centrifugation and gravity sedimentation are the processes commonly employed in harvesting of microalgae (Singh and Patidar 2018). Further extraction of pigments from microalgae is performed in two steps of pre-treating to disrupt cell walls and membranes of microalgal cells followed by actual extraction operation. Mechanical processes like ball milling, homogenisation, etc. are employed while non-mechanical processes like chemical and biological processes can also be employed (Lee et al. 2010) for achieving the extraction of pigments. After disruption of cells, the process of extraction is performed involving the solvents specifically targeted at products like pigments, lipids or other components of microalgae. While mostly conventional solvent extraction (soxhlet) approaches is commonly applied for extraction, upcoming processing technologies like ultrasound and microwave as well as processes like PEF, homogenisation, pressurised processing can also be applied as process intensifying approaches to improve efficiency of pigment extraction.

13.3 Extraction Processes for Pigment Recovery

13.3.1 Organic Solvent Extraction

The most common method used for recovery of pigments is organic solvent extraction process which employs an organic solvent to extract pigments from disrupted microalgae cells. Microalgae cells are firstly disrupted via homogenisation, ball milling, colloidal milling or sonication processes and are further subjected to organic solvent extraction. Using cell disruption is a very important step in deciding overall recovery. In a study performed by Simon and Helliwell on chlorophyll pigment extraction, only 25% pigment recovery was reported by solvent extraction performed without cell disruption (Simon and Helliwell 1998). Parameters like time of extraction, type and loading of organic solvent, time span of microalgae storage and cycles of extraction also play a major role in optimisation of this process. Organic solvents generally used are ethanol, methanol, acetone and dimethyl formamide (DMF) with ethanol and methanol typically giving better results as compared to acetone, while DMF is reported to be effective but its toxic nature limits the further usage. There have been some modifications in the overall processing of microalgae reported to enhance the yield of pigments. For example, boiling of organic solvent at its boiling point for a time period of 3 to 5 min has been reported to result in increased pigment yield after 24 h extraction process (Sartory and Grobbelaar 1984). It is also reported that freezing microalgae at lower temperatures after filtration helps in cell disruption with further increased yields of pigments from solvent extraction process (Schumann et al. 2005). High solvent consumption, selectivity and extraction efficiency are, however, the major limitations of conventional solvent extraction process.

13.3.2 Supercritical Fluid Extraction (SFE)

Supercritical fluids have same densities as fluids but their viscosity is similar to gas which helps them in diffusing into cells under higher pressures with enhanced mass transfer rates and selective extraction of desired products (Guedes et al. 2013). Carbon dioxide is one of the most preferred solvents as it can attain supercritical state easily and has advantages like high purity and low flammability, toxicity and cost. Supercritical carbon dioxide results in non-polar environment and its polarity can be varied using co-solvents like ethanol. Extraction efficiency of SFE is typically reported to increase up to an optimal CO_2 pressure and temperature values and can vary based on combined effect of pressure and temperature under different conditions. In one of the study, pressure and temperature were varied in range of 40–60 °C and 100–500 bar, respectively and the highest yield of 1.51 μg/mg pigments from *Synechococcus* sp. was reported to be obtained at 50 °C and 200 bar pressure reported as optimum with further variation resulting in decreased yield (Macías-Sánchez et al. 2007). Another study performed by Macias-Sanchez et al. (2010) on *Scenedesmus*

almeriensis reported an optimum temperature and pressure of 46 °C and 600 bar, respectively to obtain highest yield of lutein (Macías-Sánchez et al. 2010). Few examples of SFE process with the information on typical yields are also mentioned in Table 13.1.

Polarity modifiers like ethanol, vegetable oil and acetone have also been used in many studies to improve the efficiency of pigment extraction. Co-solvents cause swelling of algal cells and help in increasing the dissolution of pigments in supercritical CO_2 (Nobre et al. 2006; Poojary et al. 2016). In one of the study performed on comparison of efficacy of co-solvents like ethanol, acetone, methanol, propanol and butanol for extraction of pigments, it was reported that ethanol is the most effective co-solvent (Yen et al. 2012). In another study performed by Krichnavaruk et al. (2008) using vegetable oil as co-solvent, it was reported that using 10% olive oil increased pigment recovery up to 51% at 70 °C and 400 bar pressure (Krichnavaruk et al. 2008). An another study reported on application of SFE on sea weed established sunflower oil to be an effective co-solvent as compared to canola oil, ethanol, soybean oil and water (Saravana et al. 2017).

The processing of microalgae with disruption techniques like sonication, ball milling, homogenisation also help in extraction of pigments during SFE process. Homogenised form of microalga *Synechococcus sp* was subjected to SFE by Nobre et al. (2006) and it was reported that pigment extraction was found to increase from 58 to 91% after homogenisation as pretreatment (Nobre et al. 2006). Overall it can be said that SFE applied under optimised conditions of pressure and temperature enhances the process of pigment extraction with selectivity of desired components and addition of co-solvent further increases process efficiency. The SFE process can also be combined with cell disruption to give process intensification resulting in efficient processing.

13.3.3 Subcritical Fluid Extraction (SCF)

Subcritical fluid extraction is process similar to that SFE but requires lesser temperature and pressure conditions to operate using liquefied fuels (some typical examples include subcritical CO_2, 1, 1, 1, 2-tetrafluoroethane and dimethyl ether) as extraction solvents. Process conditions generally employed in SCF extraction are temperature in range of 40 to 45 °C, pressure upto 35 MPa with use of co-solvent such as 5% loading of ethanol (Du et al. 2015). Extraction at lower temperatures results in efficient extraction of thermally labile pigments. In one of the studies, lutein extraction from *C. pyrenoidosa* was performed with subcritical CO_2 and ethanol as co-solvent resulting in 124 mg/100 g of lutein extraction (Fan et al. 2015). Use of dimethyl ether (DME) as an extraction solvent was reported to offer advantage in that raw sample of microalgae can be used directly without drying. DME can also be evaporated at lower pressures from extraction solution making the process more efficient with lesser processing and time requirements (Kanda and Li 2011). Using DME as solvent in SCF at 25 °C and 5.9 bar pressure for 0.72 h was reported to give yield of

Table 13.1 Examples of SFE for pigment extraction from microalgae. P-pressure, T-temperature, t-time.

Microalgae	Solvent	Pre-treatment	Process Conditions	Yield	References
Chlorella vulgaris	CO_2 and 7.5% ethanol	–	P-80 bar T- 500 °C t- 3 h	Lutein- 0.145 mg/min β-carotene-0.0046 mg/min chlorophyll a-0.27 mg/min chlorophyll b-0.085 mg/min	Kitada et al. (2009)
Nannochloropsis gaditana	CO_2	Freeze drying	P-60 bar T-400 °C t- 3 h	Total Cartenoids-0.343 μg/mg, Total chlorophyll a- 2.238 μg/mg	Macías-Sánchez et al. (2005)
Nannochloropsis oculata	CO_2 and 16.7 wt% ethanol	–	P-50 bar T-350 °C	Total carotenoids- 7.61 mg/g	Liau et al. (2010)
Dunaliella salina	CO_2	Homogenisation	P-60 bar; T-400 °C, t- 3 h	Total carotenoids- 12.17 mg/g	Macias sanchez et al. (2008)
Nannochloropsis oculata	CO_2 and Ethanol	Grinding & freeze drying	P-50 bar T-350 °C	Zeaxanthin- 13.17 mg/g	Liau et al. (2011)
Haematococcus pluvialis	CO_2	Drying	P-70 bar; T-500 °C, t- 4 h	Astaxanthin- 23.04 mg/g	Thana et al. (2008)
Synechococcus sp.	CO_2	Freeze drying	P-50 bar; T-300 °C, t- 3 h	Carotenoids- 1.511 μg/mg Chlorophyll a-0.078 μg/mg	Macías-Sánchez et al. (2007)
Chlorella vulgaris	CO_2 and 5% ethanol	Crushing	P-60 bar T-400 °C	0.29% carotenoid yield with 69% recovery	Gouveia et al. (2006)

390 μg/g of fucoxanthin which was reported to be significantly higher as compared to that obtained using the conventional approach of Soxhlet extraction (50 μg/g) (Kanda et al. 2014). SCF is reported to be efficient than conventional extraction processes but finds lesser application as compared to SFE which is also observed to give higher pigment extraction yields.

13.3.4 Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction consist of using liquid solvents at higher temperatures (50–200 °C) and pressure (35–200 bar) to extract desired components from biological materials. Solvents are found to be effective at reduced surface tension and viscosity prevalent at such conditions resulting in higher solubility and mass transfer rates. PLE is reported effective in pigment extraction with use of greener solvents like ethanol or water and intensification benefits of lower extraction time and solvent requirements (Plaza and Turner 2017). Other reported solvents for PLE are ethyl acetate, methanol, dichloromethane, propane, n-hexane and ionic liquids (Poojary et al. 2016). As a specific example, *D. salina and H. pluvialis* subjected to PLE with acetone was reported to give pigment extraction same as that of traditional methods but with significant reduction in time (20 min) and solvent requirement (Denery et al. 2004). Similarly, study performed by Herrero et al. (2006) reported that PLE assisted pigment extraction from *Dunaliella salina* at 160 °C for 17.5 min gave high yield of pigments as 31.35 mg/100 g using ethanol as solvent (Herrero et al. 2006). Another study on extraction of β carotene from *C. vulgaris* reported 0.67 mg/g pigment yield at 116.8 °C and treatment time of 25 min (Kwang et al. 2010). In the same study, efficiency of PLE was reported to be higher than conventional solvent extraction process and maceration. Study performed by Koo et al. (2012) on zeaxanthin extraction from *C. ellipsoidea* using PLE resulted in 4.28 mg/g at 115.4 °C treatment for 23.3 min (Koo et al. 2012). Taucher et al. (2016) reported 60 °C and 10 min as the optimised PLE conditions for extraction of 1.48 μg/mg of lutein using dichloromethane as solvent (Taucher et al. 2016). In summary, it is evident that PLE is one of the potential processes for pigment extraction demonstrated to be quite effective at laboratory scale of operation and further investigation is needed to use PLE at commercial scale. It is also important to understand that the optimum conditions of temperature, pressure and time need to be established at laboratory scale for the specific system.

13.3.5 PEF Assisted Extraction

Electric field technologies like pulsed electric field (PEF), moderate electric field (MEF) and high voltage electric discharge (HVED) can also be effective in pigment extraction processes. The process is considered to be non-thermal and green with

main application in the cell disruption stage where passage of electrical current results in enhanced extraction of desired pigment (Golberg et al. 2016). Very few studies are reported for the MEF and HVED assisted extraction processes while PEF has found wide range applications and hence has been discussed in detail here.

PEF is a non-thermal process consisting of treatment of the material with unipolar or bipolar pulses applied in square wave or exponential shaped frequencies. Biomaterial is typically subjected to repetitive electric frequencies with intense electric field ranging from 0.1–80 kV/cm for a very short time period ranging in nano to milliseconds (Mahesha et al. 2017). PEF causes reversible or irreversible pore formation in cell membrane causing rapid solvent diffusion with enhanced mass transfer of intracellular components in the subsequent extraction stage. It is important to note that selective extraction of components can be achieved with controlled pore formation depending on electric intensity and cell characteristics (Raso et al. 2016). We now present some case studies to understand the efficacy of PEF.

C. vulgaris when subjected to PEF at 20–25 kV/cm field strength for a period of 3 μs pulse duration was reported to give irreversible pore formation yielding a higher pigment yield of 0.82 mg/g after pretreatment. A subsequent 1 h incubation was also reported to enhance the pigment yield to 1.04 mg/g (Luengo et al. 2014). Incubation of microalgae after pretreatment results in plasmolysis of the chloroplast due to osmolytic disequilibrium, which assists the process of pigment extraction. The time of PEF treatment also affects the final yield of the product. In a study performed by Luengo et al. (2015a, b), higher yield of 1.58 mg/L was reported by using microsecond (μs) range of treatment as compared to 1.09 mg/L of yield obtained at millisecond (ms) range of treatment. Similar to effect of time, operating pH also plays a major role in deciding enhancement in yields due to the PEF. Parniakov et al. (2015b) studied the carotenoid pigment extraction from *Nannochloropsis* sp. and reported that using PEF at pH of 8.5 resulted in enhanced yield of 0.2 mg/g as compared to 0.04 mg/g yield obtained using normal distilled water. Combination of PEF with biphasic mixtures of organic solvents has also been reported in few studies as an approach for intensifying the recovery of pigments. Parniakov et al. (2015c) combined PEF with biphasic mixture of dimethyl sulfoxide/ethanol and water reporting increased yield of pigment extraction as compared to conventional process of only biphasic mixture-based extraction or PEF alone (Parniakov et al. 2015c). In another reported study, *Heterochlorella luteoviridis* was subjected to MEF at 180 V and 60 Hz for 50 min combined with ethanol as solvent where higher lutein yield of 1.21 mg/g was obtained (Jaeschke et al. 2016). Application of moderate thermal treatment along-with PEF has also been reported to increase pigment extraction yield. Lutein extraction from *C. vulgaris* with PEF along-with thermal treatment at 40 °C was reported to result in 4.5 fold increase in the production (Luengo et al. 2015a). Increase in yield using PEF along-with higher temperature is attributed to the increased membrane permeability. Though PEF has been reported to be successful for variety of lab scale applications, its application at commercial scale will be limited mainly to specific high-value pigment extraction processes due to the associated higher costs of operation.

13.3.6 Microwave Assisted Extraction (MAE)

Over the last decade there has been a focus on usage of irradiations like microwave in processes of pigment or antioxidant extraction from plant sources. Microwaves are electromagnetic radiations with a frequency in range of 300 MHz to 300 GHz and transfer heat to the medium by ionic conduction and dipole rotation. Microwaves when used as irradiating source in microalgae treatment process result in rapid heating and also pressure changes across intracellular components and outer system resulting in cell wall and membrane rupture which further helps in efficient solvent diffusion giving higher pigment extraction (Leonelli et al. 2012). MAE is reported to demonstrate higher yields of pigments with lesser requirement of solvents and processing time making the process more energy efficient (Pasquet et al. 2011). In the case of lipid extraction from marine algae, MAE was reported to be the most efficient technique as compared to processes like bead-beating, autoclaving or maceration (Lee et al. 2010). MAE is generally performed in closed or open systems with the open systems used for lower temperature requirement at atmospheric pressure while closed systems are used in processes with higher temperatures and pressures.

Efficiency of MAE generally depends upon the microalgae cell structure and extraction conditions. In a study performed on fucoxanthin extraction from *Cylindrotheca closterium,* it was reported that MAE at 50 W power for a period of 3–5 min resulted in 4.24 μg/mg yield of fucoxanthin which was comparable to conventional process of cold and hot soaking extractions (4.68 and 5.23 μg/mg as the yields, respectively) though the conventional process required much higher time of 60 min (Pasquet et al. 2011). It was also reported that MAE reduced the process time significantly till an optimum condition and further increase in power or time had no effect on the yield. Another study on phycocyanin extraction from *Porphyridium purpureum* using MAE applied for 10 s at 100 °C demonstrated yield of 34.8 ± 6.4 μg/mg which was found equivalent to that obtained using the conventional solvent extraction method, though after higher time required as 60–80 min (Juin et al. 2014). Another study on fucoxanthin extraction from *U. pinnatifida* established that MAE is an effective approach giving maximum recovery of 109.3 mg/100 g under conditions of 300 W as the power, solvent (ethanol) to sample ratio of 15:1 mL/g, temperature of 60 °C and treatment time of 10 min (Xiao et al. 2012). Study on astaxanthin recovery from *H. pluvialis* performed in closed system (Ruen-ngam et al. 2011) demonstrated that MAE is effective yielding 74% recovery of astaxanthin in only 5 min of extraction time at 75 °C using acetone as the solvent. Comparison of different solvents like acetone, methanol, ethanol and acetonitrile was also performed in the same study and acetone was established to be the most effective in the case of MAE. It was also reported that an increase in temperature above 75 °C resulted in degradation of astaxanthin. Zhao et al. (2009) presented an optimisation study of MAE with response surface methodology and reported that 5.94 μg/mg as the maximum astaxanthin yield could be obtained under optimised parameters of 141 W power, 83 s as the time, 9.8 mL as solvent volume with four cycles of extraction. It was also reported that using higher power resulted in decreased astaxanthin yield, which can

be attributed to the fact that increase in power increases the temperature resulting in structural changes in the astaxanthin (Zhao et al. 2009). MAE was also reported to be efficient in another study performed on pigment extraction from *Arthrospira platensis* using mixture of methanol/ethyl acetate/light petroleum in 1:1:1 ratio under conditions of 1 bar as pressure, 400 W as power, temperature of 50 °C and 15 min as the treatment time resulting in 629 μg/mg yield of total carotenoids (Esquivel-Hernández et al. 2016). Overall it can be concluded from the studies reported that MAE is an effective extraction process giving significant benefits for the pigment extraction in terms of lesser processing time and chemical requirements.

13.3.7 Ultrasound Assisted Extraction (UAE)

Ultrasound (US) assisted extraction is a process in which an ultrasound wave of 18–200 kHz frequency is passed through a liquid medium producing rarefaction and compression cycles. During the rarefaction cycle, formation of micro-scale cavities occurs which grow to a particular size and implode during compression cycle. Implosion of these cavities results in generation of local hotspots having temperature in range of 1000–10,000 K and pressure in the range of 100–1000 bar along-with physical effects of turbulence and micro streaming (Gogate and Pandit 2000). US generated physical effects help in pigment extraction by disruption of cell wall and membranes along-with enhanced diffusion of solvents into micro algal cells. UAE offers potential to reduce process time and required chemicals making pigment extraction process economical. UAE is generally performed using different reactor configurations (US horn, US bath and continuous US flow cell) depending upon the processing volumes. We now present an overview of different studies that reported the intensification obtained using UAE.

Pigment extraction from *Nannochloropsis* sp. using UAE applied at 24 kHz frequency and 400 W power for 7.5 min was reported to result in maximum total chlorophyll yield which was 9 folds higher than that obtained using conventional process of extraction (Parniakov et al. 2015). Another study performed on UAE of pigments from *Phormidium autumnale* reported yield of pheophytin a′ *as* 371 μg/g of and that of chlorophyll as 159.3 μg/g using ethyl acetate as solvent followed by second stage application of methanol under optimum conditions of frequency of 20 kHz, 50% amplitude and 20 min (Cichoski et al. 2016). Study performed by Macías-Sánchez et al. (2008) on extraction of total carotenoids from lyophilised *D. salina* using N,N-dimethylformamide (DMF) as the solvent reported significantly higher yields of carotenoids as 27.7 μg/mg as compared to SFE which gave 15 μg/mg as the yield of carotenoids. Fucoxanthin extraction from *Dunaliella tertiolecta* was performed using UAE at 12.2 W power for 5 min and yield of pigment obtained was reported as 4.49 ± 0.08 μg/mg which was similar to that of conventional extraction process but a significant reduction in extraction time was reported for the UAE (Pasquet et al. 2011). Study performed by Jaeschke et al. (2016) on carotenoid extraction from *Heterochlorella luteoviridis* using UAE (20 kHz frequency, 50 W/cm^2

Table 13.2 Examples of UAE employed in pigment extraction from microalgae. f- Frequency, P-power, t- time

Microalgae	Solvent	Process Conditions	Yield	References
Spirulina platensis	n-heptane	f:20 kHz, P: 165 W, t: 4 min	β-carotene- 1 mg/g	Dey and Rathod (2013)
Haematococcus pluvialis	Acetone	f:38.5 kHz, P:18.4 W, t:60 min	73%-Astaxanthin recovery	Ruen-ngam et al. (2011)
Dunaliella tertiolecta	Water	P: 12.2 W, t: 5 min	β-carotene- 5 mg/g	Pasquet et al. (2011)
Chlorella vulgaris	Ethanol (90%)	F:35 kHz, Intensity:50 W/cm^2, t:10 min	3.36 mg/g-Lutein	Jaeschke et al. (2016)
Chlamydomonas reinhardtii	Water	F: 20 kHz, P:220 W, t:30 s	0.3 carotenoids μg/ mg cells	Gerde et al. (2012)

power for 10 min) using ethanol (70%) as solvent was reported to give yield of carotenoids as 1.31 mg/g (Jaeschke et al. 2016). Similarly, Phyocyanin extraction from *Spirulina platensis* performed using UAE (Hadiyanto et al. 2016) at two different frequencies of 28 kHz and 42 kHz for 20 min in ethanol (95%) at 55 °C was reported to give yield of 15.97% and 11.24%, respectively. It was reported that the time required for UAE was significantly lower as 20 min as compared to the conventional process of extraction (4 h). UAE performed at 200 W of power, 40 kHz as frequency and using 48% ethanol in ethyl acetate at 41 °C was reported to result in 27.58 mg/g as the astaxanthin extraction yield in 16 min from *Haematococcus pluvialis* (Zou et al. 2013). In one of the recent studies, alkaline pretreatment combined with UAE was also reported to be effective for micro algal cell disruption (Phong et al. 2018). Table 13.2 provides data on few more case studies of UAE.

Overall, US can be employed individually or in combination of other processes depending upon cell characteristics and specific pigment to be extracted from microalgae. UAE is one of the potential extraction processes that can be employed in pigment extraction processes with significant reduction in processing time and chemicals required for similar yield.

13.3.8 High Pressure Homogenisation (HPH)

HPH is a wet milling process where high intensity fluid mechanical stresses are applied when the process fluid passes under higher pressures (50–400 MPa). HPH has advantages like reproducibility, ease of operation, high throughput and industrial scalability as compared to other processes like ball or colloid milling and ultrasound assisted process. HPH results in non-selective release of intercellular components with disruption of cell walls and membranes due to the effects of turbulence, high pressure gradients, collision with hard surfaces, cavitation and high pressure shear.

Feed concentrations up to 25% w/w can be effectively processed using HPH. HPH are reported to demonstrate higher extents of disruption as compared to other processes like ball milling, freeze drying, colloidal milling, PEF, ultrasound and microwave (Poojary et al. 2016). Taucher et al. (2016) studied pigment extraction from *H. pluvialis* using HPH operated at 1000 bar with 3 passes and reported 4.21 μg/mg of pigment yield which was higher as compared to other processes like ball mill (3.56 μg/mg) and freeze and thaw cycles (0.02 μg/mg) (Taucher et al. 2016). Another study also reported HPH (1500 bar and 1–10 passes) to be more effective resulting in higher cell disruption and pigment extraction as compared to other processes like US, PEF and HVED (Grimi et al. 2014). Very few studies are reported on application of HPH in pigment extraction, especially considering scale up issues. HPH also has disadvantages like high capital investment in process setup and also result in rise in temperatures after few passes which further affects the yield of thermally labile pigments. Overall it can be said that more work is indeed required for developing successful applications of HPH.

13.4 Conclusions

Current chapter focused on presenting details of the intensified processes like SFE, PLE, PEF, MAE, UAE and HPH for pigment extraction from microalgae. It has been demonstrated that PLE, MAE and UAE can provide an energy efficient process with lesser requirement of time and chemicals along-with better selectivity and yield. MAE and UAE are reported as rapid extraction techniques with higher pigment recovery while PEF significantly reduces the solvent requirement. Temperature increase during MAE and UAE is one of the disadvantages and needs to be optimised while PEF is ineffective with microalgae having complex cell structures. SFE is also reported to be an efficient technique but comes with disadvantages of no specific pigment selectivity and requires dry samples for processing. Economic feasibility of these processes in terms of energy and cost needs to be assessed and further scientific investigation in terms of commercialisation is also required. Further development of these processes can lead to efficient recovery of valuable pigments playing a significant role in medicinal and food industry.

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Chapter 14
The Bioeconomy of Production of Microalgal Pigments

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Abstract The utilization of renewable biological resources from living organisms to generate food, energy, and materials is a significant driver of bioeconomies. There is a growing awareness among consumers in the utilization of natural products obtained from microorganisms and plant-based materials to deliver novel functional product technologies that can contribute to industrial bioeconomy. Photosynthetic microorganisms, especially microalgae, are beneficial in yielding highly valuable metabolites, including lipids, proteins, pigments, and carbohydrates. Among these bioactive compounds, natural organic pigments such as chlorophyll, carotenoids, phycobiliproteins, astaxanthin, and xanthophyll are gaining importance in several cosmetic, food, and textile industries. This chapter gives an overview of microalgal pigments production and profiles high-value products that are obtained from microalgal pigments. Discussions on the utilization of microalgal pigments as viable ingredients in various product formulations, recent industrial applications of natural pigments, and the future of microalgal pigment-based economy is also discussed.

Keywords Bioeconomy · Microalgae · Pigments · Chlorophyll · Natural products · Bioresource

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14.1 Introduction

The utilization of renewable biological resources from living organisms to generate food, energy, and materials is a significant driver of bioeconomies. There is a growing attention among consumers in the utilization of natural products obtained from microorganisms and plant-based materials to deliver novel functional product technologies that can contribute to industrial bioeconomy. The time required for plant growth and the tediousness in the extraction of valuable products are the drawbacks of plant-based novel functional products to boost the bio-based economies (Marchetti et al. 2014). Further, the factors of deforestation (Hoffmann et al. 2018), bioprospecting (Krishnaswamy 2018), and biopiracy (Efferth et al. 2016) also hinders the custom of using plants for the large-scale manufacture of commercial goods. These challenges in plant-based commercial products lead to the introduction of microorganisms, as a replacement, to extract valuable products (Patridge et al. 2016). Among microbes, algae are unique organisms that are similar to plants in several aspects, especially in photosynthesis. This exclusive property of algae, along with their rapid growth in favorable conditions with less nutrient requirement gained the attention of researchers to use them for natural product extraction and valuable compounds, as a better alternative to plants (Trantas et al. 2015).

Algae are known to humanity for more than a thousand years and are involved in human diet even before 14000 years, which was evident from archaeological excavation sites in Chile. Numerous pre-historic literatures from China, India, Ireland, and historic literatures by eminent scientists, including Newton and Turner, mentioned about algae, and its association with human food and medicine. It is noteworthy that the global production of seaweeds gained a profit of about USD 6.7 billion, out of which 95% are produced by commercial cultivation of aquatic lives for food called mariculture from China and Indonesia (Wells et al. 2017). Two main types of algae, namely, macro and micro algae are extensively present throughout the world, that are classified based on their size (Verawaty et al. 2017), whereas aquatic and terrestrial algae are the types that are classified based on their habitat (Bharathiraja et al. 2015; Ismail et al. 2017). In both the habitats, micro and macro algae possess the ability to grow extensively and, in several cases, the algal species helps to reduce the complex chemical compounds, especially wastes, and simplify them to reduce their toxic effect (Yu et al. 2019). Thus, the presence of algae either serves as a bioindicator of toxic content in the ecosystem (Parmar et al. 2016) or facilitates the *in situ* bioremediation processes (Vidyashankar and Ravishankar 2016).

In recent times, microalgae are widely used in several applications, compared to macroalgae, due to their rapid growth and smaller size. In addition, the advancements in the microalgal biotechnology increased their chances to be utilized in several sectors for natural product fabrication to replace toxic chemical products, food products, medicine, and nutraceuticals (Posten and Chen 2016). Photosynthetic microalgae are beneficial in yielding highly valuable metabolites such as lipids, proteins, carbohydrates, lipids, and pigments (Priyadarshani and Rath 2012). Among these bioactive compounds, natural organic pigments such as chlorophyll,

carotenoids, phycobiliproteins, astaxanthin, and xanthophyll from microalgae are gaining importance in numerous cosmetic, food, and textile industries (Dufossé et al. 2005). Thus, this chapter gives a summary of microalgal pigment production and profiles high-value products that are obtained from microalgal pigments. Additionally, utilization of microalgal pigments as viable ingredients in various product formulations, recent industrial applications of natural pigments, and the future of microalgal pigment-based economy are also discussed.

14.2 Bioeconomy Based on Microalgae

Microalgae is one of the marine organisms that are proven to have a wide economic value. Ease in culture methods, the lesser nutrient requirement for their growth and presence of highly valuable biomolecules makes microalgae as a profound agent to improve bio-based economy. These microalgae are cultured commercially for food, medicine, nutraceuticals, cosmetics, energy, and other industries as displayed in Fig. 14.1, that depend on microalgae for their growth and economic development.

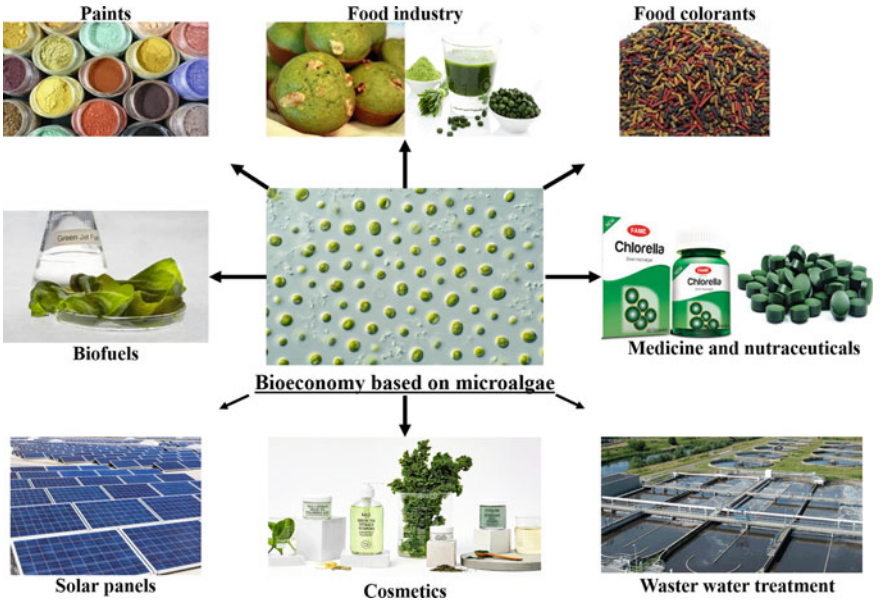

Fig. 14.1 Bioeconomy based on microalgae

14.2.1 Availability

The major photosynthesizers on earth are the microalgae that produce important various plant pigments. These natural colorants can replace the presently used synthetic colorants used in food, cosmetic, pharmaceutical, and nutraceutical industries. Cascading event of symbiosis between heterotrophic hosts gave rise to the diversified photosynthetic microalgae, which directs to the plethora of photosynthetic pigments capable of utilizing various trophic levels in submarine habitats. Nearly a quarter of the biomass in world's total vegetation is constituted by phytoplankton. Further, some microalgae, also contributes to the food web by acting as effective primary producers. In an independent study conducted in the south east Asian region disclosed the abundance and spatial distribution, the major taxon that are identified are *Chrysophyta*, *Cyanophyta,* and *Pyrrophyta* (Kadim and Arsad 2016). The growth rate and distribution of microalgae are dependent on the environmental factor and prevailing conditions, the factors that drive growth potential varies with every species (Adenan et al. 2013). The parameters that affect the distribution are temperature, salinity, and water current (Cokrowati et al. 2014). Studies involving species distribution, abundance, biological diversity index, and dominance index indicate that the growth of microalgae is better in places with pristine environment and less polluted areas.

14.2.2 Opportunities and Threats

Microalgae are considered as a contemporary attraction across the world due to its varied areas of implications. Microalgae has shown a remarkable potential as value added products in biology, but the most challenging portions in the production of microalgae are enhancing growth rate, dewatering the culture for the production of biomass, pretreating the biomass, standardizing the fermentation process (Khan et al. 2018). In certain conditions, local weather can be a major limiting factor that affects the production rates; local species show better adaptability than the exotic better yielding variants. Few species like *Scenedesmus* performs consistently in large-scale production and it is considered as the validating species to study the biotic and a biotic factor favoring and diminishing the growth dynamics (Lee et al. 2014). Agar plating methods and micromanipulation show substantial improvement in the subsequent processing of samples. Pure cultures are often established after several attempts; hence, the preparatory cultures are retained till pure cultures are obtained. The three primary algal pigments, β-carotene, astaxanthin, and phycocyanin are produced on a large-scale to be utilized as organic food colorants. Besides the fact that the industry has established, applied researches in these pigments are still developing at a rapid rate (Perumal et al. 2015). Their impact on human health is to be explored to the maximum to understand the usage spectrum and applications (Eriksen 2016).

14.2.3 Food Industry

More than a century, microalgae are utilized as a food source among humans due to their exclusive nutrient content. The microalgae was proposed as an alternate source to meet the global demand of proteins in the 1950s, which leads to the first large quantity *Chlorella* export in 1960s and used them as protein-rich food among humans. The developments in the downstream process leads to the establishment of microalgal production facility units in countries including the US, India, Israel, Australia, and several countries in Asia (Enzing et al. 2014). Lately, microalgae serves as a sustainable source of food that can yield nutrients such as fatty acids, polysaccharides, sterols and polyhydroxyalkonates along with common nutrients required for growth in humans (Vigani et al. 2015). Microalgae has potential to yield a large quantity of lipids and proteins along with a portion of carbohydrates. Both these microalgae produced biomolecules, either in their raw or decontaminated form serves as a food source and has a wide market throughout the world. Commercially grown microalgae are used to extract lipid and proteins as food, as an alternate to their plant counterparts, to maintain sustainability and reduce deforestation (Vanthoor-Koopmans et al. 2013). In recent times, *Spirulina* and *Chlorella* are extensively used as a source of nutritional food, not only for man, but also for fishes in aquarium, pets including dogs, cats, certain birds, horses, cows, and in poultry. In addition, microalgal species, including *Pavlova, Chaetoceros, Skeletonema, Tetraselmis* and *Nannochloropsis* are used as feed in aquaculture of fishes. Moreover, microalgae are gaining acceptance among public as a raw nutritional diet, compared to other food sources. Thus, they are marketed as individual food product as *Chlorella* noodles or their extracts are mixed with other food products to improve their nutritional value (García et al. 2017). The low maintenance, low energy usage for growth, and high nutritional value compounds are the significant factors to use microalgae in most of the food industries to improve their economy and profit.

14.2.4 Medicine and Nutraceuticals

Literatures of eighteenth-century revealed that the presence of microalgae in the drinking water causes undesirable tastes and odor. Later, the reason was found to be the release of metabolites such as trans-2-cis-6-nonadienal from algal blooms in drinking water. Mostly, microalgae are considered as harmful microbes that can lead to diseases until the twentieth century. The use of microalgae such as *Arthrospira* and *Nostoc* for medicinal purposes has been started in the twentieth century. In China, *Nostoc commune* and *Nostoc flagelliforme* are reported to be the only edible species in their traditional medicine literatures. The herbal values of Nostoc have been still utilized by several traditional and folklore medicinal practices for disease treatments in countries such as Mongolia, Fiji, Japan, Philippines, Mexico, and Siberia (Borowitzka 2018). After twentieth century, microalgae such

as *Chlorella*, *Arthrospira,* and *Aphanizomenon* has gained importance in the food and pharmaceutical sectors. *Chlorella* growth factor is a significant product from *Chlorella* that is proved to possess anticancer and antiaging properties (Panahi et al. 2016). In addition, *C. pyrenoidosa* with lysine and threonine are reported to be beneficial in curing famine edema (da Silva Ferreira and Sant'Anna 2017). Likewise, *Chlorella* extracts are reported to reduce cholesterol, hypertension (Fallah et al. 2018), anemia in pregnant women (Bito et al. 2016), improves ulcerative colitis (Freitas 2017) and enhance the immune system (Chidley and Davison 2018). The extracts from *Chlorella,* especially from *C. vulgaris*, possess ability to prevent harmful, disease causing bacterial growth and are used as antibiotics (Acurio et al. 2018).

The elevated protein, vitamin B12, provitamin A, linolenic acid, iron, and other minerals present in *Arthrospira* increases their potential to be beneficial as excellent microalgal nutraceutical agent (Stunda-Zujeva and Ruģele). Studies reported that the extracts obtained from *Arthrospira* species possess ability to reduce hypercholesterolemia (Sengupta et al. 2018), allergic rhinitis, including nasal congestion, sneezing and itching (Appel et al. 2018), along with anticancer (Srivastava et al. 2015), antioxidant, antidiabetic (Parveen 2016), and anti-inflammatory properties (Jensen et al. 2015). The high dietary value of these microalgae reduces nutritional deficiency, thereby elevates immunity by producing a large number of T-cells, cytokines, secretes IgA antibody, and by increasing the activity of natural killer cells (Seyidoglu et al. 2017). Moreover, *Arthrospira* possess enhanced antibacterial (Markou et al. 2016), antifungal (Keimes and Gunther 2016) and antiviral activities against disease causing microbes (Reichert et al. 2017). It has been reported that the calcium spirulan extracted from *Arthrospira platensis* facilitates the Herpes simplex virus (HSV-1) and human immunodeficiency virus (HIV-2) inhibition (Jena and Subudhi 2019). These calcium spirulan polysaccharides obtained from microalgae, also possess anticancer activity and are useful in the oral cancer theranostics (de la Jara et al. 2018). Further, it is noteworthy that the filamentous cyanobacterium *Aphanizomenon flos-aquae* are also used in the nutraceutical products due to their worthy nutrition and in pharmaceutical products due to their medicinal properties (Borowitzka 2018). The extracts of *Aphanizomenon* are reported to possess anticancer, antimicrobial (Srivastava 2015), antidiabetic, antioxidant (Hyun et al. 2016), and helps in the treatment of neurodegenerative (Nuzzo et al. 2018), cardiac and vascular ailments (Eid et al. 2018). These medicinal and nutritional values of these microalgae are produced as products by several companies which serves as a profitable bioeconomy, throughout the world.

14.2.5 Cosmetics

Apart from food and medical applications, microalgae are used to produce natural cosmetic products as it contains minerals, fatty acids, pigments, vitamins, protein,s and minerals for the structural and functional maintenance of skin. The

bioactive components that are extracted from microalgae are reported to possess ability to protect skin against harsh environment as well as microbial attack and regenerate them. Microalgal bioactive compounds, including exopolysaccharides, chrysolaminarin, glucan, carotenes, asthaxanthin, lycopene, phycocyanin, ectoine, and biopterin glucose are used in the cosmetic product formulation. These biocompounds from microalgae possess enhanced properties to be used as moisturizers, sunscreen lotions, free radical collector, antioxidants, lipsticks, deodorants, eye shadows, UV protectors, and for collagen repair which are beneficial to produce personal care products. These microalgal bioactive compounds are also used in thalassotherapy which utilizes seawater and marine elements including microalgae for skin care. Products such as Blue Retinol™ from *D. salina* for skin cell development and propagation, SILIDINE® from *Porphyridium cruentum* for improving skin aspect and decreasing redness and effects of rosacea and protein-rich *Arthrospira* extract as products for repairing skin damages. The advancements in the downstream processing of commercial microalgae have led to the emergence of skin and care, personalized cosmetic products such as GoldenChlorella™ and AlgaPur™ algae oils. In future, the bioeconomy based on microalgae for cosmetic applications are expected to increase about three times as these products are comparatively less toxic and highly effective in skin care, compared to chemical-based cosmetic products (Mourelle et al. 2017).

14.2.6 Energy

The ability of microalgae to produce highly valuable and essential lipids has been mentioned in the previous sections. The ability of microalgae to accumulate lipid similar to vegetable oil, in their cell, makes them highly beneficial in biofuel production as an alternate fuel. It is noteworthy that the microalgae possesses potential to synthesize and store, 100 times higher quantity of lipids than any other organisms, especially plants (Mubarak et al. 2015). These lipids obtained from microalgae are processed and are widely used as biodiesel to encounter the growing fuel demand throughout the world. However, the production of microalgal biodiesel, specifically dimethyl ether, is still in its infancy and lab-scale stage. After the in situ transesterification process with methanol for biodiesel production from microalgal oil, the residual microalgal oil are also used for the biogas production (Salam et al. 2016). Microalgae are used to produce ethanol, methanol, hydrogen, and biodiesel as efficient biofuels. It has been reported that the biofuel production is high in green algae, compared to red, blue-green and yellow-green algae. Microalgal species, namely, *Chlorella* and *Chlorococcum* are proved to be extensively utilized for the distinctly classifiable biofuel production (Faried et al. 2017). Due to the depletion of conventional, non-renewable fossil fuels and demand for novel green fuels, the bioeconomy based on the microalgal biofuel are expected to increase gradually in the near future.

14.2.7 Other Industrial Applications

The pigments present in microalgae are the high valuable components that can be used for enormous applications. In food sectors, these pigments are beneficial as organic coloring agent (Dufossé 2018), paints as natural colors (Danaee et al. 2018), solar energy collectors to trap more sun energy (Dufossé 2016) and as fluorescent dyes in bioimaging of diseases (Della Rosa et al. 2018). In addition, the extracts of microalgae possess biologically valuable compounds that are beneficial as high-value co-products (Chew et al. 2017), stable biochemical isotope (Allen et al. 2017), biofertilizer (Garcia-Gonzalez and Sommerfeld 2016), bio-mitigation of carbon dioxide emission (Zhou et al. 2017), co-processing of wastewater (Puyol et al. 2017), and in eliminating organic pollutants (Matamoros et al. 2015). Even though a wide variety of microalgal highly valuable compounds are available and are under extensive research, each component possesses at least a drawback which hinders their transformation process from lab to large-scale production (Rizwan et al. 2018). Microalgal pigments are currently under extensive research as they are easily extracted from microalgae and requires less processing stages to fabricate the final product. The bioeconomy associated with microalgae are mostly depending on these pigments and each pigment are significantly helpful in various applications which are discussed in consecutive sections.

14.3 Economically Important Microalgal Pigments

14.3.1 General Characteristics

Microalgae is one of the photosynthesizing organisms that produce various pigments, namely, chlorophyll, carotenoids, astaxanthins, phycobiliproteins, and other lipids. These phycopigments are in the colors of green, yellow, brown, red, and are preferred over synthetic colors, due to their non-carcinogenicity and non-toxicity (Sekar and Chandramohan 2008). These pigments are the important source of natural colors compared to synthetic colorants.

Microalgal pigments such as chlorophyll, carotenoids, phycobiliproteins, astaxanthins, and other pigments are extensively used in different industries. For example, carotenoids obtained from *Dunaliella*, phycobiliproteins from *Spirulina*, red algae, cyanobacteria, and astaxanthin from *Haematococcus* are commonly used in food, pharmaceutical, nutraceutical, aquaculture, and cosmetic industry. Additionally, these pigments are utilized in medical research as receptors that are effective as antibodies (Santiago-Santos et al. 2004; Begum et al. 2015). Apart from performing the primary role in pigmentation-based metabolism and photosynthesis in algae, these pigments also display numerous advantageous anti-inflammatory, antioxidative, anti-carcinogenic, anti-obesity, anti-angiogenic and neuroprotective activity

(Guedes et al. 2011). The major microalgal pigments, their source, biological effects and applications are mentioned in Table 14.1.

14.3.2 Chlorophylls

Chlorophyll molecules are green fat-soluble pigment, universally present in all algal groups and are classified into three major types, namely, a, b and c of chlorophyll. The basic structural unit of these molecules is a ring of porphyrin that are coordinated to a central magnesium atom. Each porphyrin ring consists of a tetrapyrrole ring having four carbon and one nitrogen in each pyrrole ring (Humphrey 2004). The structural differences in chlorophyll a (ring II has methyl group) and chl b (formyl group) is responsible for different absorption maxima (Scheer et al. 2004). Chlorophyll c is a reddish-brown pigment and similar to chlorophyll b. The pigment obtained from Chlorophyll a is blue and green in color, having an extreme optical absorption at 660–665 nm, whereas pigment from chlorophyll b is yellow and green in color with absorption maxima at 642–652 nm (Humphrey 1980; Begum et al. 2015). Chlorophyll a photosynthetic pigment is highly present in cyanobacteria and red algae. Chlorophyll b is present in *chlorophyta*, *euglenophyta* and marine microalgae, whereas chlorophyll c is present in dinoflagellates. The green alga *Chlorella* sp. is named as Emerald food as it has high (7%) chlorophyll content and is the main source of chlorophyll production (Bewicke and Potter 2009a). Chlorophyll b is the major pigment in alga *Spirogyra*, *Chlorella,* and *Euglena gracilis*, whereas Chlorophyll c is present in *Fucus vesiculosus*, *Dunaliella salina*, *Amphidinium carterae,* and *Cryptomonas* sp.

The chlorophyll extraction method traditionally involves solvents of organic origin, namely, acetone, ethanol, diethylformamide, and methanol that dissolve lipoproteins and lipids into extract phase. Pretreatment methods before extraction, including homogenization and sonication disrupts the cells, so that chlorophyll extraction process is efficient. Homogenization followed by the use of dimethyl formamide as solvent led to effectual chlorophyll a extraction from algae *Stichococcus* sp. and *Chlorella* sp. (Schumann et al. 2005). Phycopigment extraction via fluid at supercritical condition (SFE) is better than the solvent-based approaches, as no carcinogenic solvent is involved, and the extract has better purity with less processing steps. Being an inert, inflammable, cheap, and readily available, CO_2 are used as SFE in multiple studies, where chlorophyll is extracted from microalgae *Synechococcus* sp. (Macias-Sanchez et al. 2009). Following extraction, polyethylene powder in chromatography column is used in an economically patented method for separation of chlorophyll a and b from other pigments. The final purification process involves sucrose filled column that yields highly pure and crystalline chlorophyll molecules.

Table 14.1 Different microalgal pigments and their algal sources

Pigment present	Microalgae	Reference
Chlorophyll a	*Chlorella*	(Bewicke and Potter 2009a)
Chlorophyll b	*Spirogyra*, *Chlorella,* and *Euglena gracilis*	(Cunningham and Schiff 1986)
α-carotene	*Dunaliella salina*	(Christaki et al. 2013)
β-carotene	*Chlorella zofingiensis, Dunaliella salina, Coccomyxa acidophila,* and *Aphanizomenon flos-aquae*	(Sathasivam and Ki 2018; Del Campo et al. 2007; Ranga Rao et al. 2010)
Phycocyanin	*Galdiera suphuraria, Spirulina platensis, Spirulina fusiformis, Spirulina maxima, Porphyridium cruentum, Anabaena marina, Aphanizomenon flos-aquae, Arthronema africanum, Coccochloris elabens,Cyanidium caldarium,Gracilaria chilensis,Lyngbya* sp., *Mastigocladus laminosus, Microcystis, Nostoc, Phormidium, Oscillatoria quadripunctulata, Phormidium fragile, Synechocystis* sp., *Synechococcus elongates, Synechococcus lividu,s* and *Synechococcus vulcanus*	(Benedetti et al. 2004; Kuddus et al. 2013; Cuellar-Bermudez et al. 2015)
Phycoerythrin	*Rhodosorus marinus, Porphyridium cruentum*	(Sekar and Chandramohan 2008)
Astaxanthin	*Haematococcus pluvialis, Chlorella zofingiensis, Neochloris wimmeri, Chlorococcum, Botryococcus braunii, Scotiellopsis oocystiformis, Diacronema vlkianum,* and *Euglena rubida*	(Sathasivam and Ki 2018; Ranga Rao et al. 2010; Christaki et al. 2013; Durmaz et al. 2009; Zhang and Lee 1997; Del Campo et al. 2004)
Canthaxanthin	*Coelastrella striolata var., Multistriata, Chlorella vulgaris,* and *Anabaena* spp	(Sathasivam and Ki 2018; Abe et al. 2007; Cha et al. 2008)
Lutein	*Chlorella vulgaris, Coccomyxa acidophila, Auxenochlorella Protothecoides, Chlorella pyrenoidosa, Chlorella sorokiniana, Coelastrella sp., Galdieria sulphuraria, Parachlorella kessleri, Scenedesmus bijugus,* and *Vischeria stellate*	(Sathasivam and Ki 2018; Cuellar-Bermudez et al. 2015; Del Campo et al. 2000; Ranga Rao et al. 2010)
Echinenone	*Botryococcus braunii*	(Sathasivam and Ki 2018; Tonegawa et al. 1998)

(continued)

Table 14.1 (continued)

Pigment present	Microalgae	Reference
Fucoxanthin	*Cyclotella cf. cryptic, Cyclotella meneghiniana, Cylindrotheca closterium, Isochrysis aff. Galbana, Mallomonas sp. SBV13, Nitzschia cf. carinospeciosa, Odontella aurita, Paralia longispina, Phaeodactylum tricornutum, Fucus vesiculosus,* and *Odontella aurita*	(Sathasivam and Ki 2018; Li et al. 2012a, b; Kim et al. 2012; Galasso et al. 2017)
Violaxanthin	*Chlorella ellipsodea*	(Sathasivam and Ki 2018)
Zeaxanthin	*Porphyridium cruentum* and *Chlamydomonas acidophila*	(Sathasivam and Ki 2018; Christaki et al. 2013; Ranga Rao et al. 2010)

14.3.2.1 Application of Chlorophylls

Chlorophyll-rich foods provide good nutritional value to humans as they contain heme, and thus increases the production of red blood cells (RBCs) (Solymosi and Mysliwa-Kurdziel 2017). Chlorophyll molecules are broadly utilized in cosmetic, pharmaceutical, and food sectors. Chlorophyll a, being a stable molecule has been used as a natural colorant, alternative to artificial colors. *Spirulina platensis* contains large amounts of chlorophyll, which can be utilized as an organic green color in food products and cosmetics (Godoy Danesi et al. 2011). In addition, Chlorophyllin which is a chlorophyll derivative is used as a fabric dye to add colors in wool and cotton.

Chlorophyll derivatives have also exhibited various health promoting activities, apart from being primarily used as natural colors. (Ferruzzi and Blakeslee 2007) have documented the medicinal use of these compounds owing to their anti-inflammatory and wound healing entities. Another experiment (Balder et al. 2006) revealed the characteristic of chlorophylls in decreasing the colorectal cancer risk. Chlorophyll molecules also exhibit antimutagenic property, due to production of carcinogen detoxifying enzyme and thus reducing the risk of cancer.

14.3.3 Carotenoids

In nature, carotenoids are widely spread as yellow, orange, or red colored pigments with aliphatic or alicyclic structures (Prasanna et al. 2007). There are more than 1000 carotenoids that are identified till date, and it comprises of about 0.1% of the total algal dry weight. Carotenoids are accessory light harvesting pigments as they transfer the energy to chlorophyll. Carotenoids are antioxidative molecules that deactivate reactive singlet oxygen that are released, when the cells are experiencing stress due to environmental conditions (Ciccone et al. 2013). Carotenoids can be classified

based on their structural cyclization, hydrogenation, and chemical groups that are responsible for the bioactivity (Dutta et al. 2005).

Further, these colored pigments are categorized as major and minor carotenoid groups. Carotenoids that constitute the physical characteristics of xanthophylls are called major carotenoids, whereas the minor carotenoids are formed in large quantities by the microalgae due to firm external environmental stimulus (Eonseon et al. 2003). Most carotenoids contain C40 hydrocarbon backbone, having basic eight isoprenoid units with double bonds that are conjugated in the series. These carotenoids could be cyclic or linear molecules with or without oxygen. Further, carotene and xanthophylls are considered as two major carotenoids groups. Hydrocarbon carotenoids are called carotenes as they do not contain any oxygen compound or its subordinates in their structures, whereas oxygenated derivatives of carotene are xanthophylls with oxygen being in the group of hydroxyl form (e.g., Zeaxanthin), oxi-groups (e.g., echinenone) or in combinations (e.g., astaxanthin) (Del Campo et al. 2000). Other substituent groups such as epoxy is present in violaxanthin and diadinoxanthin; acetyl group is present in dinoxanthin and fucoxanthin in the structures. Additionally, xanthophylls being hydrophobic molecules are either membrane-linked or have exclusive protein interactions via non-covalent bonds and are commonly present in the membrane of thylakoids. Minor carotenoids are present in vesicles formed by lipids and possess extraplastidic property (Grossman et al. 1995).

For most carotenoids, three major absorption maxima have been recorded. The absorption maximum is affected by cyclization and oxygenation. The common cyanobacterial carotenoids are β-carotene (only cyclic compounds), zeaxanthin, ketocarotenoid, echinenone, and carotenoid glycoside myxoxanthophyll. Primary carotenoids of green algae include β-carotene, lutein, and violaxanthnin that are present within the chloroplasts along with chlorophyll. Till date, more than 600 xanthophylls have been reported with different oxygen containing groups and their combinations (Lemoine and Schoefs 2010). Certain green algae, including *Chlorella* sp. accumulates biomass of carotenoids and are considered as good sources of carotenoids (Bhosale and Bernstein 2005).

Dunaliella salina is the preferred microalgae for β-carotene production due to its highest carotenoid content (10% dry weight) (Prieto et al. 2011a). Under critical conditions such as nutrient limitation, high salt and light, these microalgae can produce 14% of β-carotene in its dry weight. Growth rate and yield of β-carotene by *Dunaliella* is increased, when seaweeds are cultivated at higher concentrations (Raja et al. 2004). *Dunaliella* cultures are used to extract β-carotene using biphasic aqueous or organic systems (Hejazi et al. 2003, 2004). Cyanobacterial species *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC702 are good candidates that accumulates β-carotene after genetic modification (Macias-Sanchez et al. 2009). Moreover, the different strains of *Synechococcus* are reported to contain β-carotene, equinone and other phycopigments at varying extents (Kaur et al. 2009). Extraction of carotenoids is performed using organic solvents including acetone, methanol, or dimethyl sulfoxide (DMSO). Commercially, *Dunaliella* is cultivated throughout

the world (Australia, Israel, USA, India, and China) for β-carotene production with Australia being the major producer (Kleinegris et al. 2011)

The initial step of carotenoid extraction and purification involves separation of algal biomass from liquid media by centrifugation, flocculation, and filtration. Later, the biomasses were processed by specific drying methods. Carotenoids are extracted from various microalgae, namely, *D. salina*, *C. vulgaris*, *Spirulina pacifica* and *Nannochloropsis gaditana* by supercritical fluid extraction approach (Macias-Sanchez et al. 2009; Careri et al. 2001). Likewise, the supercritical CO_2 crystallization method with organic solvent and edible are utilized for the extraction of β-carotene from *Dunaliella* biomass. The marine algae *Synechococcus* sp. is evaluated in terms of effective operating pressure and temperature on efficiency of β-carotene extraction using supercritical CO_2 (Montero et al. 2005). Multiple studies have shown that extraction processes that are operated at the temperature between 40–80 °C and pressure between 20–40 MPa, selectively separates carotenoid molecule from algal biomass (Khanra et al. 2018). Moreover, supercritical dimethyl ether is utilized for efficient fucoxanthin extraction from microalgae *U. pinnatifida* (Kanda et al. 2014).

14.3.3.1 Applications of Carotenoids

Commercially available carotenoids include β-carotene, zeaxanthin, lycopene, canthaxanthin, lutein, and astaxanthin (Sathasivam and Ki 2018), that are used as provitamins, antioxidants, immune boosters, antiageing, and anticancer agents. Out of all carotenoids, β-carotene is the most commonly occurring, first commercialized carotenoid with diverse biological functions. Carotenoids are not vitamins, but these molecules have provitamin A activity and performs diverse biological functions in humans (Pisal and Lele 2005; Vilchez et al. 2011). There are approximately 60 carotenoids that can act as precursors of vitamin (1 ug retinol = 2 ug β-carotene). In humans, β-carotene is converted to vitamin A through the activity of skin tissues. Further, vitamin A highly necessary and efficient in the human body, as it prevents cataracts, night blindness, skin diseases and boosts the immune system (Agarwal and Rao 2000). Multivitamin and healthy food formulations also contain β-carotene as provitamin A (Spolaore et al. 2006a)

β-carotene from *Dunaliella* has been approved as a natural colorant by the United States Food and Drug Administration (FDA) to be beneficial in food and cosmetic industries as well as drugs to cure diseases. These carotenes possess ability to elevate the appearances of food products. Furthermore, it is beneficial as a food supplement, as feed in poultry and aquaculture (Christaki et al. 2013) in powder form. Additionally, it is also used as a colorant and a provitamin supplement in pet foods (Cantrell et al. 2003). Further, β-carotene gained potential significance in the cosmetic industry as sunscreen lotions, nail paints, lipsticks, and anti-aging creams. An interesting property of carotenoids is their antioxidant activity to protect the cells from harmful free radicals by quenching and scavenging processes. The antioxidant property is due to the physical and chemical interactions of these pigments with cell membranes. Thus, these pigments act as immune modulators, prevents the onset of

cancers, protects against life menacing diseases, including different forms of cancer (Rock 1997; Albanes et al. 1997; Tornwall et al. 2004). Certain carotenoids including astaxanthins also reduce the risk and progression of cancers. In particular, *Dunaliella* extracted β-carotene have 40% of 9-cis and 50% of all-trans stereoisomers that helps them in lowering the risks of certain cancers and degenerative diseases (Ausich 1992; Sandmann 2001).

The antioxidative properties of carotenoids allow them to protect brain cells from getting deteriorated and hence, prevents the process of aging. The specific role played by β-carotene in stimulating the immune system also prevent several other diseases such as cancers, premature aging, and arthritis. Additionally, β-carotene declines the memory loss in Alzheimer's disease by acting as a better antioxidant to facilitate brain cells to survive (Mattson 2004). Further, a detailed health aid of β-carotene is investigated in in vivo models. Cognitive impairment was found to be prevented, when transgenic mice fed with β-carotene and lutein of *Chlorella sp.* (Nakashima et al. 2009). Furthermore, extracts of *C. ellipsoidea* and *C. vulgaris* containing β-carotene prevented development of colon cancer (Plaza et al. 2009). *D. salina* extracted β-carotene has been commercialized by an Australian company named Western Biotechnology, since 1986. Similarly, huge quantities of β-carotene are produced in India from cyanobacteria. Microalgal carotenoids are good competitors of synthetic carotenoid forms as they provide superior natural isomers, compared to the synthetic form. Along with carotene, other carotenoids help in decreasing the premenopausal cancer prevalence in the breast; cryptoxanthin lowers the chances of cervical cancer. Lycopene can reduce the menace of prostate and digestive tract cancers. Additionally, combination of β-carotene, lycopene, vitamin E, and C help the patients under cancer therapy in developing immunity. Lycopene with β-carotene helps in preventing coronary heart disease by inhibiting the harmful cholesterol and low-density lipoprotein (LDL) formation. Lycopene also reduces the risk of type II diabetes with β-carotene and cryptoxanthin. Oxygenated carotenoids (Xanthophyll) obtained from *Dunaliella sp.*, have better anticancer properties and high bioactivity (Roodenburg et al. 2000). Lutein and Zeaxanthin inhibits the harmful UV radiations from sun and prevent loss of vision. Also, these carotenoids protect skin from photooxidation and hence can be used as nutraceutical or cosmetic ingredients that prevent oxidative stress-related diseases.

14.3.4 Phycobiliproteins

Phycobiliproteins (PBS), belongs to the family of water soluble, brilliantly colored, primary protein pigments in microalgal species (Glazer 1994). These pigments are arranged as macromolecular phycobilisomes complexes and are devoted to the outer thylakoid membrane surface for photosynthesis (Hemlata and Fatma 2009). These pigments constitute up to 50% of the soluble protein and comprise about 20% of the cell dry weight (Grossman et al. 1993). Bluish green allophycocyanin, bluish phycocyanin, purple colored phycoerythrin, and orange colored phycoerythrocyanin

are the major microalgal phycobiliproteins (Sekar and Chandramohan 2008). The optical absorbance of allophycocyanin is 650–655 nm, phycocyanin is 615–640 nm, phycoerythrin is 565 ~ 575 nm and phycoerythrocyanin is 577 nm. Thus, phycobiliproteins absorb light in the visible spectrum and acts as accessory light collecting pigments during photosynthesis (Batista et al. 2006). Phycobiliproteins are made of α and β polypeptides, having a molecular weight of 15 kDa and 22 kDa, respectively, that associates non-covalently to form heterodimers (Glazer 1994).

Two polypeptide chains with methionine was present in c-phycocyanin protein that is isolated from *Oscillatoria agardhii* microalgae (Peters et al. 1992). These two polypeptide chains are linked by disulphide bonds and each chain contains a single chromophore group. The composition of amino acids and N-terminal sequences of both polypeptides are also found to be similar. In complete phycobilisome, Allophycocyanin is present in the core of phycobiliproteins that are joined to the disc-shaped hexameric phycocyanins to the proximal side and distal side phycoerythrins present in the core. The prosthetic groups in covalent arrangement with phycobilins led to exclusive phycobiliprotein colors (Sekar and Chandramohan 2008). About 10–15% of total phycobiliprotein consists of uncolored or linker polypeptides which are not only the structural components that stabilizes PBS, but also helps in the efficient energy flow to the reaction center of photosynthesis.

Most abundant phycobiliproteins produced by red algae *Porphyridium* are phycoerythrins, while cyanobacteria *Arthrospira Spirulina* have phycocyanins as the major pigment (Glazer 1994; Bermejo Roman et al. 2002). The core pigment in *Nostoc* strains is C-phycoerythrin and it makes 10% of the microalgal dry weight. A strain of *Ananbena* having 8.3% dry weight of phycoerythrin was isolated from coastal lagoons of Spain (Rodriguez et al. 1991). Heterocyst containing strains of cyanobacteria were found to be high producers of phycobiliproteins among 41 cyanobacterial species, including unicellular, colonial, filamentous (heterocystous and non-heterocystous) and heterotrichous strains (Kaushik 2000). Phycoerythrin and phycocyanin also have a direct effect on total phycobiliprotein production. Analysis of 20 *Tolypothrix* strains of their phycobilin content revealed significant differences at both inter- and intraspecies level (Prasanna et al. 2003).

Various potential algal species for phycobiliprotein production are screened and it was found that *Spirulina fusiformis* produced 6 to 46% of c-phycocyanin and about 26% in *Spirulina platensis* (Zhu et al. 2007; Madhyastha et al. 2006). Moreover, the high heterotrophic cell density, continuous and fed batch unicellular red algae culture of *Galdieria sulphuraria* also produced large quantities of phycocyanin (Graverholt and Eriksen 2007). For extraction of phycobiliproteins, cell disruption methods such as osmotic shock, enzyme treatment, or high-pressure homogenization is used. The crude extract is then directly used for purification of pigments. Moreover, a combination of methods is also used depending on the desired purity and source organism. The purified pigment is dried by lyophilization to prevent its denaturation. Recently, high-pressure homogenization is utilized for extraction of phycocyanin from *S. platensis* (Seo et al. 2013). Likewise, chromatography coupled with expanded bed adsorption

technique has been used to extract phycocyanin in recent times (Ramoz et al. 2010). Various methods, including lysozyme treatment and nitrogen cavitation are utilized to obtain *Synechococcus* sp. extracted phycobiliproteins (Viskari and Colyer 2003; Gupta and Sainis 2010). Phycoerythrin isolation is performed by methods including sonication and freeze thaw method, using different cyanobacterial species (Pumas et al. 2011; Mishra et al. 2011).

14.3.4.1 Applications of Phycobiliproteins

Phycobiliproteins possess enhanced antioxidative, hepatoprotective, and neuroprotective entities (Spolaore et al. 2006a). These pigments are commercially used as organic dyes in various countries. They form an important component of fluorescent labeling for flow cytometry and histochemistry, due to their exclusive spectral entities (Kronick and Grossman 1983). Phycoerythrin due to its absorbance spectra is utilized as a secondary color in antibodies that are labeled in fluorescent colors (Sekar and Chandramohan 2008). Further, DNA and protein probes can be detected by using phycoerythrin labeled streptavidin (Diwu et al. 2012). Low-molecular weight cryptomonad derived phycobiliproteins (termed cryptoFluortrade mark dyes) finds applications in both extracellular and intracellular flow-based labeling systems. Also, these phycobiliproteins are beneficial as electrophoresis markers, focusers in chromatograph and iso-electric experiments, since they exhibit optical absorption in the visible light wavelength.

PBS is used as natural food colorants over synthetic dyes which exhibits carcinogenicity. Other benefits comprise their strong colors and elevated water solubility. The yellow fluorescent color of phycoerthrin is used for making transparent sugar-based lollipops and in other food decorations (Dufosse et al. 2005). *S. platensis* and *Phorphyridium aerugineum* extracted phycoerythrin is utilized as confectionary colors, gelatin desserts, and in cosmetic products. Another phycobiliprotein, *S. platensis* extracted C-phycocyanin is widely preferred as a colorant, compared to synthetic ones (Sekar and Chandramohan 2008) and marketed in Japan. It is beneficial as a chewing gum coloring agent, popsicles, and soft drinks to name a few. PBS is not permitted by FDA, however, desert lake technologies applied to grant generally regarded as safe (GRAS) status to CyaninPlus (C-PC) (FDA GRAS 2012). Additionally, it is used in cosmetic industries as lipsticks (Santiago-Santos et al. 2004). Phycocyanin is a pharmaceutically active phycopigment due to its antioxidant, neuroprotective, and hepatoprotective properties (Sekar and Chandramohan 2008). For instance, *Aphanizomenon flos-aquae* extracted phycocyanin has been found to be a robust antioxidative agent and is proven to reduce oxidative damage (Benedetti et al. 2004). It is capable of scavenging free radicals and also inhibits in vitro peroxidation of microsomal lipids (Romay et al. 2003). Further, phycocyanin significantly reduced hippocampal cell death in rats (Thaakur and Sravanthi 2010; Penton-Rol et al. 2011), decreased hepatic inflammation (González et al. 2003; Sekar and Chandramohan 2008) and decreased phagocytosis in Kupffer cell (Remirez et al. 2002).

Phycocyanin reduces edema, release of histamines, myeloperoxidase activity, prostaglandins and leukotriene in the inflamed tissues. Thus, phycocyanin is potentially beneficial in pathological conditions involving oxidative stress and inflammation. Phycocyanin also exhibits anticancer properties by reducing the tumor necrosis factor (TNF-α) in serum of the endotoxin treated mice and it also exhibited a neuroprotective effect in the cell cultures of rat cerebella granules (Sekar and Chandramohan 2008). In addition, phycocyanin derived from *S. platensis* exhibited dose and time-dependent growth inhibition of human leukemic K562 cells (Subhashini et al. 2004). Phycocyanin also led to prostate cancer cell apoptosis by enhancing the effect of anticancer medication topotecan (Gantar et al. 2012). Further, Phycocyanin reduced the spread of human hepatoma HepG2 cell line in another study by (Basha et al. 2008). Furthermore, R-phycoerythrin subunits were used for treating mouse tumor S180 cells and human SMC 7721 carcinoma cells (Huang et al. 2002). Allophycocyanin (APC) at concentrations not toxic to the host cells, inhibited cytopathic effects by inducing enterovirus-71 as reported by (Shih et al. 2003). Along with phycocyanin, allophycocyanin also has antioxidative and anti-inflammatory properties and hence, APC is also a potential therapeutic agent.

14.3.5 Astaxanthin

Astaxanthin with $C_{40}H_{52}O_4$ as molecular formula is a xanthophyll carotenoid and a red fat-soluble pigment. *Haematococcus pluvialis* is one of the best green algal sources of organic astaxanthin as it represents 90% of the total carotenoids (Ranga et al. 2009). The basic astaxanthin structure has two rings in terminal linked by a chain of polyene. It possesses keto (C = O) and hydroxyl groups on each ring of ionone that are responsible for its unique esterification ability and other exclusive properties (Ma and Chen 2001). Likewise, conjugated double bonds (hydroxyl and keto group) are accountable for the red astaxanthin color. These bonds make this pigment a better antioxidant as it donates electrons and lead to free radical and stable product formation, thus terminating the free radical chain reactions in living organisms. Closed tubular bioreactors are industrially explored for astaxanthin production from *H. pluvialis* (Lopez et al. 2006). Actively growing cells of *haematococcus* biomass are fragile and breaks easily, therefore the recovery of astaxanthin from hard walls of haematocysts is studied by (Mendes-Pinto et al. 2001). Settling and centrifugation are the steps involved in the biomass harvesting of microalgae. Finally, the biomass are broken to improve astaxanthin bioavailability and then dried. The biomass product, that are dried, is either encapsulated directly or used for extraction of astaxanthin (Olaizola and Huntley 2003).

14.3.5.1 Applications of Astaxanthins

Astaxanthin serve as the superior vitamin E, having a strong antioxidative activity (10X), compared to β-carotene (Jyonouchi et al. 1995). Higher concentration of various antioxidative enzymes, including peroxidases are found to be present in plasma and liver of rat fed with *Haematococcus* biomass as an astaxanthin source (Ranga Rao et al. 2010). The levels of these antioxidant enzymes were found to be increased, when model animals (rabbits and ethanol-induced gastric ulcer rats) were supplemented with astaxanthin (Augusti et al. 2012; Kamath et al. 2008). Likewise, astaxanthin is a superior antioxidant agent, compared to other carotenoids as seen in the dermal region of human fibroblasts and hence used as theranostic agents for diseases related to skin (Camera et al. 2009). In deep burn model, early progression of burn wounds is prevented by astaxanthin by reducing ROS production and oxidative stress (Fang et al. 2017). The exclusive molecular astaxanthin structure permits it to remain stable in both inside and outside the cell membrane and hence, it can aflatoxin carcinogenicity (Rao et al. 2013).

Astaxanthin is an antioxidant that acts against inflammation in biological systems. *Haematococcus* extract containing astaxanthin, reduced the *H. pylori* growth in gastric inflammation infected mice (Bennedsen et al. 1999; Park et al. 2010). The lower rate of respiratory inflammation enhances secondary messenger cGMP and cAMP levels, which were found in extracts of *Ginkgo biloba*, astaxanthin, and Vitamin C supplemented lung tissues (Haines et al. 2011). Another study demonstrated that *Haematococcus* sp. extracted astaxanthin from preventing gastric ulcers by inhibiting H_1K_1ATPase activity, elevating mucin content and antioxidative activity (Kamath et al. 2008). Further, astaxanthin are proved to be useful for the ocular inflammation treatment in rats as it inhibits the NFkB signaling pathway (Suzuki et al. 2006). The mechanism of continuous stress via singlet oxygen leading to inflammation is responsible for neurodegeneration, cancers, and skin damage is well known. Astaxanthin treatment has been proved to prevent the UV induced skin damage by reducing the levels of prostaglandin E2 after UV exposure (Yoshihisa et al. 2014). This inhibitory astaxanthin effect is important for the anti-inflammatory drug development, for skin infections including psoriasis. Astaxanthin also prevents skin thickening and inhibits collagenase, inflammatory mediators, and thus, responsible for the anti-wrinkle effects (Davinelli et al. 2018).

Numerous studies have demonstrated the purpose of astaxanthin in oxidative stress reduction, damages renal cells and thus prevents diabetic nephropathy in diabetic mice (Naito et al. 2004; Kim et al. 2009; Manabe et al. 2008). Astaxanthin, being an antioxidant, also serves as an immune modulator by providing protection from free radical damage. This pigment significantly alters immune functions in several studies using live models (Lin et al. 2015). In murine models, astaxanthin showed a higher immune modulating effect, compared to β-carotene (Jyonouchi et al. 1991). A human lymphocyte experiment showed enhanced responsorial production of immunoglobulins to T-cell dependent stimuli (Jyonouchi et al. 1995). In humans, astaxanthin supplementation for 8 weeks, improved the activity of NK cells that targets and

destroys virus infected cells was observed (Park et al. 2010). Along with cell mediated immunity, astaxanthin has stimulated humoral immunity in murine model by inducing production of polyclonal IgG and IgM antibodies (Jyonouchi et al. 1995; Okai and Higashi-Okai 1996). Main pathological features of cardiovascular diseases are reduced by the antioxidative properties of astaxanthin in experimental model systems and humans. Thus, this pigment can be considered as latent agents for cardiovascular atherosclerotic disease treatments (Fassett and Coombes 2011).

Antioxidants by inhibiting oxidative damage of the cells decrease their rate of mutagenesis and carcinogenesis. In human tumors, cell to cell contact through gap junction is decreased. Gap restoration junctions by elevating the connexin-43 protein levels tend to decrease tumor cell progression. Natural carotenoids, specifically canthaxanthin and astaxanthin, increased gap junctions in mouse embryo and human skin fibroblasts (Hanusch et al. 1995; Daubrawa et al. 2005; Hix et al. 2004). Astaxanthin showed better anti-tumor activity by inhibiting the growth several tumor cells (Palozza et al. 2009). Further studies showed that astaxanthin lead to cell death and inhibited cell proliferation of mammary tumors in models of chemically induced murine (Tanaka et al. 1995; Jyonouchi et al. 2000; Nakao et al. 2010). The peroxynitrite reaction with lutein and astaxanthin leads to the formation of Nitrolutein and Nitroastaxanthin which are proved to possess enhanced anticancer properties as they inhibited mouse skin papillomas (Maoka et al. 2012).

H. pluvialis extracts containing astaxanthin have been accepted as a supplement in humans or animal diets and reduces the risk of various disorders (Kidd 2011; Yuan et al. 2011). Dietary astaxanthin enhances the fish and shrimp immunity system which eventually elevates their survival and growth. Additionally, it plays a crucial part in the markets of feeds for aquaculture and livestock (Dufosse et al. 2005; Cysewski and Todd 2004; Guerin et al. 2003). Further, Astaxanthin is utilized in food, feed, nutraceutical, medical and cosmetic industries. In addition, market availability of astaxanthin in distinct powder and capsule forms reveals their enhanced applications in food industries. The patented benefits of this algal pigment include its use in preventing inflammation, cancer, bacterial infections, cardiovascular diseases, improving brain functioning, and skin thickness.

14.3.6 Secondary Pigments, Essential Lipids, and Other Pigments

The secondary pigments in algae include the secondary carotenoids such as fucoxanthin, canxanthin, cryptoxanthin, violaxanthin, and echinone (Lemoine and Schoefs 2010). Fucoxanthin is the primary light harvesting carotenoid that efficiently performs the energy transfer and plays a significant role in photoprotection and antioxidation in algae. Fucoxanthin has shown activities of anti-proliferation and prevention of cancer by inhibiting the cell cycle process (Kumar et al. 2013). Anti-angiogenic effect of fucoxanthin has proven to prevent the development of cancer

in endothelial cells (HUVECs). The anticancer activities of fucoxanthin with astaxanthin and β-carotene are reported towards HL-60 cancer cells (Kim et al. 2012). The role of several secondary pigments including zeaxanthin on cancer cell lines of prostate region is documented in the experiment by (Kotake-Nara et al. 2001) and fucoxanthin exhibited the highest growth inhibition rates. It is noteworthy that the effects of fucoxanthin and its metabolites varies depending upon the type of cancer cells. Moreover, Fucoxanthin also possesses antidiabetic activity. Obese mice treated with fucoxanthin restored normal blood glucose and insulin levels by elevating glucose transporter 4 (GLUT4) levels in skeletal muscle cells (Maeda et al. 2009). A carotenoid cryptoxanthin also inhibited colon cancer cell proliferation, whereas combinations with oxaliplatin induced cancer cells apoptosis (Sathasivam and Ki 2018). These are important applications of some of the secondary pigments of microalgae.

The essential lipid fraction of microalgae includes fatty acids, waxes, and sterols (Spolaore et al. 2006a). Based on head group polarity, fatty acids are divided into two groups, namely, neutral and amphipathic lipids (includes phospholipids and glycolipids) (Cuellar-Bermudez et al. 2015). Acylglycerols is characterized into monoacylglycerols, diacylglycerols and triacylglycerols depending on the number of fatty acids (Halim et al. 2012). Production of microalgal lipids is based on its species and the culture conditions, including pH and salinity (Guschina and Harwood 2006). The content of neutral lipids in microalgae is efficiently increased by nitrogen starvation (Breuer et al. 2012; Yeesang and Cheirsilp 2011; Cuellar-Bermudez et al. 2015). Important commercial source of DHA includes dinoflagellate *Crypthecodinium cohnii*, and *Schizochytrium* and can be extracted algal oil extraction methods includes mechanical pressing, homogenization, milling, and solvent extraction (Palmquist and Jenkins 2003).

Among the fatty acids of polyunsaturated nature, omega-3 PUFA is considered as a fish oil substitution (Ryckebosch et al. 2014). Microalgal fatty acids of omega-3 type have several medicinal benefits in reducing high blood pressure, stroke, myocardial infarction, and cardiac arrhythmia. Adequate intake of major omega-3 PUFA, namely, Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by pregnant women is highly essential for the enhanced fetal brain growth (Adarme-Vega et al. 2012). Algal classes producing EPA include *Bacillariophyeae* and several other microalgal species (Cuellar-Bermudez et al. 2015). Thus, algal lipids are used as an alternate source of nutritionally significant PUFA and are composed of functional food formulations (Cuellar-Bermudez et al. 2015). Another important application of algal lipid is their benefits in aquaculture as documented in various studies (Gladue and Maxey 1994; Birkou et al. 2012).

14.4 Microalgal Pigments as a Potential Economic Booster

The evolution of biotechnology commenced to flourish during the last century, with a steep increase in the microalgal production for aquaculture, human as well as animal food and fodder, and cosmetics. This section deals with the application of

various algal pigments with a great prospect of being an economic booster. This has resulted in the enhanced methodology in production and genetic microalgal strain modification. Increased application of pigments as natural dyes and polyunsaturated fatty acid oil in food, increase the need of diversifying the quest of microalgal biome for their potential economic implication (Spolaore et al. 2006b).

14.4.1 Commerce Behind Bioindustrialization

Commercial microalgae production started in the early 1970s in Japan, with *Dunaliella salina*, as a β-carotene source which later became a rapidly growing bioproduct industry, when Australia pioneered in mass outputs. The species showing higher growth rates, invasion resistance, and the presence of optimal lipids are the triad of selecting the best seeding culture. The biofuel production and utilization of bioactive compounds from microalgae is hindered by the increased industrial slab. Improvement of extraction techniques is mandatory for the efficient usage; this can be achieved by alternate methods of extraction such as microwave irradiation methods (Kapoore et al. 2018). Microalgal cells pose a challenge while disrupting the intracellular polymers such as sporopollenin and algaenan. In a few species such as *Dunaliella tertiolecta*, the absence of frustules led to rapid extraction of phycopigments, in contrast, the presence of frustules in *Cylindrotheca closterium* renders as a mechanical barrier to pigment extraction. Solvent-based disruption techniques require massive protocols to obtain products of interest. Conventional techniques such as cold and hot soaking technique, ultrasound assisted technique, are often very expensive and contribute to low efficiency. MAE is found to be a better alternative as it is a combination of rapidity, better reproducibility, uniform heating, and increased yields (Pasquet et al. 2011). The process of harvesting involves enormous energy and finance; the techniques include centrifugation, foam fractioning, chemo-flocculation, electro-flocculation, osmotic separation, and ultrasonic separation. Whereas, certain low energy and low budget harvesting process like gravity sedimentation are not economically feasible. Scientists are expected to come up with a solution to address the problems affixed to harvesting microalgae for the commercial isolation of valuable metabolites (Ghosh et al. 2016).

14.4.2 Potential of Various Microalgal Pigments in Industries

The markets were seen inviting microalgal components in the past, in this backdrop, a marked increase in research is expected to expand the horizon for various microalgal products. The quality of such products is maintained by the downstream processing technique.

14.4.2.1 β-Carotenoids

Fat soluble of β-carotenoid pigments obtained from certain photosynthetic bacteria, algae, and plants, they perform as an important photosynthetic component. It is predominantly isolated from the stenohaline species *Dunaliella salina*, the dried biomass of these algae composes of almost 10% of dried biomass, this is the highest among all species (Prieto et al. 2011b). After a successful pilot study in culturing these organisms by the USSR in 1960, many nations followed the lead in successful commercialization of *Dunaliella salina.* Annual production rates across the globe ranges around 1200 tons, with Australia as the leading producer. The worldwide market for this species is expected to be 1.8 billion dollars in this current year, this is a leap of 0.3 billion dollars from the levels in past demi-decade. The global demand of cyanobacteria-based pigments is predicted to increase in the next fiscal year and it is expected to shoot in the future to meet 1-billion-pound mark.

14.4.2.2 Chlorophyll and Astaxanthin

Chlorella microalgal species are considered as the Emerald food due to its richness in chlorophyll along with 7% of the biomass, which is roughly five times of the Spirulina chlorophyll content. It is an organic colorant of food that contains rich antioxidant content and acts as an antimutagenic agent. The Weiwang company in Taiwan was the first to commercially produce *Chlorella* at diverse scales in industries (Bewicke and Potter 2009b) and they are grown in large plastic tubes to harvest more solar radiation for efficient growth.

Astaxanthin is considered an efficient medicinal agent that can cure neurodegenerative diseases, high-cholesterol, hepatic dysfunctions, geriatric muscular atrophy, and for the prevention of malignancy. It is also used as a therapeutic agent for metabolic syndromes. In certain conditions, it is given as a supplement for increasing muscle fitness, endurance, increasing the exercise performance, decreasing muscle soreness after exercise. Oral intake of astaxanthin prevents sunburn, reduces sleep apnea, insomnia, dyspepsia, infertility disorders, carpal tunnel syndrome, rheumatoid arthritis, and menopausal syndrome. Direct application on the skin, reduces sunburn and wrinkles; it is also used as a colorant in few other cosmetics. It is beneficial as a supplement in food for egg laying chicken in the poultry industry. It works as an antioxidant and prevents cell damage. The investment return for distinct astaxanthin market prices are assessed to understand the economic viability of these phycopigments. Market astaxanthin value varies between $2500–7000/kg with a global market value of 280 metric tons/year (Panis and Carreon 2016).

14.4.2.3 Xanthophyll

Xanthophyll is a form of oxygenated carotenoids found in a plethora of photosynthetic organisms, there has been major advancements in molecular biology for the last few years, which has paved way for efficient isolation and production and bioutilization of xanthophylls. Recently, molecular biology developments have paved way for an effective genetic microalgae manipulation to increase xanthophyll yield and helps in their commercial production (Jin et al. 2003). A commercially significant fucoxanthin- xanthophyll type is obtained from *Fucus* sp. and *Phaeodactylum tricornutum,* it is a protein bound component associated with chlorophyll. Antioxidant assay of *Chaetoceros calcitrans* and *Isochrysia galbana* showed an amazingly high number of antioxidants, primarily bound to the pigment (Foo et al. 2017).

14.4.3 Economic Boosters of Bioeconomy

The pigments derived from microalgae and microalgae themselves have unique vital characteristics that can be translated into advanced technical and commercial products. This contributes to the production of a variety of beneficial lipids, carbohydrates, bioactive compounds, etc. At a very cost-reducing way, they can incorporate the stable isotopes of 13C, 15 N, and 2H into their biomass and produce various other components they produce. They comprise huge unexplored taxa of organisms, providing a virtually unexplored source of products with potential implicational significance.

Industrial and scientific advancement in effectual microalgal product fabrication plays a vital role in influencing the monetary feasibility of microalgal products (Roux et al. 2017). However, limited articles focused on the large-scale microalgal utilization to obtain valuable compounds, especially pigments. It is inevitable to understand a few aspects of microalgae before starting a commercial spin-off using microalgae. Factors include the analysis of microalgae-based product production, market expectancy, demand, and production scale. The analysis must include a pilot study on the technological advances in the downstream processing of microalgal products, search on patented and protected inventions in the specific field, and a global screening on the present situation of a global market that depends on microalgal biomass with future outlooks.

During the last decade, the research focusing on microalgal pigments has made the noteworthy advancements. Novel methodology for purification permits large-scale harvesting of pigments with high purity. Microalgal PBP pigment generates extra demand in the pharmaceuticals and nutraceutical markets. It is essential to develop novel approaches for heterotrophic and mixotrophic production to meet the growing demand of such algal pigments. More widespread research in microalgal application is still needed to explicate the complete action of microalgal pigments using various model organisms. Studies focusing on the productivity involving various species

producing a particular pigment have to be explored thoroughly to choose the best source of culture for effective productivity and increased financial output. The market for the pigments derived from various sources are kept at a high due to the increased awareness about the benefits of bio-products, the demand is rather satisfied with the alternative and synthetic substitutes. This bottleneck is mainly due to the difficulties associated with the mass production of microalgae, resource cost and comparative cheaper production cost in the case of synthetic pigments (Ambati et al. 2018).

Although there is a wide demand for plant pigments in the International market, 95% of this is satisfied by the synthetic substitutes. Synthetic pigments are often derived from petrochemical products and often raise alarming food safety issues with a potential toxicity in the end product. Besides, the hazard of environmental distress and sustainability issues also arise with the synthetic production. In fact, to date, the usage of synthetic pigments is restricted to its usage in aquaculture and are not permitted to consume by humans. Increasing awareness has spearheaded the market improvement for the naturally derived pigments due to the transition towards "green alternatives" and organic products, the market is expected to hit a $1.5 billion by the end of 2020 with all the natural pigments gaining an upper hand in the market (Bhat and Madyastha 2000; Begum et al. 2016).

14.5 Current and Future Perspective

The current status of microalgal pigments is concisely elaborated in this chapter. It is noteworthy that the microalgal pigments are extensively beneficial in improving the bioeconomy of algae-based companies and as a potential economic booster. The market size of microalgal products is increasing rapidly, especially the products with microalgal pigments are easily reaching the break-even point in a short time, due to low production cost and high yield (Singh et al. 2017). Among the microalgal/phycopigments, carotenoids are widely in several companies for the production of natural pigments and use them in finished products. In the US, Astaxanthin has been used by Mera Pharmaceuticals Inc., and Valensa International, β-carotene by aquacarotene Ltd., *Spirulina* by Nutrex Hawaii Inc., *Chlorella* by Maypro Industries Inc. for large quantities of organic pigment production. In addition, Cyanotech Corporation in US is the producing large quantities of astaxanthin and β-carotene for commercial applications that are extracted from *Spirulina* and *Chlorella.* Likewise, Fuji Chemicals Industry Co., Ltd., in Japan and Sweden extracted astaxanthin pigments from microalgal species for various applications. Other Japanese companies such as Nikken Sohonsha Corporation extracted β-carotene, Sun Chlorella Corporation and Yaeyama Shokusan Co., Ltd., used *Chlorella* to extract phycopigments to produce commercial products. In India, astaxanthin and β-carotene is produced in large quantities by E.I.D Parry Ltd., and the same company along with Hydrolina Biotech Pvt., Ltd., produced phycopigments with *Spirulina* for medicinal applications. Other countries such as China, Australia, Israel, Taiwan, and Germany also produces large quantities of phycopigments for unique and valuable commercial

products (Ambati et al. 2018). Recently, BlueBioTech, a company from Germany, has been producing a wide variety of natural, pharmaceutical and dietary supplements via microalgal extracts, especially using their pigments (Misra et al. 2016).

In recent times, the association of nanotechnology with microalgal products to enhance their value and properties is the trend in product formulations. The emergence of nanoparticles in the formulation of phycopigments has led to reduced usage and elevates their value, compared to standalone phycopigments. Recently, biocompatible nanosized ovalbumin has been functionalized using microalgal phycocyanin proteins as functional groups and these nanoparticles are used to formulate cefpirome which is a derivative of cephalosporin. The results revealed that complex nanoformulated cefpirome with microalgal pigment possess potential to inhibit bacterial pathogens with high biocompatibility, compared to the non-formulated cefpirome, ovalbumin, and phycocyanin (Namasivayam 2017). Other than nanoformulations, several studies showed ample evidences that the addition of nanoparticles improves the production of bioactive contents in microalgae, especially bioactive pigments (Miazek et al. 2015; Lee et al. 2015; Hazeem et al. 2015). Moreover, the phycopigments along with microalgal bioactive molecules possess ability to synthesize biocompatible nanoparticles that can be utilized for several applications (Patel et al. 2015). Silver nanoparticles have recently been synthesized using the microalgal pigments of diatom *Amphora* sp. as the biogenic synthesis agent and the result showed that the obtained silver nanoparticles possess enhanced antimicrobial activity against a wide range of microbes (Jena et al. 2015). Silver, copper, gold and iron oxide nanoparticles are synthesized using extracts of microalgae with phycopigments in recent times (Salas-Herrera et al. 2019; Dahoumane et al. 2016; Siddiqi and Husen 2016; Shankar et al. 2016). Sometimes, the nanoparticles also may cause adverse effect in microalgae and may affect the ecosystem dynamics, if nanoparticles are exposed directly to the growth site of microalgae (Amanda et al. 2017). Nanoformulation of phycopigments into liposomes, micelles, polymers, dendrimers, and biocompatible encapsulations will further enhance their properties and can be used for controlled release of these phycopigments at the target site to elevate their pharmaceutical and nutraceutical purposes (Jeevanandam et al. 2016). Thus, nanoformulated phycopigments will help to boost the bioeconomy that depends on microalgal production, especially the pharma and nutraceutical industries in future.

14.6 Conclusion

This chapter shows the wide industrial applications of microalgae and the valuable bioactive compounds, especially pigments that are extracted from microalgae. These phycopigments are usually termed to be better in lab-scale production and applications. However, the list of companies that uses microalgal pigments in the large-scale production of commercial products revealed that the phycopigments slowly moving from lab to large-scale phase. The advancements in the microalgal

biotechnology are the factors that contribute to the large-scale extraction of microalgal pigments and use them to produce highly valuable commercial products. In addition, the market size of the microalgal products also increased gradually over the years as the quest and marker demand for natural products has globally increased. In future, it is expected that the bioeconomy that depends on the plant products will eventually replace and increased by microalgae, provided the proper funding by governments, approval of products by regulatory bodies and increased acceptance from the public.

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Chapter 15
Sustainability Metrics in the Microalgae-Based Pigments Production: A Life Cycle Assessment Approach

Mariany C. Deprá, Aline M. dos Santos, and Eduardo Jacob-Lopes

Abstract The purpose of this chapter is to provide a comparative life cycle assessment with a focus on the establishment of energy resource, global warming potential, land use, acidification potential, water footprint, eutrophication potential, photochemical ozone creation potential, ozone depletion potential, and ecotoxicity of the current production scenarios of synthetic and microalgae-based pigments. Although the functionality of the microalgae-based pigments are greater and the commercial production is practicable, the environmental impacts for these natural pigments output are higher compared to the synthetic pigments.

Keywords Microalgae · Natural colorants · β-carotene · Astaxanthin · Phycocyanin

15.1 Introduction

The global pigments market is poised to grow with a compound annual growth rate of around 5.1% over the next decade to reach approximately $1.95 billion by 2025 (Markets and Markets 2019). Currently, chemical synthesis is the dominant route for the production of pigments, with 55% of the global market mastered by two main chemical manufacturers BASF and DSM (Deinove 2019).

Changes in legislation regarding the use of synthetic dyes and increasing consumer awareness of the health benefits associated with natural food colors are increasing the demand for natural products, making synthetic routes less desirable, which justifies the considerable efforts that is being paid on the promotion of biotechnological alternatives with environmentally friendly production systems (Grand View 2019; Pérez-López et al. 2014a).

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Given this scenario, boosted by the favorable market, the intensification of the research resulted in important discoveries, which made it possible to open up alternative or additional biotechnological processes for the industrial manufacture of natural food colorants with enhanced functionalities (Tuli et al. 2015).

Thus, microalgae have emerged as an attractive alternative, since they are microorganisms capable of naturally synthesized pigments such as chlorophylls, carotenoids, and phycobiliproteins that exhibit several health-relevant properties besides colorant, such as anti-viral, antimicrobial, anti-inflammatory, antioxidant, pro-vitamin A, immunomodulatory and anticancer activities (Viera et al. 2018). In terms of microalgae-based pigments that reached the commercial level, β-carotene from *Dunaliella salina*, astaxanthin from *Haematococcus pluvialis* and phycocyanin from *Arthrospira platensis* are molecules already approved as food ingredients by main world regulatory organizations (EC EFSA 1997; MHLW 1996; ANVISA 2018; FDA 2019).

However, in spite of the perceived advantages, a number of techno-economic barriers need to be overcome for the consolidating of microalgae-based processes on a commercial scale. In order to understand this processes performance, comprehensive analyses addressing environmental, economic and social indicators should be carried out following a life cycle perspective (Gong and Bassi 2016).

In this sense, the objective of this chapter is to perform a comparative life cycle assessment of the current production scenarios of synthetic and microalgae-based pigments with a focus on the establishment of sustainable metrics.

15.2 Synthetic Pigments

Chemical synthesis of pigments was developed by three teams independently in the year 1950, being considered a milestone in the research of pigments (Karrer and Eugster 1950; Inhoffen et al. 1950, Milas et al. 1950). In 1976, more than 90 naturally occurring pigments were synthesized based on this early work, through the principle of coupling appropriate end groups to a central building block. During this time some methods of chemical synthesis have been successfully developed to obtain pigments and they have since been used repeatedly (Britton et al. 1996). Two examples of these methods are illustrated in Figs. 15.1 and 15.2.

The first scaled up method developed by Hoffman-La Roche was the Roche synthesis in 1954. With a yield of 60%, this method was based on the Grignard reaction (enol ether condensation) and follows the $C_{19} + C_2 + C_{19}$ synthesis principle (Fig. 15.1) (Ribeiro et al. 2011). By the glycidic ester synthesis, the ß-ionone is lengthened to ß-C14-aldehyde. Through an alkali treatment, a CH_2-group link to the side chain of this aldehyde, thus undoing the double-bonded conjugate system of the ring. Thus, the ß-C14-aldehyde is transformed into its acetal for chain lengthening, being condensed with vinyl ether in the catalytic presence of an acid. Subsequent hydrolysis gives the crystalline ß-C16-aldehyde. By a similar process, the β-C16 is converted with propenyl ether to the crystalline ß-C19-aldehyde. Through the

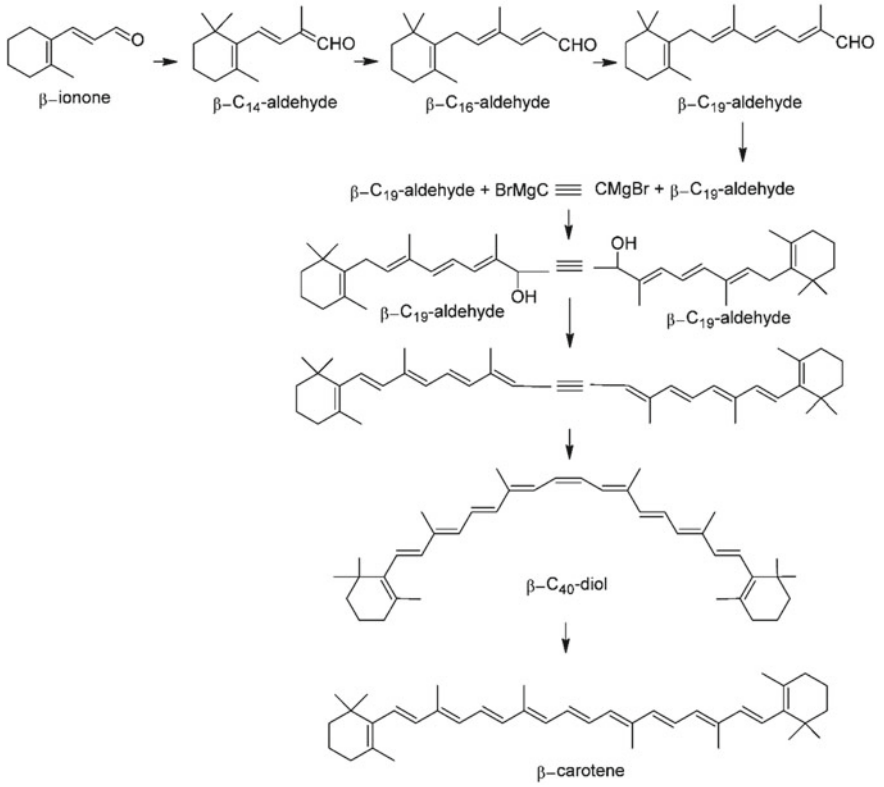

Fig. 15.1 Synthetic pathway for obtaining ß-carotene developed by Hoffman-La Roche

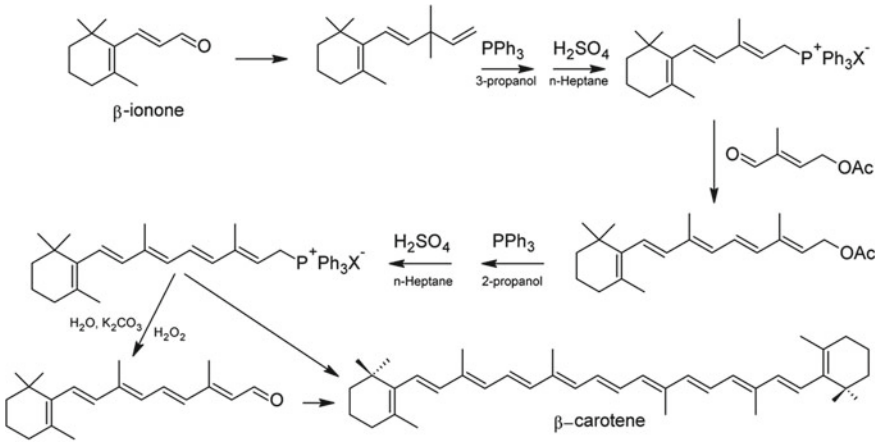

Fig. 15.2 Synthetic pathway for obtaining ß-carotene developed by BASF

Grignard reaction, two moles of ß-C19-aldehyde are condensed with acetylene to form ß-C40-diol. By elimination of two molecules of water with simultaneous allylic rearrangement, the ß-C40-diol is transformed into ß-carotene. Partial hydrogenation in catalyst presence gives 15, 15' -*cis*-ß-carotene, which is isomerized in elevated boiling petroleum ether to crystalline all-*trans*-ß-carotene (Isler et al. 1967; Pfander et al. 1997; Ernst 2002).

In 1960, a higher yield method (85%) was developed by BASF. This method was underpinned on the Wittig condensation and follows $C_{20} + C_{20}$ synthesis principle, at where phosphonium salt, preliminarily derivatized with triphenylphosphine, react with an aldehyde, resulting in a double bond and increasing the polyenic chain. In Fig. 15.2, the reaction occurs between retinal and retinyltriphenylphosphonium salt (Ribeiro et al. 2011). The Wittig reaction is today the most important reaction for the synthesis of carotenoids, as it allows the formation of carbon–carbon double bonds in a specific location in the molecule. However, this reaction has a disadvantage, such as the environmentally undesirable formation of triphenylphosphine oxide (Britton et al. 1996; Ribeiro et al. 2011; Gong and Bassi 2016).

Without exception, the C40-carotenoids produced by Hoffman-La Roche and BASF synthetic pathways have symmetrical structures, once that they have identical end-groups (Ernst 2002).

The astaxanthin is a xanthophyll carotenoid subclass, characterized by the presence of oxygen molecule in their structure, that is similar to β-carotene and other xanthophylls, such as lutein, canthaxanthin, and zeaxanthin, in that they share a common semi-symmetric layout with two terminal carbon rings flanking a polyene chain. However, astaxanthin is distinctive in its structure from other carotenoids due to the presence of hydroxyl and keto moieties on both ends (Yang et al. 2013).

Many strategies have been developed to the astaxanthin synthetic synthesis from the symmetrical C40-carotenoids, being Wittig reaction of two C15-phosphonium salts with a C10-dialdehyde the most commonly used method (Fig. 15.3a) (Widmer et al. 1981). Other methods include the hydroxylation of canthaxanthin (Fig. 15.3b) (Bernhard et al. 1984), a C10 + C20 + C10 synthesis via dienolether condensation (Rüttimann 1999), and the isomerization of lutein to zeaxanthin and then oxidation to astaxanthin (Fig. 15.3c) (Schloemer and Davis 2002).

Given the technological aspect, the process of synthetic production of these pigments involves more compact and optimized equipment which reflects in a processing plant with less occupation of the physical space and with lower spending on electricity (Fig. 15.4) (Isler 1971; Pommer 1977).

15.3 Microalgae-Based Pigments

The synthetic pigments proved extremely successful, their availability and reasonable price led to more extensive use, and to attempts to find new applications and open up new markets (Hu 2019). However, a popular view sustains that natural pigments are innately superior to synthetic form with respect to their effects on human health,

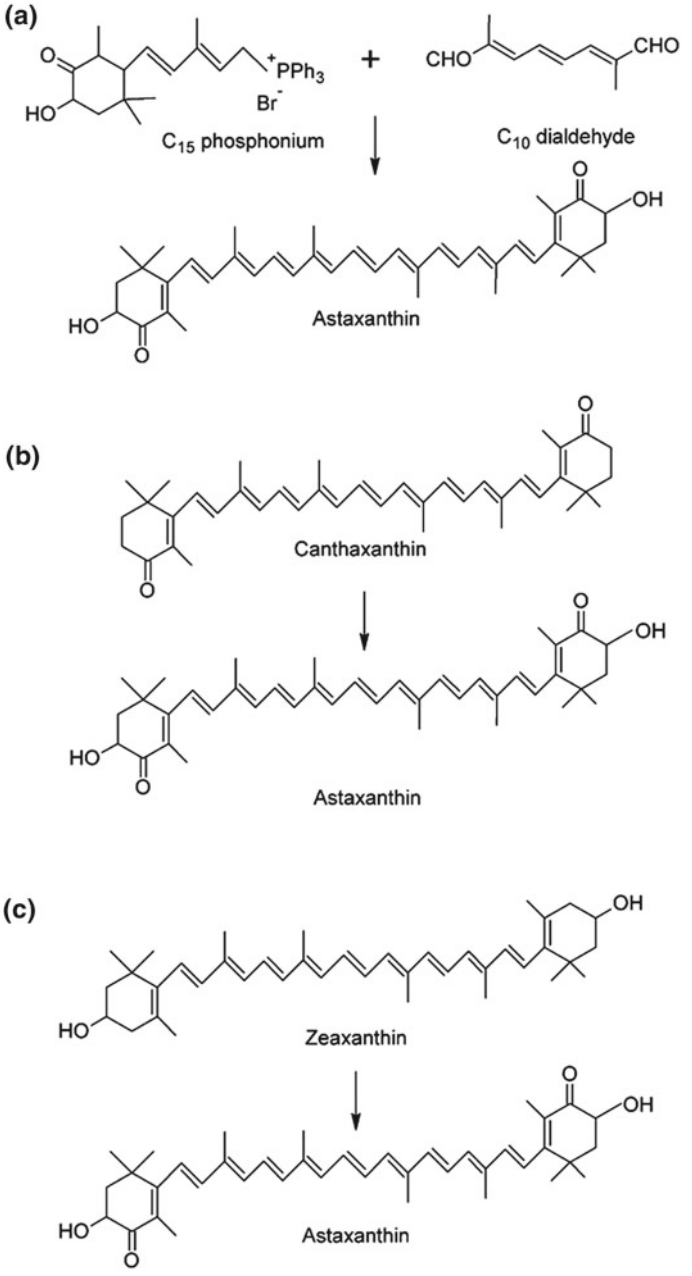

Fig. 15.3 Synthetic pathways for obtaining astaxanthin. **a** Wittig reaction **b** Hydroxylation of canthaxanthin **c** Oxidation of zeaxanthin

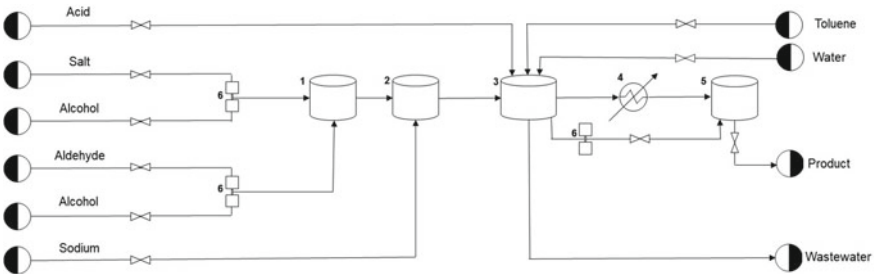

Fig. 15.4 Process flow diagram of synthetic ß-carotene and astaxanthin production. (1) Mixing tank of salts, aldehyde and alcohols; (2) Mixing tank of sodium; (3) Reaction and equalization tank; (4) Condenser; (5) Tank; (6) Pumps

due to the presence of 9-*cis* isomers, more efficient in protecting against oxidative damage (Britton et al. 2017).

Natural pigments comprise one of the most interesting components produced in microalgae-based systems that can produce until three classes of pigments (chlorophylls, carotenoids, and phycobiliproteins) (Jacob-Lopes et al. 2019). These pigments are in competition with your synthetic counterparts once that has the advantage of supplying an approximately equal proportion of *trans* and *cis* isomers compared to 100% all-*trans* stereoisomers of synthetic forms (Guedes et al. 2011).

Considering the high structural diversity of pigments, it is possible to obtain high 9-*cis* to all-*trans* ratio that has been claimed to be a direct function of microalgae growth conditions. Thus, the potential of microalgae as a source of molecules of bioactive interest is very high (Rodriguez-Concepcion et al. 2018).

Facing the worldwide demand for bioactive to be applied in food, cosmetics, and other oxidizable goods, the search for natural alternative sources increases yearly, and microalgal biotechnology has gained a prominent share as a pathway for the production of these compounds (Guedes et al. 2011).

15.3.1 Microalgae Carotenoids Production

Natural synthesis of carotenoids starts off in a general primary metabolic pathway to all photosynthetic microalgae classes, as shown in Fig. 15.5. This pathway initiates from the common five-carbon (C5) construction units, isopentenyl pyrophosphate (IPP) or its isomer, dimethylallyl diphosphate (DMAPP). The C5 building block can be obtained via the cytosolic mevalonic acid pathway (MVA), produced from either acetyl-CoA or the plastidic methylerythritol 4-phosphate pathway (MEP), produced from pyruvate and glyceraldehyde 3-phosphate (G3P) (Paniagua-Michel et al. 2012; Gong and Bassi 2016).

Then, the chain stretching proceeds by sequential head-to-tail condensation of three IPP molecules to DMAPP, catalyzed by prenyltransferases that synthesize

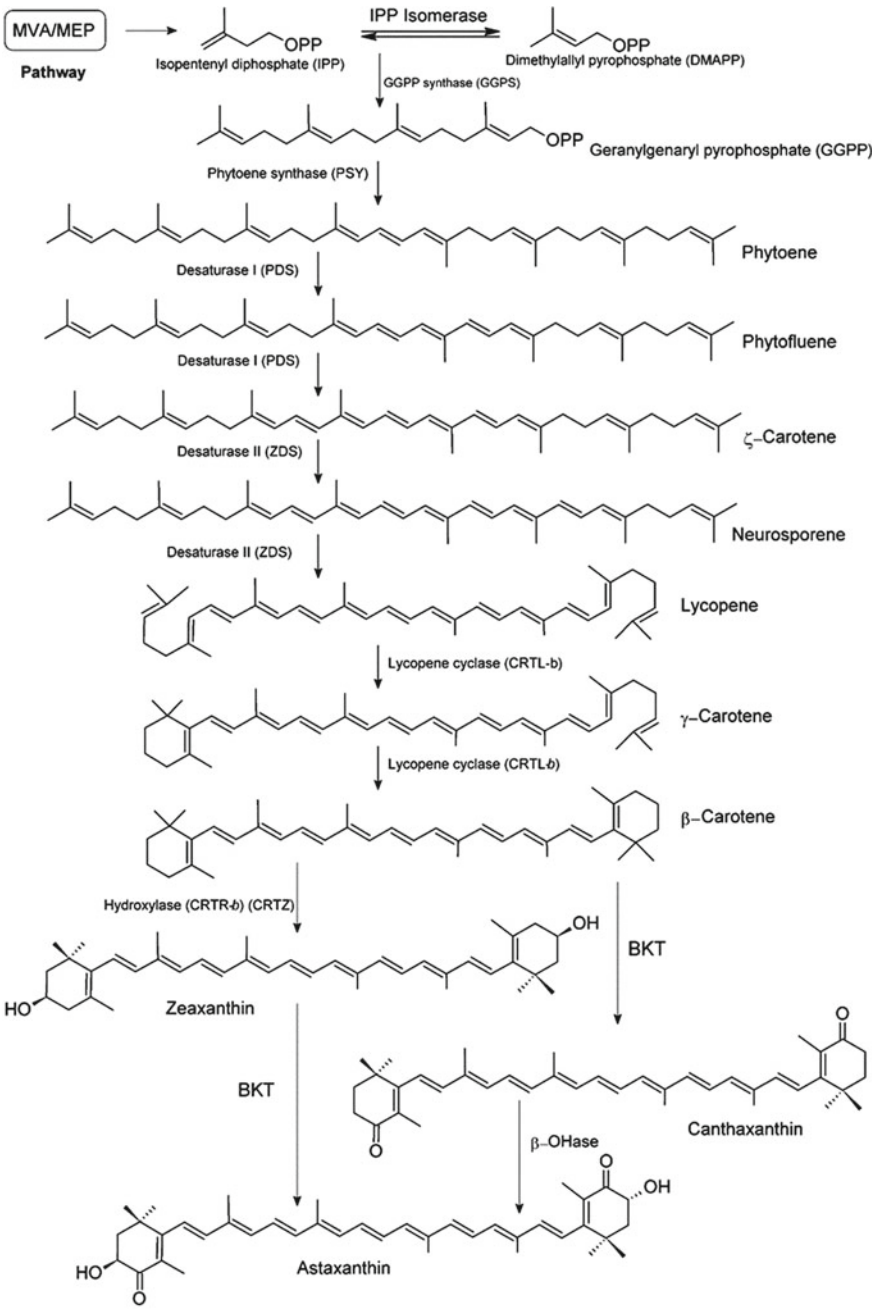

Fig. 15.5 Biosynthesis of carotenoids in microalgae

geranyl pyrophosphate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate, which are the precursors of mono-, di-, and tri-terpenes and carotenoids (Hunter 2007; Lemoine and Schoefs 2010). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are the immediate forerunners of C30 and C40 carotenoids (Lee and Schmidt-Dannert 2002; Lu and Li 2008).

Through the head-to-tail condensation of two geranylgeranyl pyrophosphate molecules, the phytoene (C40) is obtained. In this process, two diphosphate groups are rejected, being catalyzed by phytoene synthase (PSY) (Lemoine and Schoefs 2010). PSY is among the more important enzymes for carotenoid biosynthesis in photosynthetic organisms and can be controlled by environmental stresses (Bertrand 2010; Nisar et al. 2015). The enzymes phytoene desaturase (PDS) and ζ-carotene desaturase catalyze successive desaturation reactions of phytoene, enhancing the number of conjugated carbon–carbon double bonds and ensue in the formation of the first phytofluene, zeta-carotene (ζ-carotene), neurosporene and finally the first colored carotenoid, lycopene is formed (Varela et al. 2015).

The formation of α and β-carotene occurs after two cyclization reactions of biosynthesized lycopene. The α-carotene is transformed in two steps to lutein, while the β-carotene can then be transformed to zeaxanthin by two successive hydroxylation steps (Paniagua-Michel et al. 2012).

Astaxanthin synthesis can occur by the oxidation of β-carotene, having echinenone, canthaxanthin and phoenicoxanthin as intermediates or by the hydroxylation of β-carotene, having β-cryptoxanthin, zeaxanthin and adonixanthin as intermediates (Lemoine and Schoefs 2010).

15.3.2 Microalgae Phycobiliproteins Production

Among commercially important pigments of microalgae, phycobiliproteins stand out not only for their commercial value but also because these pigments are exclusive of some microalgae classes (Cyanophyta, Rhodophyta, Cryptophyta, and Glaucophyta) (Guiry and Guiry 2019). In addition, there are no existing derived counterparts of the synthetic route (Klein and Buchholz 2013).

The initial step of phycobiliproteins biosynthesis is the cleavage of heme macrocycle by the action of heme oxygenase (HO), to afford the first linear molecule biliverdin IXα (BV) (Fig. 15.6) (Dammeyer and Frankenberg-Dinkel 2008). A complete heme oxygenase catalytic cycle requires a total of seven electrons and three molecules of molecular oxygen to yield biliverdin IXα (Wilks 2002).

Then, the enzyme biliverdin reductase (BvdR) reduces the methine bridge between ring B and C of biliverdin IXα (BV) resulting in the formation of bilirubin IXα (BR) in the presence of cofactor NADH and NADPH. Biliverdin reductase (BvdR) displays a high substrate specificity to prevent cross-reactions with other bilin metabolites like phycocyanobilin (PCB) and phycoerythrobilin (PEB) that occur in the same organisms (Sugishima et al. 2005).

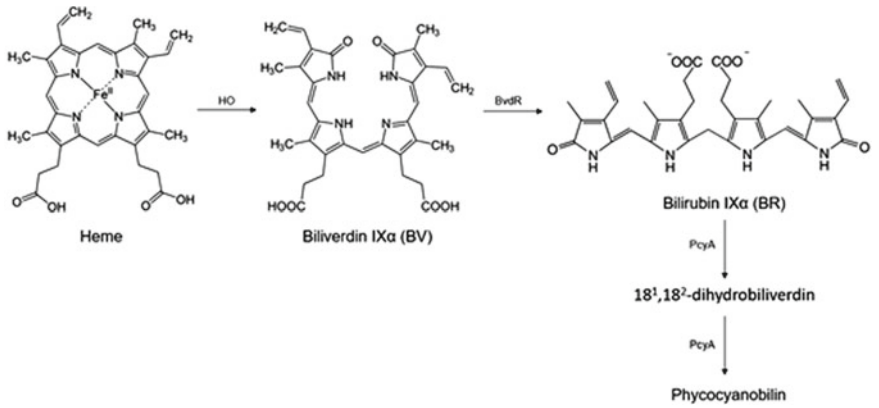

Fig. 15.6 Biosynthesis of phycobiliproteins in microalgae

Finally, the bilirubin IXα (BR) can be reduced either through ferredoxin oxidoreductase (PcyA) to 18^1,18^2-dihydrobiliverdin. By a similar process, 18^1,18^2-dihydrobiliverdin are reduced to phycocyanobilin (Beale and Weinstein 1991). This compound is present in the chromophore of the biliprotein phycocyanin and also in allophycocyanin, and it is covalently linked to the phycobiliproteins by a thioether bond (Pagels et al. 2019).

15.4 β-Carotene from *Dunaliella Salina*

Given the commercially exploited natural sources, the microalgae *Dunaliella salina* is a classical source of β-carotene, producing quantities above 14% dry weight at a selling price of US$790/kg (Acién et al. 2017). This is 10 to 100 times the amount found in carrots, which is the second natural source of this pigment (D'Alessandro and Filho 2016). Currently, there are large *Dunaliella salina* production plants that produce natural β-carotene in the form of suspensions in oil, beadlets, and water-soluble powder for pharmaceutical and nutraceutical applications and dried biomass for use in animal feed (Cezare-Gomes et al. 2019).

The accumulates of massive amounts of β-carotene by halophilic microalgae *Dunaliella salina* is due to unbalanced physiological conditions of the cell, created due to various stress factors such as salinity and the total irradiance received by the cells. The optimum salinity for growth is about 22% of NaCl, whereas the optimum salinity for carotenoid accumulation is greater than 30% NaCl (Borowitzka et al. 1990).

Once prefers to grow in the hypersaline culture medium, the *Dunaliella salina* presents low contamination risk. In this sense, raceway ponds are recommended for large-scale β-carotene production, since these systems imply in culture medium treatment requirements lower (Fig. 15.7a) (Acién et al. 2017).

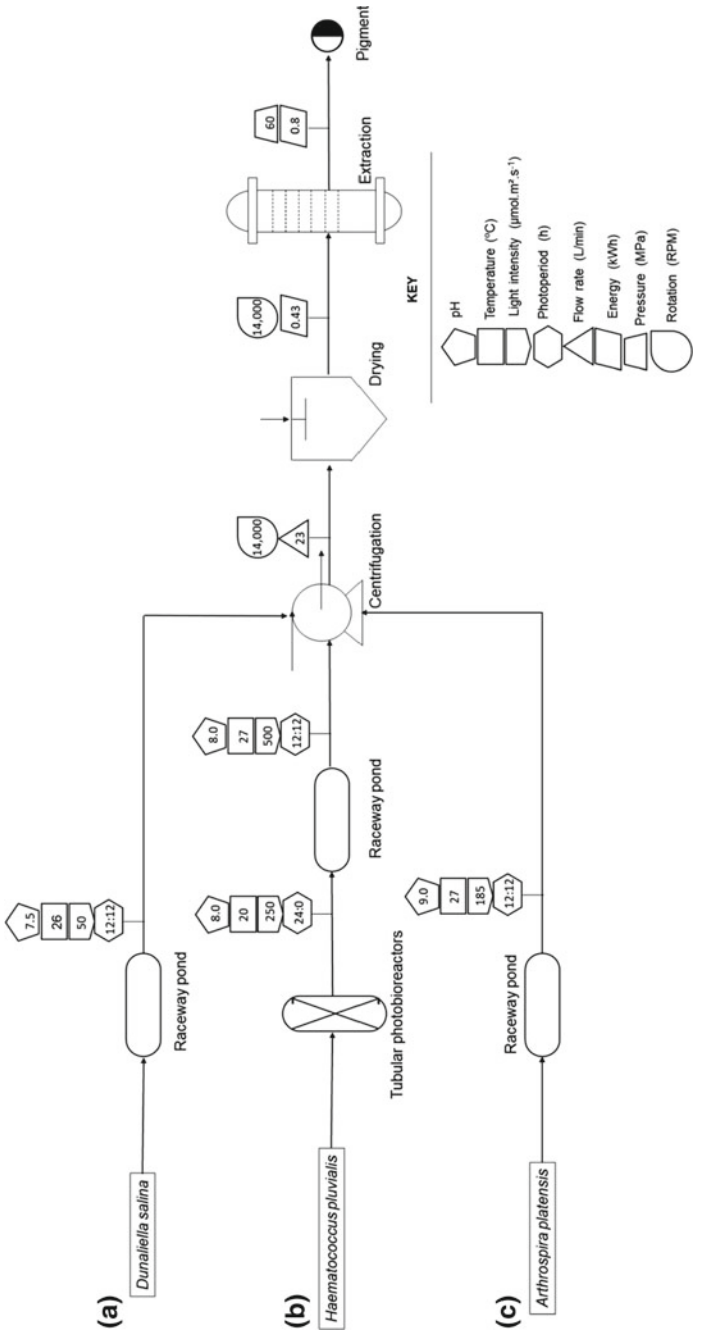

Fig. 15.7 Process flow diagram of the commercial production of pigments through *Dunaliella salina*, *Haematococcus pluvialis*, and *Arthrospira platensis*

15.5 Astaxanthin from *Haematococcus Pluvialis*

The cultivation of *Haematococcus pluvialis* shows lower pigment productivity compared to *Dunaliella salina.* This microorganism can produce and accumulate astaxanthin up to concentrations of 1–8% per dry weight. Once the cost of the final product also depends on productivity, the astaxanthin is produced from *Haematococcus pluvialis* at a selling price of US$2,500/kg (Acién et al. 2017).

Moreover, the cultivation of *Haematococcus pluvialis* is more complex than *Dunaliella salina*, once this microorganism does not accumulate astaxanthin under optimal growth conditions. Thus, commercial producers, they use a two-system strategy for astaxanthin production (Fig. 15.7b). In the growth phase, vegetative cells are produced under optimal growth conditions for a period of 2 days. Once the cultures of *Haematococcus pluvialis* are easily contaminated with other fast-growing strains such as *Scenedesmus* or *Chlorella*, the growth phase is ideally performed in a batch mode using tubular photobioreactors to avoid external contamination. The carotenogenesis phase is performed for a period of 5–10 days, being induced under nutrient deprivation conditions and high irradiance in raceway ponds (Olaizola and Huntley 2003; Acién et al. 2014).

The two-system strategy is successfully used to commercially produce *Haematococcus* in the large-scale (Cezare-Gomes et al. 2019). Notoriously, some parameters require optimization for an optimal economic and environmental performance front these production systems. Thus, by the way, this adopts strategies for the improvement of the production process such as the use of deep ocean water at 14 °C to cool culture systems using heat exchangers, besides sedimentation for the harvesting of the haematocysts (Lorenz and Cysewski 2000).

Over 95% of the astaxanthin available on the market is produced synthetically, whereas astaxanthin from *Haematococcus* comprises <5% of the commercial products (Hu 2019). This is reflex of the relatively lower production costs of synthetic astaxanthin (US$1,000/kg) compared to astaxanthin produced naturally (US$2,500/kg). However, the synthetic astaxanthin is not allowed in human consumption or other human-related applications, making biologically produced astaxanthin the preferred option in these specific markets (Acién et al. 2017).

15.6 Phycocyanin from *Arthrospira Platensis*

Phycocyanin is the subunit most commonly used among phycobiliproteins (Kannaujiya et al. 2019). It is the blue pigment extracted from *Arthrospira platensis* that in 2013 became the first natural blue food coloring to be approved by the FDA. Since then, demand for phycocyanin has increased drastically, particularly in the Americas and Europe, which have seen a rapid shift in consumer demand toward natural food colorings (Buchweitz 2016).

The first and most important application of phycocyanin is as a blue pigment in food and cosmetics, replacing current synthetic pigments (DIC 2019). The phycocyanin was also shown to have therapeutic value, with immunomodulating activity and anticancer activity (Wu et al. 2016). Owing to its fluorescence properties it has gained importance in the development of phycofluor probes for immunodiagnostics (Raja et al. 2016). The price of phycocyanin products depends on the purity ratio, which is defined as the relationship between the absorbance of phycocyanin and protein (A_{620}/A_{280}). The price of food grade phycocyanin (purity > 0.7) is approximately US$ 500/kg, whereas the price of the analytical grade (purity $\geq$ 4.0) can reach US$ 15/mg (Insights 2019).

Arthrospira platensis is cultured in raceway ponds, especially in tropical and subtropical regions, once that your productivity strongly depends on optimal light conditions (Fig. 15.7c). Notably, in open systems, biomass levels reach low levels of dry biomass (0.05 g L^{-1} day^{-1}) and phycocyanin (0.003 g L^{-1} day^{-1}). In addition, such culture systems favor external contamination. Conversely, productivity can be improved through the use of closed systems such as photobioreactors. Studies have shown that, in tubular photobioreactors, the microalgae *Arthrospira platensis* presented values of dry biomass and phycocyanin of 1.32 g L^{-1} day^{-1} and phycocyanin 0.092 g L^{-1} day^{-1}, respectively. However, the scale-up of operation in photobioreactors have not yet been implemented due to its cost-uncompetitiveness (Kannaujiya et al. 2017).

15.7 Life Cycle Assessment of Commercial Pigments

Recent developments and innovations in the current state of the art of pigment production boost the large corporations and consumers, for the search for more efficient alternative systems in front of the environmental footprints. In addressing the environmental sustainability of a process, specific indicators are of interest. In this sense, the establishment of sustainability metrics capable of guiding and motivating actions toward sustainable development is necessary.

A solution to establish sustainability in microalgae-based pigment production is a life cycle assessment. Life cycle assessment (LCA), in definition, is a mathematical tool that allows the quantification of the inputs and outputs of systems, evaluating their environmental performance through a series of impact categories. Besides, this methodology provides a consistent data structure, in order to assist administration agencies and companies for strategic planning, product improvement, and marketing, with an environmental focus.

Nowadays, after 25 years coming, its popularization has expanded worldwide. Ever since, its application to manufacturing strategies for sustainable products has demonstrated high success among the companies.

Structurally, the principles of application of the LCA are directed by the International Standardization Organization (ISO) 14040. This normative establishes four stages, known as the definition of objectives and scope, the development of life cycle

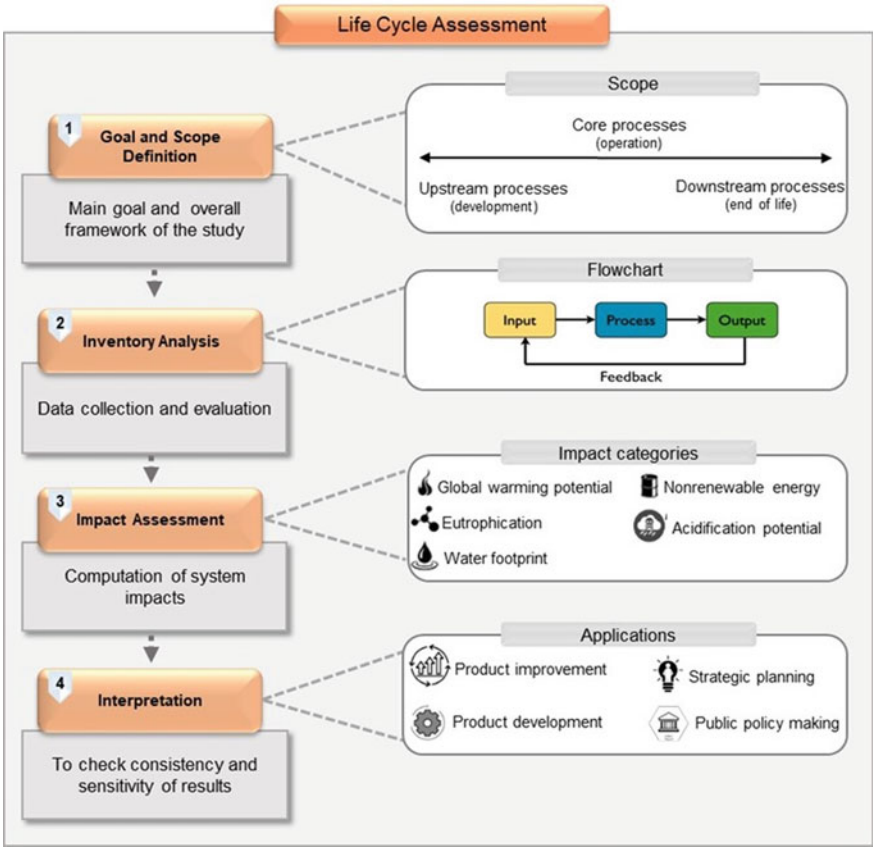

Fig. 15.8 Summary framework for application of life cycle assessment

inventory (LCI), life cycle impact assessment (LCIA), and interpretation (Fig. 15.8). These divisions act as guiding stages for the application of the LCA.

In short, both the scope definition steps and the inventory analysis present critical points of conduct. However, it is known that the greater relevance is attributed to the stage of impact assessment since this stage is aimed at transforming inputs and processes in equivalent indicators. Thus, in view of the need to establish sustainability metrics in the production of the microalgae-based pigment, altogether ten categories of impacts are divided according to their individualities (Table 15.1).

Numerous studies can be found in the literature on the cultivation of microalgae, specifically for the purpose of obtaining renewable energy. However, the field of research on the environmental sustainability assessment of bioactive compounds, such as microalgae-based pigments, to date has not been extensively explored (Table 15.2).

Table 15.1 Categories of impact and their respective principles

Impact categories	Principles of application
Ozone depletion potential	Diminution of the stratospheric ozone layer due to anthropogenic emissions of ozone depleting substances
Photochemical oxidation potential	Type of smog created from the effect of sunlight, heat, and non-methane volatile organic compounds (NMVOC) and NOx
Ecotoxicity	Toxicological responses of different species and nature of the chemical in the ecosystem
Acidification	Reduction of the pH due to the acidifying effects of anthropogenic emissions
Eutrophication	Accumulation of nutrients in aquatic systems
Energy resource	The decrease in the availability of energy resource as a result of their unsustainable use
Water footprint	The decrease in the availability of water as a result of their unsustainable use
Land use	Impact on the land due to agriculture, anthropogenic settlement, and resource extractions
Global warming potential	Alteration of global temperature caused by greenhouse gases
Human toxicity potential	Toxic effects of chemicals on humans

Adapted from ILCD (2010)

In this sense, the objective and scope of the application of this LCA was to evaluate the demand requirements and environmental footprint, in order to reveal the sustainable metrics of the current state of the art of the production of the microalgae-based pigments comparing with conventional synthetic pigments obtention. The functional unit of the 1 kg of pigment was established (Bogacz-Radomska and Harasym 2018; Boussiba et al. 2000; Pagels et al. 2019; Herloff and Ferdinand 1954; Schloemer and Davis 2002).

For the inventory assessment step, the definition of the limits of the systems, as well as the inputs and outputs of the processes for the production of β-carotene and astaxanthin chemically synthesized and their microalgae-based pigment counterparts are presented in Tables 15.3 and 15.4, respectively, and for microalgae-based phycocyanin production is presented in Table 15.5.

Thus, on the impact assessment step, the collected data in the inventory analysis was characterized into corresponding environmental impacts. In this step, were applicated none categories of characterization for the scenarios, being the energy resource, global warming potential, land use, acidification potential, water footprint, eutrophication potential, photochemical ozone creation potential (SMOG), ozone depletion potential and ecotoxicity. The selected impact categories are directly related to the requirements of the process, as they encompass water and energy quantifications. Subsequently, the categories of impacts were normalised according to Sala et al. (2017).

Table 15.2 Studies in literature of life cycle assessment in microalgae-based pigments

Parameters	[1]Scenario 1	[2]Scenario 2	[3]Scenario 3
Microalgae	*Tetraselmis suecica*	*Dunaliella salina*	*Haematococcus pluvialis*
Cultivation system	Closed	Open	Closed
Photobioreactor configuration	Bubble column	Raceway ponds	Tubular photobioreactor
Pigment	β-carotene	β-carotene	Astaxanthin
Unit functional	1 kg	1 kg	1 kg
Abiotic depletion potential (kg Sb eq)	4.71	4.07	716.00
Acidification potential (kg SO_2 eq)	18.30	4.63	447.00
Eutrophication potential (kg PO_4^{-3})	4.67	1.37×10^{-1}	150.00
Global warming potential (kgCO_2 eq)	650.00	4.87×10^{2}	87×10^{3}
Ozone layer depletion potential (mg CFC-11 eq)	65.40	7.32×10^{-5}	5×10^{6}
Photochemical oxidants formation potential (g C_2H_4 eq)	250.00	2.22×10^{-1}	15×10^{3}
Non-renewable fossil (MJ eq)	13.78×10^{3}	–	–

[1]Pérez-López et al. (2014a), [2]Kyriakopoulou et al. (2015), [3]Pérez-López et al. (2014b)

15.7.1 Life Cycle Impact Assessment for Carotenoids

The results of the characterization of environmental impacts of the current production scenarios of synthetic and microalgae-based β-carotene and astaxanthin are shown in Fig. 15.9 and the normalised results are shown in Fig. 15.10.

For energy resource category, the production of the microalgae-based β-carotene and astaxanthin showed average values of 1,836.19 MJ and 6,044.55 MJ of energy input, respectively. In contrast, the values for β-carotene and astaxanthin synthetics were 185.11 MJ and 155.95 MJ of energy input, respectively. For microalgae-based pigments production, the scenarios showed one of the major contributions of energy resource that was assigned to the cultivation step. When compared to the cultivation of *Dunaliella salina* for the production of β-carotene, the operation in two-step for the production of microalgae-based astaxanthin presents high energy consumption. This is due to the fact that tubular photobioreactors used in the first step of cultivation of *Haematococcus pluvialis* require higher energy input, both for the operation of the reactor itself and for artificial illumination. Open systems do not require a high energy supply and use light energy to perform photosynthesis (Jorquera et al. 2010). Still,

Table 15.3 Elementary flows of the process to produce 1 kg of synthetic pigment

Process	Unit	β-carotene	Astaxanthin
Input			
Equipments			
Storage tank (2)	kWh	11.52	5.76
Mixing reactor (1)	kWh	0.06	0.04
Metering pumps (2)	kWh	33.48	34.34
Condenser (1)	kWh	4.26	2.16
Evaporator (1)	kWh	1.05	0.53
Extractor (1)	kWh	1.05	0.53
Chemicals products			
β-ionone	kg	1.06	–
Phosphonium salt	kg	4.80	2.83
Aldehyde	kg	0.34	0.35
Methanol	m^3	–	0.01
Solution of sodium methoxide	kg	–	0.39
Acid acetic	m^3	0.009	–
Petroleum ether	m^3	0.030	–
Ammonia acetate	m^3	0.004	–
Others			
Water	m^3	0.038	0.01
Output			
Pigment	kg	1	1

Source Scholoemer and Davis (2002), Herloff and Ferdinand (1954)

in the processes for obtaining microalgae-based pigments, the drying and extraction steps represent about 80% of the total energy required. Conversely, in synthetic production, most of the energy is attributed to the mixing and cooling steps. In addition, the energy requirements are also responsible for the environmental loads related to greenhouse gas, since for each 1kWh it is equivalent to 0.545 kgCO_2.

Global warming potential is an indicator of greenhouse gas generated. Thus, the production of the microalgae-based β-carotene and astaxanthin showed average values of 852.49 and 2,804.52 kg CO_{2eq}, respectively. In contrast, the values for β-carotene and astaxanthin synthetics were 118.22 and 36.62 kg CO_{2eq}, respectively. At the level of comparison among scenarios, the microalgae-based pigment production process showed higher values when compared to the synthetic processes. The consumption of energy resources is accounted for most of the GHG emissions in the microalgae-based production scenarios since it involves a more complex process than chemical synthesis. In the synthetic routes, the main factor associated with the GHG emissions is referring to the use of chemical reagents that are inevitable in these technological routes. At the level of comparison, among the microalgae-based pigment

Table 15.4 Elementary flows of the process to produce 1 kg of microalgae-based pigment

Process	Unit	β-carotene	Astaxanthin
Cultivation			
Input			
Nutrients enrichment			
Phosphate	kg	0.22	0.66
Nitrate	kg	0.32	0.96
Tubular photobioreactor	m^3	–	3.0
Power consumption for cooling	kWh	–	45
Aeration power	kWh	–	3.0
CO_2 consumption	kg	–	1.5
Water evaporation	m^3	–	0.006
Raceway pond	m^3	27.77	80.0
Electric power for paddle wheel	kW	15.0	43.2
Electric power for water pumping	kW	2.77	8.0
Electric power for CO_2 injection	kW	9.21	26.56
CO_2 consumption	kg	2.0	2.0
Biomass productivity	kg/m^3	0.45	0.4
Output			
Water evaporation	m^3	3.61	10.40
Algae liquid	kg	83.3	222.2
Harvest			
Input			
Power consumption centrifugation	kWh	56.33	168.40
Drying			
Input			
Wet biomass	kg	49.98	133.32
Spray-dryer	kWh	21.49	57.32
Extraction			
Input			
Dry biomass	kg	12.5	33.33
sCO_2	kWh	10.0	26.66
Output			
Oil rich pigments	kg	1	1

Source Bogacz-Radomska and Harasym (2018), Boussiba et al. (2000)

Table 15.5 Elementary flows of the process to produce 1 kg of microalgae-based phycocyanin

Process	Unit	Phycocyanin
Cultivation		
Input		
Nutrients enrichment		
Phosphate	kg	0.33
Nitrate	kg	0.48
Tubular photobioreactor	m^3	–
Power consumption for cooling	kWh	–
Aeration power	kWh	–
CO_2 consumption	kg	–
Water evaporation	m^3	–
Raceway pond	m^3	41.66
Electric power for paddle wheel	kW	22.50
Electric power for water pumping	kW	4.16
Electric power for CO_2 injection	kW	13.83
CO_2 consumption	kg	2.0
Biomass productivity	kg/m^3	0.32
Output		
Water evaporation	m^3	5.41
Algae liquid	kg	71.09
Harvest		
Input		
Power consumption centrifugation	kWh	84.52
Drying		
Input		
Wet biomass	kg	53.31
Spray-dryer	kWh	22.92
Extraction		
Input		
Dry biomass	kg	13.33
sCO_2	kWh	10.66
Output		
Oil rich pigments	kg	1

Source Pagels et al. (2019)

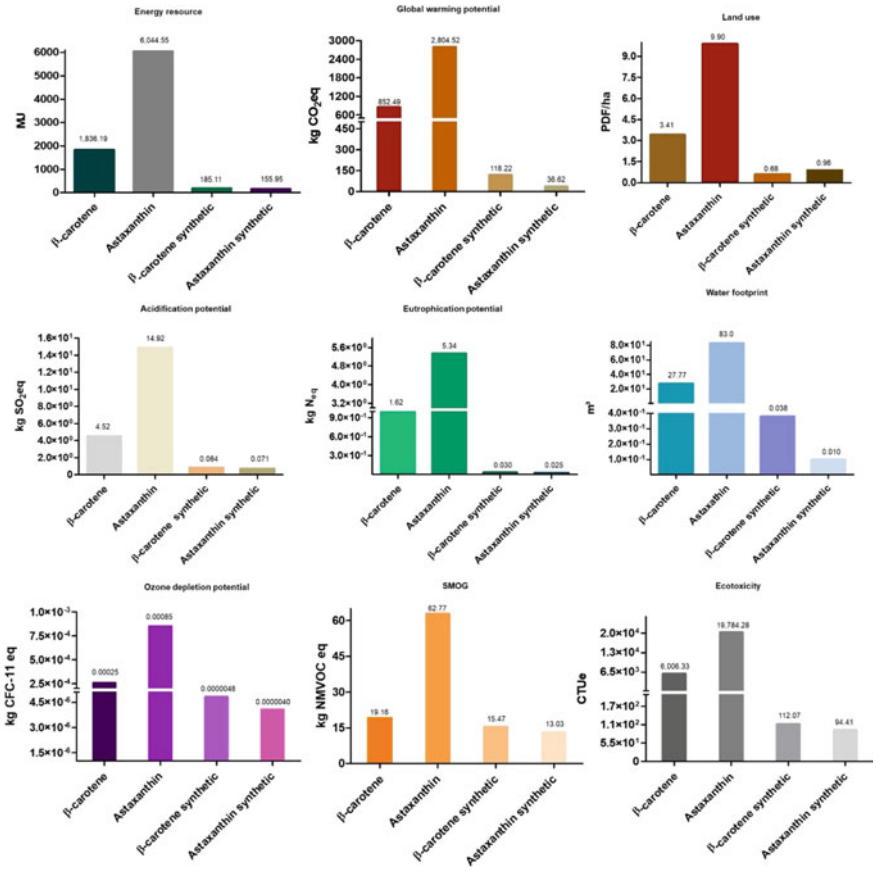

Fig. 15.9 Results of the impact assessment (characterization stage) associated with the industrial production of synthetic and microalgae-based β-carotene and astaxanthin

production scenarios, the microalgae-based astaxanthin showed higher values when compared to the microalgae-based β-carotene. The results are related to the consumption of fossil energy resources for the cultivation in two-stage of *Haematococcus pluvialis.*

Land use and land cover are considered primary factors affecting ecosystems. Thus, among the identified impacts, the use of land occupation by the potentially missing fraction of species was evaluated. Therefore, for the microalgae-based pigment production scenarios, values on the order of 3.41 PDF/ha for β-carotene and 9.90 PDF/ha for astaxanthin were found. The highest values for astaxanthin production are attributed to the great land use required for the cultivation of *Haematococcus pluvialis* in a two-step system. In comparison with synthetic production scenarios, lower values were found, being 0.68 PDF/ha for β-carotene and 0.96 PDF/ha for astaxanthin. The process of synthetic production of pigments involves

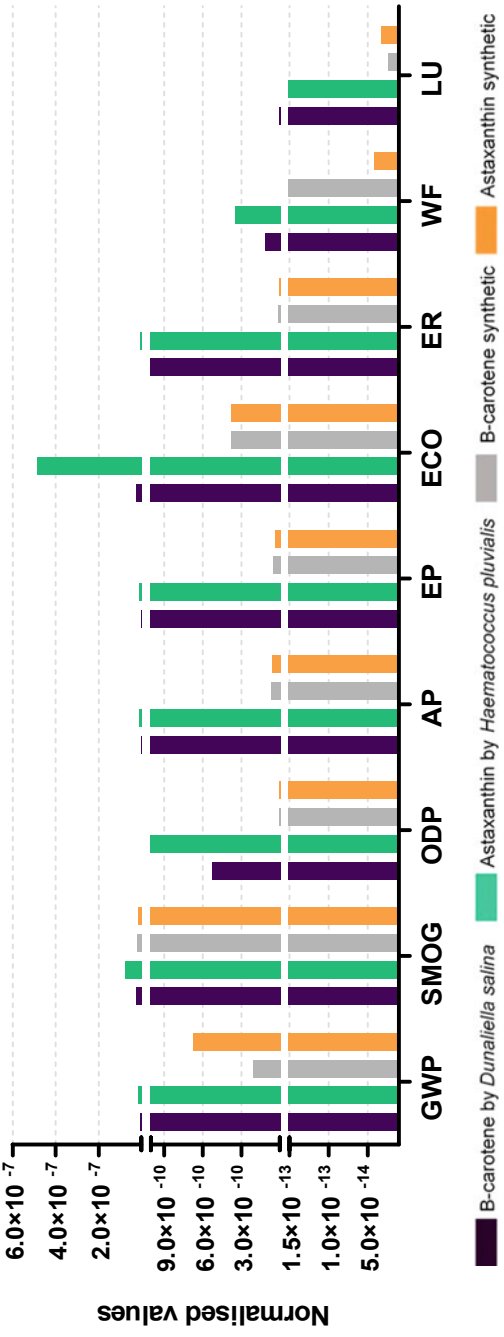

Fig. 15.10 Normalised results of impact assessment related to the industrial production of synthetic and microalgae-based β-carotene and astaxanthin

more compact and optimized equipments, which reflects in a processing plant with less occupation of land use and with lower spending on electricity.

Terrestrial acidification is an indicator related to emissions of air pollutants that result in acid rain (Reuss and Johnson 2012). Thus, for microalgae-based β-carotene and astaxanthin, the values found were in the order 4.52 and 14.92 kg SO_{2eq}, respectively. On the other hand, to obtain β-carotene and astaxanthin synthetics the values found were in the order of 0.084 kg SO_{2eq} and 0.071 kg SO_{2eq}, respectively. The highest results for the production of the microalgae-based pigment have related to the greater fossil energy resources for the operation of the production plant. Fossil fuels consumption is found to be the largest contributing parameter for gaseous pollutants emissions in industrial processes; once only 20–30% of the chemical energy of the fuel burned is typically transformed into useful work or heating, the rest being dissipated into the atmosphere as polluting gases (Bilgen et al. 2014). In this sense, in order to reduce the acidification potential of microalgae-based processes, it is necessary to increase the energy efficiency of these technological routes.

The eutrophication potential represents the contribution of the nutrients as phosphates and nitrates to ecosystems in river and lakes. In the present study, were found values in order to 1.62 and 5.34 kg N_{eq} for microalgae-based β-carotene and astaxanthin, respectively. However, for β-carotene and astaxanthin synthetics, the emissions of eutrophication potential were next to zero, with values of 0.030 and 0.025 kg N_{eq}, respectively. These values are related to the low fossil energy consumption summed to the high yields of chemical reagents in pigments of the processing plant of synthetic pigments.

The water footprint is a metric that counts the direct and indirect use of water as well as pollution. Given this aspect, the water footprints were quantified and 27.77 m^3 for the production of microalgae-based β-carotene was required. While for production of microalgae-based astaxanthin, 83.0 m^3 was required. In contrast, in synthetic production, the values for β-carotene and astaxanthin were 0.03 m^3 and 0.01 m^3, respectively. Water use represents a challenge of particular significance for commercial microalgae cultivation. According to Guieysse et al. (2013), more than 1 metric ton of process water must be handled for each kg of microalgae biomass produced; being able to suffer from variability arising from process operation. This variability is evident in the comparison among the microalgae-based pigment production scenarios; the microalgae-based astaxanthin showed higher values when compared to the microalgae-based β-carotene. The results are related to the water used for two-system operation on the cultivation of *Haematococcus pluvialis*. Some companies adopt strategies for the improvement of the microalgae-based pigments production processes such as using ocean water as a water source (Lorenz and Cysewski 2000). According to Yang et al. (2011) using seawater or wastewater can reduce the life cycle freshwater usage by as much as 90%.

The ozone depletion potential (ODP) is an impact category related to emissions of air pollutants that result in degradation to the ozone layer. Thus, the production of the microalgae-based β-carotene and astaxanthin showed average values of 0.00025 and 0.00085 kg CFC-11 eq, respectively. Comparatively, were determined for synthetic production of β-carotene and astaxanthin, values in the order of 0.0000048 and

0.0000040 kg CFC-11 eq, respectively. The photochemical creation potential or SMOG is often used in conjunction with compounds of ozone depletion potential (ODP) as a measure of how environmentally detrimental it can be. SMOG represents the potential of a substance to contribute to photochemical ozone creation. Thus, for the production of β-carotene and astaxanthin by microalgae-based processes, the mean values were 19.16 and 62.77 kg $NMVOC_{eq}$ respectively. On the other hand, the synthetic production of β-carotene presented 15.47 kg $NMVOC_{eq}$, while for astaxanthin the values were in the order of 13.03 kg $NMVOC_{eq}$. The results of ODP and SMOG are related to the input of fossil energy resources for the operation of the processing plant of synthetic and microalgae-based pigments.

Ecotoxicity freshwater is estimates of toxic effects on the ecological system caused by natural or synthetic pollutants. In this case, for the production of β-carotene and astaxanthin by microalgae, the values were in order to 6,006.36 and 19,784.28 CTUe, respectively. In comparison, synthetic β-carotene presented mean values of 112.07 CTUe and synthetic astaxanthin of 94.41CTUe. In both scenarios, the input of fossil energy resources was a determining factor for the high values found. However, for the scenarios of obtaining natural pigments from microalgae, the uses of sodium bicarbonate and nitrates were main factors that caused higher ecotoxicity. Furthermore, it should be considered that the effects on the ecosystems are related to the densities, concentrations, exposure, background/local conditions, and thresholds, and it is difficult to assess if CTU could cause significant environmental impacts.

Finally, in brief, the categories of ozone depletion, photochemical creation (SMOG), and ecotoxicity freshwater potential were dominated by electricity in all cases. These impacts can be reduced by using sustainable engineering and design practices by optimizing process platform construction for the intended purpose as well as sustainable energy systems (Harris et al. 2019).

The normalised results indicate that the highest pollutants emissions are related to categories of the photochemical ozone creation, ecotoxicity freshwater, and energy resource potentials for all scenarios (Fig. 15.10). These emissions are related to the indirect effects of fossil energy consumption. According to Gao et al. (2019), the total of pollutants emitted per unit of energy produced by fossil fuels is typically two orders of magnitude bigger than those emitted by non-fossil energy.

Therefore, the transition from fossil energy matrix to non-fossil energy (including nuclear and renewable energy) has become an urgent issue for all countries; once that these energies can supply two-thirds of the total global energy demand, and contribute to the bulk of the greenhouse gas emissions reduction (Gielen et al. 2019). Policies and incentives of governments improve the prospects for clean energy. Total generation from renewable resources increases by 2.8% annually, and the renewable share of world electricity generation increases from 21% in 2010 to 25% in 2040 (IEA 2013). Although it is reasonable to expect that renewables will come to provide a growing share of the global energy supply, it should be noted that the replacing fossil energy with non-fossil fuels remains challenging, especially for future global policy (Cheng et al. 2019).

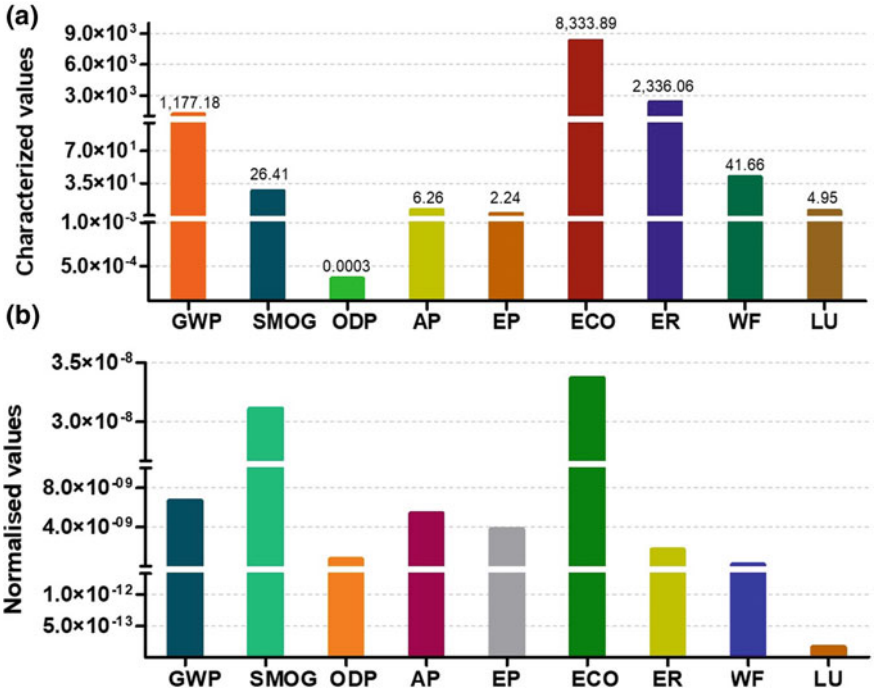

Fig. 15.11 Characterization (a) and normalization (**b**) values of the process of obtention of the microalgae-based phycocyanin

15.7.2 Life Cycle Impact Assessment for Phycobiliproteins

Life cycle assessment was restricted to the microalgae-based phycocyanin, since this is the subunit most commercially important among phycobiliproteins. The characterization and normalized results of environmental impacts for the metrics of production of phycocyanin from *Arthrospira platensis* are presented in Fig. 15.11.

The characterization of impact categories for the production of 1 kg of microalgae-based phycocyanin results in 1,177.18 kg CO_{2eq} of global warming potential, 26.41 kg $NMVOC_{eq}$ of SMOG, 0.0003 kg CFC-11 $_{eq}$ of ozone depletion potential, 6.26 kg SO_{2eq} of terrestrial acidification, 2.24 kg N_{eq} of eutrophication potential, 8,333.89 CTUe of ecotoxicity, 2,336.06 MJ of consumption of energy resource, 41.66 m^3 of water footprint, and 4.95 PDF/ha of land use. The normalized results indicate that the highest pollutants emissions are related to categories of the SMOG and ecotoxicity potentials (Fig. 15.11b).

The environmental impacts results for the production of microalgae-based phycocyanin corroborate with the results of the microalgae-based carotenoid production since they share similar production facilities. In this sense, regardless of the pigment synthesized biologically, new engineering approaches to this type of process must be

implemented, so that in the near future the microalgae-based pigments have lower environmental impacts compared to synthetic pigments.

15.8 Final Considerations and Future Prospects

The ability to naturally synthesize pigments with properties relevant to health makes microalgae as a source potential for exploitation in the natural food colorants industry. However, regardless of these potentialities, the establishment of life cycle analysis has shown the weaknesses of these systems front the competition with the consolidated chemical synthesis of pigments, based on fossil and non-renewable inputs. In this way, new approaches to process engineering must be oriented toward the development of economic and environmentally sustainable of the microalgae-based processes. Process integration, process intensification, and the implementation of the biorefinery concept have been considered as the main process engineering strategies that, in the medium term, will consolidate processes and products based on microalgae.

The process integration mediated by microalgae is an appropriate and innovative approach to complying with green engineering requirements, through the recovery and reuse of surpluses energy, mass, water, and effluent from industrial processes. Thus, different mass integration methodologies can be implemented to mitigate the effects of economic and environmental impacts. This can be reflected in the use of seawater/wastewater as an efficient way to contribute nutrients to cultures, with parallel reuse of water, either directly or partially treated for use in other processes. Additionally, the use of gaseous effluent streams from industrial combustion processes, rich in CO_2 and NOx, allowing the input of inorganic carbon and nitrogen (N_2) in microalgae photosynthetic cultures. Since the microalgae can also be grown heterotrophically, the wastewater integration allows the supply of essential nutrients, such as organic carbon, nitrogen, and phosphorus, which are efficiently assimilated by these microorganisms. These bioprocesses offer a pathway for biomass production using low-cost substrates and reducing the carbon and water footprint. However, so that the different process integration strategies are developed in practice, and it is necessary to couple a microalgae-based process in other industrial facilities already established with the aim of reducing operating expenses.

In addition, the process intensification is related to the overall increase in the efficiency of the production process, by dramatically reducing inventory through smaller equipment to improve reactor/yield, minimizing feedstock, energy consumption, and waste generation. This progress may result in the extinction of some traditional unit operations and even entire processes. Microalgal biomolecules, in most cases present at low concentrations, only will be commercially viable through of extraction techniques based on process intensification.

Finally, the chemical constitution of microalgal biomass has a potential of exploration of multiple products, besides feed and foods, such as fertilizers, fuels, and bulk chemicals. The full exploration of this bioresource, however, requires a refinery

approach, in which different operations of pre-treatments, thermo-chemical, biological and catalytic processes in addition to separation operations should be used to obtain products and co-products, allowing the effective use of resources.

These approaches are still in its infancy, with the large majority of studies being conducted at laboratory scale and few at pilot scale. Perhaps, the greatest challenge is the understanding of material flows and their value in biological systems and biopr–ocesses, for the expansion of these circuits based on the modern bioeconomy aspirations.

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Chapter 16
Chlorophylls as Food Additives

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Abstract There is a growing consensus in the world food industry that aims at replacing synthetic ingredients by natural ingredients. In this respect, the industrial relevance of microalgae as sources of a broad spectrum of bioproducts and as promising feedstocks for natural additives production systems is constantly increasing. Among the numerous chemical specialties present in the constitution of the biomass of these microorganisms, chlorophylls molecules, and their derivatives are emerging compounds in research and development to achieve greater commercial exploitation. Although there is a broad application of chlorophylls molecules, its strong green colour is gaining importance as food colouring. Consequently, microalgae as production systems for obtaining natural chlorophylls and derived compounds, they are highly sustainable sources, consisting of a series of unique, including chlorophylls *c*, *d,* and *f* with remarkable biological properties and relevant technological characteristics. In this sense, the present chapter describes the characteristic structures of chlorophylls and their derivatives, distribution, including aspects related the biological properties of these compounds. Finally, it presents a comprehensive overview of its participation in the food industry and the current legal regulations of different countries for its application in foodstuffs.

Keywords Chlorophyll compounds · Microalgae · Green colourant · Food colouring

16.1 Introduction

In recent decades, the changes in dietary habits and the modification of nutritional demands promoted to considerable alterations in food formulation, directing the market trends, and consequently the industrial interest for foods with the addition of natural additives in replacement and/or reduction of artificial additives (Martins et al. 2019).

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The natural colourants of food are estimated as the largest segment in the food colouring market, representing more than 80% of the total revenue of this sector. Thus, the global food colours market was set at USD 1.79 billion in 2016, with revenue growth estimated at USD 2.97 billion by 2025 (Grand View Research 2019).

In this context, to meet these requirements, the global microalgae market has emerged as a consistent alternative to gain market share in the food additive segment (Molino et al. 2018). Currently, the largest field of application of microalgae is in the food sector, where proteins, carotenoids, phycobiliproteins, and fatty acids are the products that stand out at the level of commercialization (Khan et al. 2018; Jacob-Lopes et al. 2019). However, recently, a growing proportion of promising microalgae research focuses on the production of pigments, including chlorophylls class, since these bioactive compounds are present in these microorganisms in abundance and with wide structural diversity (Begum et al. 2016; Solymosi and Mysliwa-Kurdziel 2016; Fernandes et al. 2017; Dufossé 2018).

Chlorophylls are fundamental molecules of life and probably the most important and widely distributed of all natural pigments, responsible for conferring several green hues (Sigurdson et al. 2017). Constitute a diversified group of molecules, with structures similar to each other, with five species characterized as chlorophyll *a*, *b*, *c*, *d,* and *f* (Scheer 2013; Roca et al. 2016). Conversely, one of the most significant chemical aspects of the structure of chlorophylls is their degree of instability, being susceptible to several transformations in their chemical structure in the presence of enzymes, acids, oxygen, light, and heat, giving rise to several derivative compounds. Accordingly, may present modifications in their oxidation state or degree of saturation of the aromatic system of the macrocyclic, associated with different transition metals (such as copper and zinc complexes replacing the central magnesium), epimerization in the isocyclic rings, as well the changes in the side chain substituents of these structures (Senge et al. 2014; Roca et al. 2016). Thus, are formed their respective breakdown metabolites like pheophytins, chlorophyllides, chlorophyllins, and pheophorbides, which have to present some small differences in their chemistry stability, their absorption spectra, and consequently in their tonality and bioactive potential (Pérez-Gálvez et al. 2017).

Although chlorophylls and their compounds derivative have a broad wide range of technological applications, such as in the monitoring of agricultural production and primary productivity of the oceans and use as nutraceutical and pharmaceutical area, the main economic attribute of these molecules today is their application as the food colourant. Accordingly, these molecules, deepen, or renew the food colour if it has been lost in the course of technological processing (Martins et al. 2019; Vieira et al. 2019).

Hence, your application as food colourant it is not only related to its colour attribute but their potential of the health benefits associated with their unique individual photochemical properties (Solymosi and Mysliwa-Kurdziel 2016; Pérez-Gálvez et al. 2017). Epidemiological, in cell, animal, and human intervention studies (Ferruzzi and Blaskelee 2007; Abd-Elhakim et al. 2018; Vieira et al. 2018) are in increasing constant development in order to broaden the knowledge about potential

health benefits for humans, such as antioxidant, antimutagenic, antigenotoxic, and anti-inflammatory activities (Pérez-Gálvez et al. 2017).

Consequently, the use of these molecules as an additive of colour in food is strictly regulated in the United States, the European Union, and many other countries in the world, such as China, India, Canada, Australia, New Zealand, Brazil, and Japan. Currently, commercially available chlorophyll compounds consist of structures basically related to chlorophylls *a* and *b* (Rodriguez-Amaya 2019), being emerging the exploration of no explored structures as species *c*, *d,* and *f* and their derivatives as alternatives to natural green dyes. Additionally, as a colour stabilization strategy, microalgae biomass appears to be a potential alternative as a functional ingredient with green colouring properties as secondary effect.

Thus, the purpose of this chapter is to provide a comprehensive overview of the application of chlorophylls and their compounds derived in foods, with emphasis on microalgae as potential sources for obtaining these compounds. Moreover, it is revised chemical structure of chlorophylls, distribution, biological properties of these compounds, including regulatory milestones in force in different countries for your application.

16.2 Structure and Distribution Chlorophylls

Natural chlorophylls are the most abundant pigments in nature, occurring more than 100 different structures, found in oxygenic photosynthetic organisms such as higher plants, microalgae, algae, and some bacteria (Lanfer-Marquez and Borrmann 2009; Pérez-Gálvez et al. 2017).

The structure of chlorophylls is tetrapyrrole with a system of coplanar conjugated double bonds that forms the chromophore with delocalization of electron density in π orbitals, formed from one magnesium atom as the central metal and a phytyl chain ($C_{20}H_{40}$) esterifying the propionic acid at $C17_3$, gives chlorophyll a hydrophobic character, as shown in Fig. 16.1 (Yilmaz and Gökmen 2016).

Chlorophylls *a*, *b,* and *c* were reported in the nineteenth century (Govindjee 2004), chlorophyll *d* was identified more than 70 years after the other chlorophylls (Manning and Strain 1943), and chlorophyll *f* was reported in 67 years after (Chen et al. 2010). However, the major chlorophylls in food additives are chlorophyll *a* and chlorophyll *b* (Viera et al. 2019).

Chlorophyll *a* (Fig. 16.2a, b) are different at position C_7, where chlorophyll *a* is composed of methyl group and chlorophyll *b* is composed of formyl group. The chlorophylls *d* and f are similar to chlorophyll *a*, where chlorophyll *d* (Fig. 16.2f) exhibits a formyl group at C_3 meanwhile chlorophyll *f* (Fig. 16.2g) contains a formyl group at C_2 (Chen and Blankenship 2011).

The members of the chlorophyll *c* family differ from other Chls in that they are porphyrins (i.e. they have a fully unsaturated tetrapyrrole macrocycle), which usually are non-esterified with phytol at $C17^3$. Since 1990, the number of members of the chlorophyll *c* family increased from seven (c_1, c_2, c_3, $c_{CS\text{-}170}$, c_2-like pigment

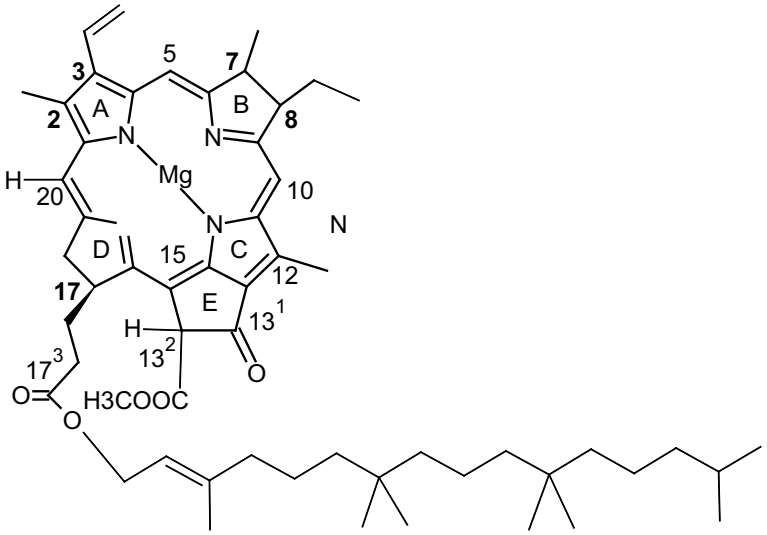


Fig. 16.1 Structure of chlorophyll *a* (a) including the IUPAC-IUB carbon-atom and rings numbering system

from Pavlova gyrans, [DV]-Pchlorophyllides, and a non-polar chlorophyll *c*-like pigment) to eleven compounds (Zapata et al. 2006). However, the most reported in the literature are the Chl c_1 (Fig. 16.2c) carries a characteristic ethyl group at C8, Chl c_2 a vinyl group at C8 (Fig. 16.2d), and Chl c_3 show of a methoxycarbonyl group at C7 (Fig. 16.2e) (Zapata et al. 2006).

Independently of the chlorophyll series (*a*, *b, c*, *d,* or *f*) are relatively unstable, the degradation of these compounds is a combination of enzymatic activity and chemical changes, converting as expected, chlorophylls into pheophytins, chlorophyllides, and pheophorbide (Lanfer-Marquez and Sinnecker 2008; Pérez-Gálvez et al. 2018).

The pheophytinization reaction is the substitution of the central magnesium atom of the tetrapyrrole by two hydrogen atoms. When this reaction starts from chlorophylls, it generates pheophytins; when starting from chlorophyllides, it generates pheophorbides. The de-esterification reaction is the hydrolysis of the phytol chain, chlorophyllides are produced if the reaction starts with chlorophyll, and the process yields pheophorbides if the dephytylation starts from pheophytin (Schwartz and Lorenzo 1990; Roca et al. 2016; Pérez-Gálvez et al. 2017).

In nature, higher plants contain only chlorophylls *a* and *b* and their respective catabolites like pheophytins, chlorophyllides, and pheophorbides. In addition to chlorophylls *a* and *b*, and chlorophylls *c*, *d,* and *f* show a wide occurring in aquatic photosynthetic organisms (Table 16.1), the currently known distribution patterns of chlorophylls pigments in bioresources are shown in Fig. 16.3 (Hendry 2000; Lanfer-Marquez and Borrmann 2009).

About the polar chlorophylls *c*, an overview of Chl *c* shows a major distribution in microalgae such as diatoms and brown algae. The Chl c_2 was considered the universal

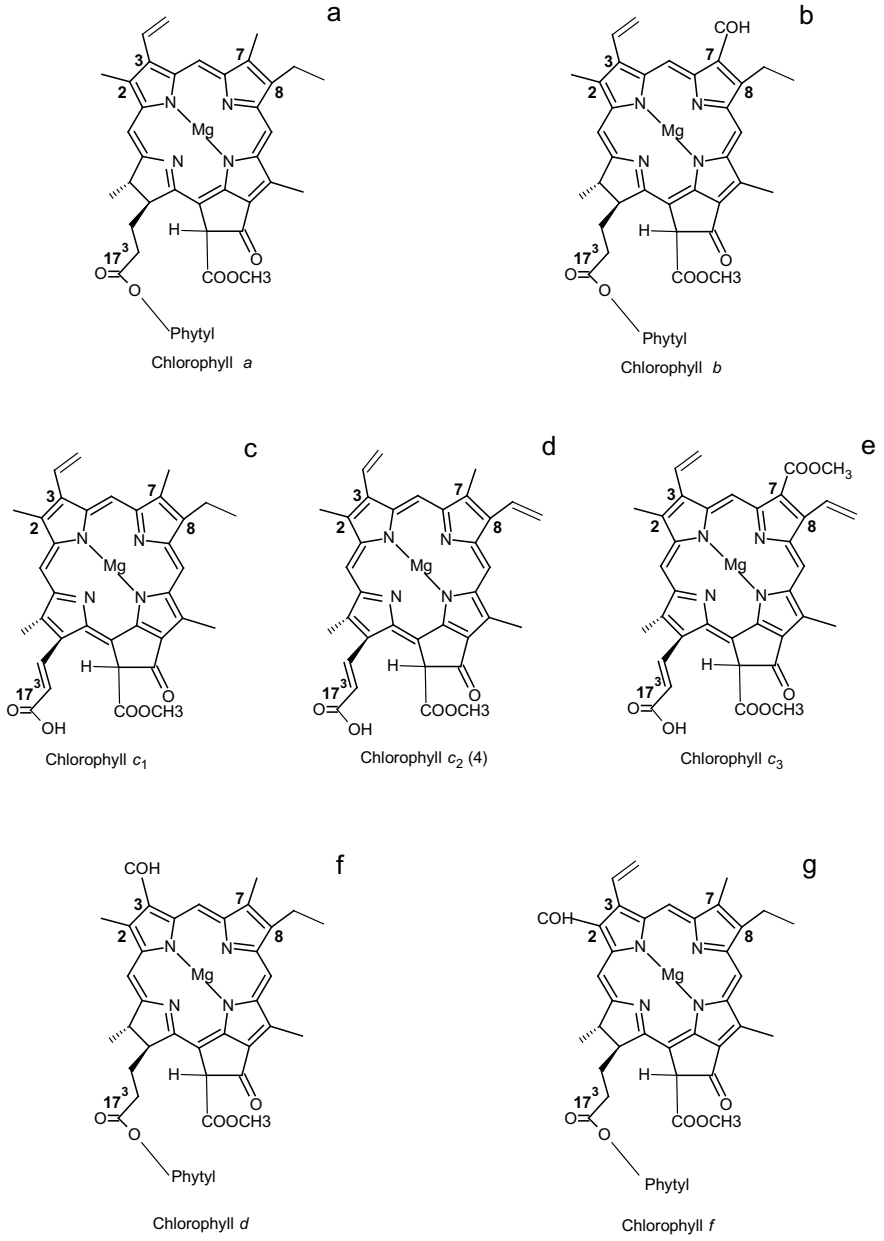


Fig. 16.2 Structures of chlorophylls *a* (**a**), *b* (**b**), c_1 (**c**), c_2 (**d**), c_3 (**e**), *d* (**f**), and *f* (**g**) including the IUPAC-IUB carbon-atom numbering system

Table 16.1 Possible phylum, classes, and species

Phylum	Macroalgae	Microalgae	Classes	Species
Rhodophyta	+	+	8	7,250
Chlorophyta	+	+	12	6,626
Charophyta	+	–	6	4,782
Dinophyta	–	+	5	3,560
Haptophyta	–	+	3	759
Euglenophyta	–	+	8	1,493
Glaucophyta	–	+	1	25
Cryptophyta	–	+	2	219
Ochrophyta	+	+	16	4,140
Cyanophyta	–	+	1	4,663
Total			67	33,511

According to Guiry and Guiry (2019): www.algaebase.org

component, since only one algae were found with Chl c_1, usually, Chl c_3 replaced Chl c_1, but the three pigments Chls c_1, c_2, and c_3 were found together in the species of haptophytes and one diatom (Stauber and Jeffrey 1988; Zapata et al. 2006).

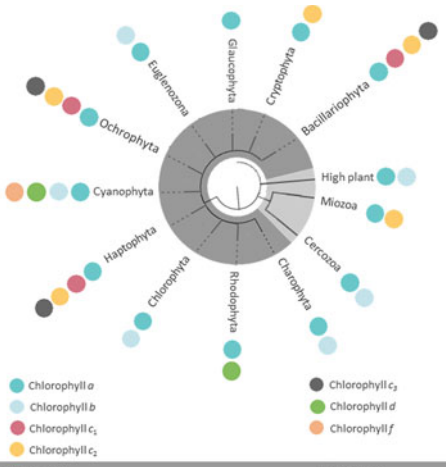
The main source of chlorophyll *d* is cyanobacterium *Acaryochloris marina*, that contains around 95% of chlorophyll *d* and only traces of chlorophyll *a*, and recently new chlorophyll *f*, was discovered in a filamentous cyanobacterium, *Halomicronema hongdechloris*, however, the proportion of Chl *f* is only 10–15% (Chen and Blankenship 2011; Zapata et al. 2006; Chen et al. 2010; Airs et al. 2014; Zepka et al. 2019).

The structural configuration of a molecule determines its mode of chemical action. In this way, it is important to know the chemical structure of chlorophylls to relate to their biological activities (Pérez-Gálvez et al. 2018).

16.3 Chlorophylls as Food Additive

With the increasing trend of the use of natural food additives, chlorophylls, and their derived compounds obtained from biological sources, is a reality that which has been gaining prominence and becoming popular in at present, as alternatives to the usual artificial chemical additives (Gárcia et al. 2017). These emerging changes are fundamentally justified about undesired and potential toxicological effects associated with artificial additives, responsible for allergic reactions, hyperactivity, and even bad taste (Oplatowska-Stachowiak and Elliott 2015).

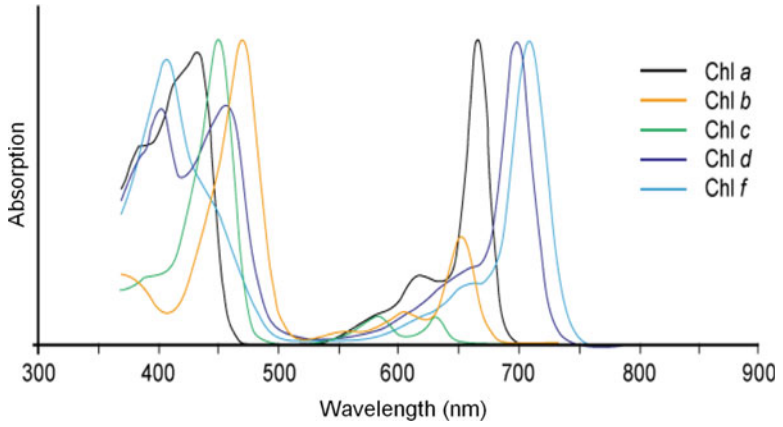
According to the *Codex Alimentarius* (FAO/WHO 2019) food additives are any substance not typically consumed as a food by itself and not normally used as an ingredient in the food but is intentionally added in the manufacture, preparation,



Representative species	Reference
Cyanophyta	
Acaryochloris marina	Miyashita, 1996; Miyashita et al., 1997; Chen et al., 2010
Rhodophyta	
Erythrophyllum delesserioides	Manning and Strain, 1943
Jania rubens	Chen et al., 2017
Chlorophyta	
Ulva spp.	Chen et al., 2017
Dunaliella Salina	El-Baz et al., 2017
Charophyta	
Chara aspera C.L.Willdenow	Blindow et al., 2003
Chara canescens	Kuster et al., 2005
Haptophyta	
Prymnesium parvum	Zapata et al., 2006
Euglenozona	
Euglena gracilis	Tolivia et al., 2013; Koziol et al., 2007
Eutreptiella gymnastica	Bjørnland, 1981
Glaucophyta	
Glaucocystis nostochinearum	Champman, 1966
Cyanophora paradoxa	Champman, 1966
Cryptophyta	
Rhodomonas báltica	Zapata et al., 2000
Ochrophyta	
Pulvinaria sp.	Zapata et al., 2006
Bacillariophyta	
Navicula jeffreyi	Zapata et al., 2006
Miozoa	
Alexandrium minutum	Zapata et al., 2000
Alexandrium tamarense	Leong and Taguchi, 2004
Cercozoa	
Bigelowiella natans	Koziol et al., 2007
Chlorarachnion reptans	Hibberd and Norris, 1984
High plant	
Lactuca sativa	Agüero et al., 2007
Brassica oleracea L. var. acephala	Kopsell et al., 2004

Fig. 16.3 Distribution of chlorophylls types in bioresources

processing, treatment, packing, packaging, transport, and holding of the food, to perform a technological function (including sensorial). In agreement with the use in the industry food, the additives are classified into 25 classes, which include about 230 different compounds. Among the different classes of food additives, chlorophylls, and their derivative compounds (chlorophyllins) are classified and are widely used as colour additives (Carocho et al. 2015; Martins et al. 2019). Thus, these molecules, may give, deepen, or renew the food colour if it has been lost in the course of technological processing, being responsible for various bluish-greenish hues (Fig. 16.4) (Zapata et al. 2011; Pareek et al. 2017; Sawicki et al. 2019).



Pigment	λ máx (nm)[a]	Colour descriptor
Chl *a*	665	blue-green
Chl *b*	652	brilliant green
Chl c_1	445	yellow-green
Chl c_2	449	yellow-green
Chl c_3	445	yellow-green
Chl *d*	696	brilliant/forest green
Chl *f*	706	emerald green

a: 100%methanol

Fig. 16.4 Chlorophyll structures and their absorption spectra

However, due to outstanding beneficial biological effects of these molecules in addition to their potential action dyes, these compounds can be used as additives in functional or nutraceuticals foods, aiming at promoting health (Humphrey 2004; Christaki et al. 2015; Janiszewska-Turak et al. 2016; Kang et al. 2018; Martins et al. 2019).

Predominantly, liposoluble chlorophyll *a* and *b* as well as their pheophytins directly extracted from natural sources are widely used in the food industry as a basis for obtaining green dyes (Rodriguez-Amaya 2019; Vieira et al. 2019). At the same time, it is known that these compounds have an inherent instability and want greater care as the physical and chemical changes during the processing (Delgado-Vargas and Paredes-López 2002; Lanfer-Marquez and Borrmann 2009). There are two key structural characteristics of chlorophyll pigments responsible for their properties, especially as it relates to their solubility and colour (Wrolstad and Culver 2012). More intense changes in the colour of chlorophylls are closely linked to the pH of the medium or enzymatic activity and can be observed when the central Mg^{2+} ion is replaced. On the other hand, the solubility of chlorophyll molecules is totally dependent on the phytol isoprenoid alcohol, since this when present in the structures gives confer the characteristic molecules more nonpolar (Galaffu et al. 2015; Pareek et al. 2017).

Consequently, to expand the applicability and reach successful application of natural chlorophylls extracted for commercial applications, some modifications are induced out in their structures and derivative compounds are formed as a strategy to increase their chemical stability and colour potential (Fig. 16.5) (Solymosi and Mysliwa-Kurdziel 2016).

Among these modifications, the copper complexes (Cu-chlorophyll), replacing the central magnesium, are frequently marketed as the green dye for the food industry, because it maintains the bright green colour and chemical stability of the molecules formed. These compounds are basically obtained by the addition of copper sulfate in acidic medium soon after extracting chlorophyll extracts (Vieira et al. 2019). More rarely, divalent cations such as Fe^{2+} and Zn^{2+} can be also used to replace the central Mg^{2+} ion during food processing (Canjura et al. 1999; Kang et al. 2018).

As well as, modifications in the hydrophobicity of the pigment molecule conferred by its long hydrophobic phytol chain and by a fifth ring (cyclopentanone) in the macrocycle are carried out starts with the alkaline hydrolization (saponification), and subsequent neutralization by K and/or Na salts, or enzymatic action. With this, are obtained chlorophyll derivatives with greater solubility in water and with similar spectral properties to those of chlorophyll, called chlorophyllins by scientists and the food colour industry. For this purpose, the term chlorophyllins refer to a complex mixture of various chlorine compounds (mainly chlorin e_6 and rhodin g_7), chlorophyllides, and pheophorbides, however its composition is not clearly defined because it is highly variable (Pérez-Gálvez et al. 2017).

Moreover, both processes of chemical transformations mentioned above (replacement of central magnesium by copper, and phytyl group removal) can be induced simultaneously in order to obtain Cu-Chlorophyllin. Several purification steps are necessary to remove interferents (Lanfer-Marquez and Sinnecker 2008). However,

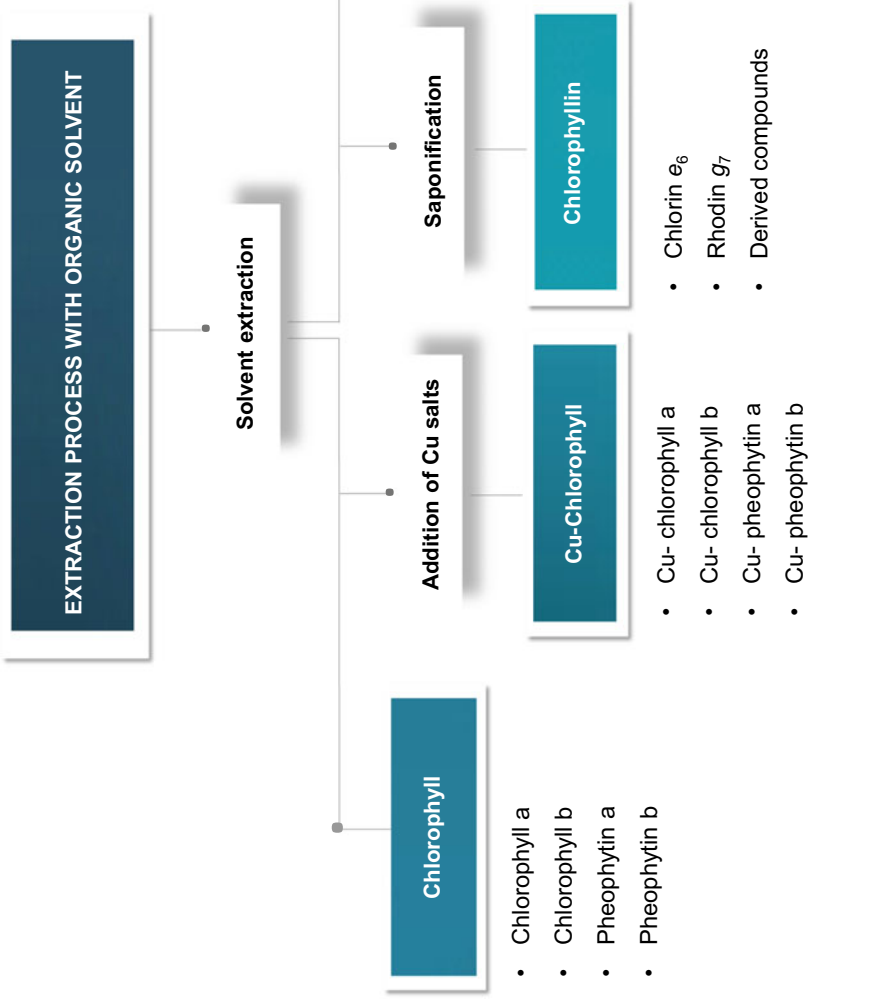


Fig. 16.5 Simplified process for obtaining chlorophylls

as with chlorophyllin synthesis, these chemical changes result in the formation of a complex mixture of various chlorine compounds including the most commonly found copper isochlorin e_4, copper chlorins e_4 (derived from chlorophyll *a*), and e_6 (derived from chlorophyll *b*), among other molecules (Mortensen and Geppel 2007; Tumolo and Lanfer-Marquez 2012).

Indeed, these chlorophyll derivatives (specifically, Cu-chlorophyll, complexes chlorophyllins, and Cu-chlorophyllin) previously mentioned not only exhibit outstanding stability to the pigment but also cause expression of more desirable green colours. Which justifies the fact that they, together with extracts of natural chlorophyll, are the approved worldwide green colourants since they present greater chemical stability to pH change, temperature, and prone to light, heat, and oxygen disintegration. In addition to these important characteristics, as a result of changes in their polarity, these formed compounds can be applied in liposoluble and hydrosoluble matrices (Shahid and Mohammad 2013; Sigurdson et al. 2017).

Although chemical methods are widely utilized to increase the stability against oxidation and aqueous solubility of green natural dyes, alternative techniques have been reported. In parallel, physical methods such as microencapsulation are currently under development (Raei et al. 2017; Ozkan et al. 2019; Kang et al. 2019). However, each type of encapsulating agent has its own specific advantages and drawbacks for encapsulation, protection as well as cost, ease of use, the biocompatibility among other aggravating factors (Ozkan et al. 2019). Recently, sodium caseinate (NaCas), a commercially available casein-rich ingredient, has been reported as a natural the chlorophyll *a* and *b* colour stabilizer the same time as increasing the solubility of these compounds in aqueous dispersion (He et al. 2019).

Regardless of these aspects, among the five different chlorophylls that exist, it is well known that green food dyes from natural sources are relatively limited to chlorophyll *a* and *b* species (Solymosi and Mysliwa-Kurdziel 2016). This is reflected by the use of higher plants as the primary source of obtaining these pigments (Wrolstad and Culver 2012). At the same time, some food industries have already explored the use of green dyes obtained from microalgae as well as other pigments widely marketed from these microorganisms (phycobilins from *Spirulina*; β-carotene from *Dunaliella*; astaxanthin from *Haematococc*us) (D'Alessandro and Filho 2016; Matos 2017). However, these extracts are obtained from microalgae that present species mostly of chlorophyll *a* and *b*, which equals, at the level of chemical constitution, the extracts obtained from higher plants.

Independent of species of chlorophylls commercially consolidated other chlorophylls (chlorophyll *c, d,* and *f*) and their derivative compounds, present exclusively in microalgae, can be implemented as alternative sources for green natural pigments considering the chemical stability of these compounds and their colour potential. Due to the structural chemical characteristics, chlorophyll *c* species, for example, indicated in the literature to be more stable and presents greater solubility in polar matrices than chlorophylls *a* and *b* (Lanfer-Marquez and Sinnecker 2008; Zapata et al. 2011, Chen et al. 2018). Considering these aspects, a greater number of molecules can be obtained to compose the green dyes class.

Although the chemical instability of these compounds is the major limitation for the use of chlorophyll as food additives, factors related to obtaining these compounds also restrict their application. In this way, sources that present high productivity, low levels of contaminants, together with simplified extraction methods are the main parameters to leverage the obtainment of natural green dyes and their possible applications (Mulders et al. 2014; Martins et al. 2016).

In addition, as an alternative to chlorophyll compounds used as food additives, the microalgae biomass is being applied as a food ingredient whose secondary effect is the green colouration. In this sense, research developed over the last years presented excellent results regarding stable green staining in all formulations, as evidenced by the high content of chlorophyll present in these microorganisms. Some examples are presented in Table 16.2.

To overcome the limitations of green dyes application, the purpose of these studies, mentioned above, is to find potential alternative sources for obtaining chlorophylls, targeting compounds that have the green colour intensity and chemical stability higher than those currently marketed. Consequently, new sources of natural chlorophylls or modified chlorophylls extracts are supported by regulations covering food additives to be marketed, which must be strictly followed.

16.4 Biological Properties

Physicochemical properties are the first resource for assuming and confirming the potential activities of a compound to be studied. Structurally, chlorophyll molecules and their derivatives are a group of compounds with different chemical structures (as can be seen in Sect. 16.2), which contemplate distinct physicochemical properties. These compounds have a significant function in the photosynthetic system, whom this versatility involves specific molecular arrangements and diversified chemical exchanges of the functional groups, serving as descriptors of the compound demonstrating its mode of action and biological activity, reaching a result that may or may not be beneficial (Agostiano et al. 2002).

Regardless of the presence of the central metal ion and the phytol chain, chemical influences and interactions also occur through other structural residues, such as the peripheral substituents and the isocyclic ring that directly influence the chromophore/absorption and the differentiated chemical and in biological behaviour (Henderson et al. 1997; Fiedor et al. 2003).

Table 16.2 Microalgae-based products that reached colouring green natural. Considering these aspects, natural and commercial chlorophylls, such as copper and sodium chlorophyllins, are widely investigated above biologically beneficial health activities, where some of these benefits are present in the healing process, antioxidant, anti-inflammatory, antimutagenic, and antimicrobial properties, as shown in Table 16.3 (Edwards 1954; Ferruzzi et al. 2002).

Accordingly, the human organism is exposed to various compounds that can damage important biological molecules such as DNA, proteins, carbohydrates, and

Table 16.2 Microalgae-based products that reached colouring green natural

Microalgae specie	Product	Application	Colouring characteristics	References
Chlorella vulgaris	Pea protein stabilized emulsions	Natural green dye and antioxidant	Stable colour for six weeks and oxidation reduction	(Gouveia et al. 2006)
Chlorella vulgaris	Butter cookies	Natural green dye and food supplement	Stable colour for three months	(Gouveia et al. 2007)
Isochrysis galbana	Biscuits	Natural green dye and food supplement	High stability of colour	(Gouveia et al. 2008)
Chlorella vulgaris and Spirulina maxima	Pasta	Increase of quality parameters and natural green dye	Stable colouring after cooking (103 °C)	(Fradique et al. 2010)
Arthrospira platensis	Pasta	Natural green dye, sensory quality, and nutraceutical potential	Stable colour during cooking	(Zouari et al. 2011)
Spirulina platensis	Pasta	Natural green dye and food supplement	Stable colour during cooking; appealing green tone	(Özyurt et al. 2015)
Spirulina platensis	Kiwifruit pastille	Natural green dye	Instability in colour over high temperatures (90 ºC)	(Pool et al. 2016)
Arthrospira platensis, Chlorella vulgaris, Tetraselmis suecica, and	Cookies	Natural green dye and food supplement	Stable colour (100ºC–120ºC)	(Batista et al. 2017)
Phaeodactylum tricornutum			and along conservation time (eight weeks); 6% microalgae seem to have more intense green colour.	
Isochrysis galbana and Nannochloropsis oculata	Chewing gum	Natural green dye	Stable and effective colour	(Palabiyik et al. 2018)

Table 16.3 Bioactive properties of different types of chlorophylls and their catabolites

Chlorophylls/catabolites	Bioactive properties	References
Chlorophyll *a*	Antimutagenic, chemopreventive, antioxidant, chemoprevention of aflatoxin B1 (AFB1), prevents DNA-mutagen intercalation, treatment of chronic ulcers and impetigo contagiosa	(Guskin 1940; Ferruzzi et al. 2001, 2002; Lanfer-Marquez et al. 2005; Castro et al. 2008; Osowski et al. 2010)
Pheophytin *a*	Antimutagenic, chemopreventive, anti-inflammatory, suppressor activity against genotoxins, inhibition of edema formation, inhibition of hepatitis C virus (HCV) proteins (NS3 protease)	(Okai and Higashi-Okai 1997; Ferruzzi et al. 2001, 2002; Wang et al. 2009; Islam et al. 2013)
Pheophorbide *a*	Antioxidant, chemopreventive, antigenotoxic, antimutagenic activity, ABCG2 inhibition, human pancreatic carcinoma in athymic mice	(Nakamura et al. 1996; Hajri et al. 1999; Ferruzzi et al. 2001; Robey et al. 2004; Ferruzzi et al. 2007)
Chlorophyll *b*	Antimutagenic, antioxidant, treatment of chronic ulcers, and impetigo contagiosa	(Ferruzzi et al. 2001; Islam et al. 2013)
Pheophytin *b*	Antimutagenic, anti-inflammatory, antioxidant, HuH-7 human hepatocellular carcinoma cells, inhibition of edema formation	(Higashi-Okai et al. 1998; Ferruzzi et al. 2001, 2002; Ferruzzi et al. 2007; Li et al. 2007)
Pheophorbide *b*	Antioxidante, HuH-7 human hepatocelular carcinoma cells	(Lanfer-Marquez et al. 2005; Li et al. 2007)

lipids. Indeed, most neurodegenerative diseases, cancer, aging is associated with the formation of excess reactive species, which results in oxidative stress and reduction of the body's antioxidant system (Ulbricht et al. 2014). Conversely, these diseases can be prevented, and the symptoms can be reduced with the use of these natural antioxidants. It is well described that chlorophyll pigments, especially pheophorbide *a* act as antioxidant compounds, acting positively under reactive oxygen species (ROS), hydrogen peroxide, free hydroxyl radicals, and lipid peroxidation, in vitro and in vivo (Nakamura et al. 1996; Kamat et al. 2000; Kumar et al. 2001; Lanfer-Marquez et al. 2005).

Among the chlorophyll compounds, Ferruzzi et al., (2002) report that chlorophyll *a* is one of the inhibitors of free radicals of greater action, this is due to the presence of Mg, Zn, Cu central ions in these structures analyzed, compared to chlorophyll *b* and pheophorbide *a* (phytol free structures), which present smaller antioxidant activity.

In addition, in vitro study of natural chlorophyll derivatives in protective action against lipid oxidation, Lanfer-Márquez et al. (2005) concluded that pheophorbide *b* and pheophytin *b*, had higher antioxidant activity than chlorine derivatives, but chlorophyllin had the highest antioxidant property all.

Likewise, pheophorbide is a potent antitumour component because of its high antioxidant mechanism. Pheophorbide *a*, present in *Capsosiphon fulvenscens*, has been shown high activity on reactive species of intracellular oxygen, inhibiting and preventing endothelial inflammation (Pangestuti and Kim 2011; Hong et al. 2016).

Al so, chlorophyllins, especially Na-Cu-chlorophyllins, are proven safe and their soluble derivatives can be applied in the wound healing process (Guskin 1940; Cady 1948; Horwitz 1951; Larato 1970; Solymosi et al. 2016). Are indicated as ideal for photodynamic therapy, photosensitization, aids in the selective destruction of tumour cells, which is obtained by activating the light phototoxicity performed by these agents, which transfer the excitation energy to produce reactive oxygen species and free radicals (Rapozzi et al. 2009; You et al. 2011).

Compounds such as Pheophorbide *a*, pheophorbide *b*, pheophytin *a* and *b*, chlorophyllin *f*, have been shown to decrease lipid peroxidation and decrease tumour cells inducing apoptosis by reactive oxygen species (ROS) (Li et al. 2007; Rapozzi et al. 2009; Du et al. 2014). Other functions of chlorophyll and its derivatives are related to the control of viral infections (Jenkins et al. 2016; Srivatsan et al. 2015; Wohellebe et al. 2011).

The chlorophyll derivatives were also tested as antimicrobials, where chlorophyllins supplemented with probiotics were ingested by dogs, where a reduction of coliforms and clostridium was evaluated, and suggesting chlorophyll additives in the canine feed to prevent bacterial infections without reducing the beneficial ones on lactic acid bacteria. These results demonstrated that chlorophyll and its derivatives are potentially beneficial in the application as supplementation and additive for functional foods (Strompfová et al. 2015).

Although the studies showed above exhibition the broad applicability of chlorophylls and their derivatives for medical and pharmaceutical purposes, the potential of these pigments with significant biological activities, including antioxidant activity, antimutagenic activity, and enzyme modulation, further stimulates the exploration of these compounds for food applications (Kephart 1955; Ferruzi and Blakeslee 2007).

Thus, interest in pursuing absorption results based on the notion that chlorophyll derivatives may be bioavailable is of paramount importance. These efforts serve to better understand the mechanism by which chlorophyll and derivatives act in the food diet, thus exploiting the real effects by absorbing foods supplemented with these biocompounds (Ferruzi and Blakeslee 2007).

Egner et al. (2000), concluded in a study with humans that Na-Cu-chlorophylls compounds are in fact absorbable and actually bioavailable in the body, but selectively. Chen and Roca (2018), found that edible algal chlorophylls *a* and *c* are better micellized than chlorophyll *b*, that is, they are more resistant to in vitro digestion of the food matrix. Also concluded that pheoforbide *c* is the most absorbable chlorophyll derivative by Caco-2 cells, which are typical research tools for nutrient absorption and demonstrate the accumulation of the compound studied by human intestinal cells and its basolateral flow by differentiated cells grown in inserts (Ferruzzi et al. 2002).

In this regard, it is assumed that chlorophylls have a high potential to be exploited as a food additive, same as today a large part of their market in food is related to

its application as a dye and not using the exploratory field of its biological activities (Humphrey 2004; Streit et al. 2005; Volp et al. 2009; Rocha and Reed 2014).

16.5 Production and World Market

The global economy is constantly changing, seeking more and more the exploitation of natural products and biorefinery products, becoming a biologically based economy, where the microalgae biomass enters with a high value and can be applied in several areas, mainly in the food industry (Barsanti & Gualtieri 2018).

Consumers' interest and the pursuit of healthy foods, including food products, functional ingredients, or dietary supplements that in some way benefit health, preventing, treating, or healing certain diseases have been increasing (Coopers 2009). Thus, the use of microalgae biomass and the exploration of derived metabolites such as chlorophylls becomes an innovative approach applied to the development of functional foods (Gouveia et al. 2008).

One of the main problems affecting the use of these pigments as food additives is the lack of globally reconciled legislation, normally the food additive regulations include the list of authorized standards, their specifications, as well as the conditions of use, but they are different rules for each country, making global production difficult (Viera et al. 2019). The main raw materials for obtaining chlorophyll are leafy foods and other green leaves and plants that produce large quantities and generally value as food or feed (Attokaran 2017).

According to Transparency Market Research (2017), the global market for chlorophylls is divided into the regions of North America, Latin America, Asia-Pacific, Western Europe, Eastern Europe, Japan, and the Middle East and Africa (MEA). Especially in China, is expected to expand, already in Latin America, it is robust due to the high demand for natural dyes. Global adoption of chlorophyll and chlorophyllin is lower in the MEA than in any other region of the world due to the low availability of raw materials in the region. In Fig. 16.6, we can observe some companies responsible for the production and commercialization of chlorophyll in the world.

Production of microalgae-based intracellular products on an industrial scale enters as a commercial opportunity that aims to cede market share in the part of bioactive molecules. Thus, in a short period of time, the microalgae biotechnology industry has grown significantly. Currently, the microalgae biomass market produces about 9000 t of dry matter/year and their global market value was US$ 2.8 billion in 2011. His market is regarded to be worth US$ 3.32 billion by 2022, with a compounded average growth rate (CAGR) growth of 6.7% (Spolaore et al. 2006; Enzing et al. 2014; García et al. 2017).

Estimates of the world production values of food colouring vary greatly with the increasing demand of this market. The global food colouring market was estimated at US$ 3.71 billion in 2017, growing at a CAGR of 5.7%, to reach US$ 5.12 billion by 2023. Estimates put the production of chlorophylls in Europe more specifically in the United Kingdom the value of approximately US$ 5.5 million per year, suggesting

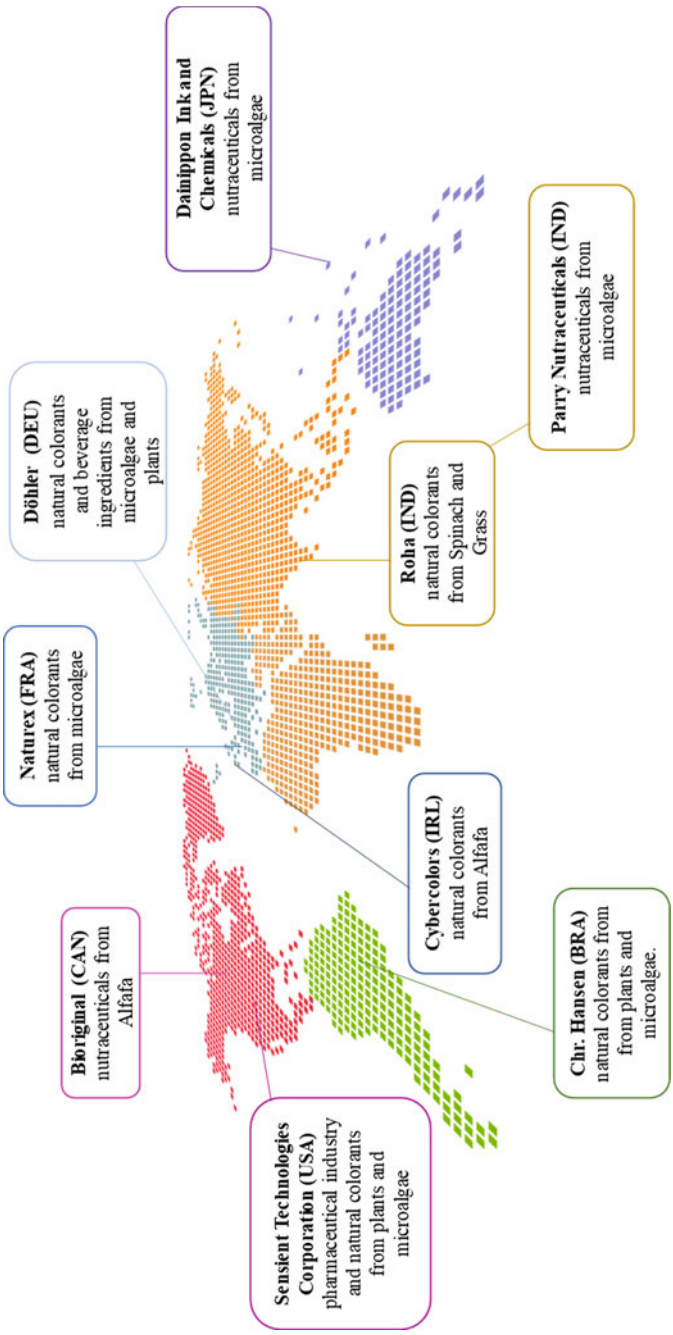


Fig. 16.6 Examples of chlorophyll and derivatives companies, applications, and sources used

that it could represent up to third of world production. In 2018, according to the Markets and Markets reports, Europe was a leader in the food colouring market, a market driven by the demand for awareness of safe products among consumers and by the inclination and pursuit of health-food natural dyes.

In order, to be considered suitable for commercial pigment production, strains must meet certain criteria such as ease of cultivation, low toxicity, nutritional value, and the presence of digestible cell walls to provide nutrients (Borowitzka 2013).

Among the species of microalgae considered to be commercially safe through the GRAS status (generally recognized as safe), which is granted by the Food and Drug Administration (FDA), are *Spirulina/Arthrospira* sp., *Chlorella* sp., *Porphyridium cruentum, Crypthecodinium cohnii*, *Haematococcus pluvialis*, *Phaeodactylum tricornutum*, *Dunaliella* sp., *Nannocloropsis* sp., *Nitzschia* sp., and *Schizochytrium* sp., some of its by-products are already authorized as ingredients by the European Union, Japan, USA, and Brazil, thus, they can be exploited as a source of natural chlorophyll (Jacob-Lopes et al. 2019).

Algae, silkworm droppings, alfalfa, pine needles, and various other types of grass are exploited for chlorophyll extraction. However, the concentration of this compound in plant sources varies, as they depend on climatic conditions and geographical position, varying the quantities over a wide range, showing one more advantage in the exploitation of these pigments in microalgae. Large-scale cultivation of microalgae has grown in recent decades, especially *Chlorella* and *Spirulina. Spirulina*'s fresh biomass is one of the largest sources of chlorophyll in nature, containing ten times more than spinach (Henrickson 1989; Lanfer-Marquez and Borrmann 2009).

Spirulina and *Chlorella* microalgae have significant benefits as potential sources of chlorophyll and are widely sold as supplements, *Spirulina* was sold at a price of US$ 20/kg and *Chlorella* at a price of US$ 44/kg in 2017, these two microalgal species gain prominence by producing the highest volumes of biomass. *Spirulina* production is widely distributed in Asia and the USA, while *Chlorella* is produced mostly in Asia. China is the country with the highest production of *Spirulina* in the world, with a total production of around 3500 t (dry weight) in 2009 (Lu et al. 2011; Voort et al. 2015; Oilgae 2016; Matos 2017).

In order to obtain chlorophyll in a given species, intracellular chlorophyll must first be extracted, and the traditional method that has been employed through extraction by organic solvents as shown in Fig. 16.7. It is a process in which it must be carried out quickly, without the presence of much light, thus avoiding the processes of photodegradation. Acetone, methanol, ethanol, hexane, chlorinated solvents, and others, are applied in the extraction (Lee 2012).

Filtration and centrifugation serve to remove the solids remaining in the solvent. After separation of the solvent, the extraction yield can reach around 20%, being chlorophylls and their derivatives.

Currently, natural pigments are the most strictly regulated additives for food applications worldwide, but there are still many obstacles to a worldwide adaptation of this type of natural product, interfering in outsourcing, import, export, and has negative economic effects. Adoption of different categories of additives is difficult, as laws

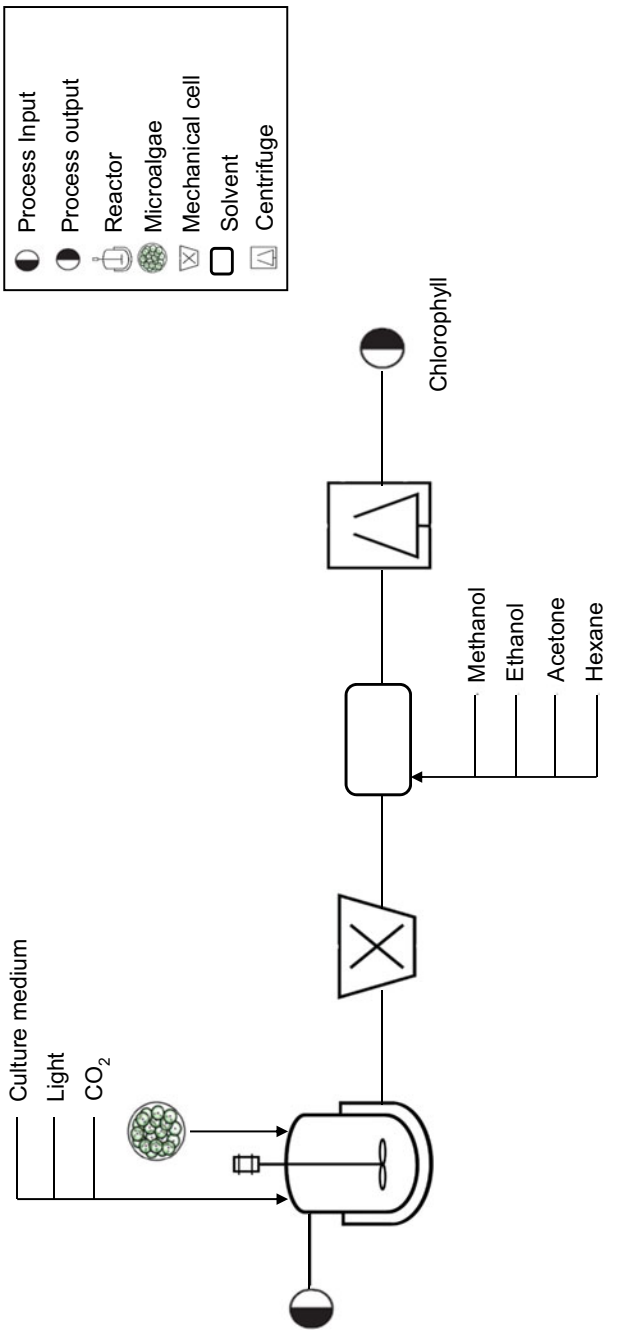


Fig. 16.7 Sketch of chlorophyll extraction process

have different limits and rules around the world, but there are also commonalities in these legislations, ensuring food safety, and ensuring procedures for risk assessment and management measures, which demonstrates that it is becoming easier to market these natural metabolites with their broad benefits to human health (Magnuson et al. 2013; Lehto et al. 2016).

16.6 Legal Aspects

The regulatory frameworks that control the application of chlorophylls as a colour additive, their specifications, as well as the conditions of use (limitations on specific foodstuff and maximum amounts) differ substantially in different regions of the world. Basically, five types of natural chlorophyll colours are authorized worldwide, although a few of them are produced by synthesis, but considered 'nature-identical' (described in Sect. 16.3). Anyhow, in each country they have a different terminology and are marketed according to their stability as to solubility and colour intensity (Table 16.4).

Based on the latest version of the commodities *Codex Alimentarius* published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as the international body responsible for food additive safety assessment, are chlorophylls (INS 140i-140ii) and the copper chlorophylls (INS 141i-141ii) registered as dyes for application in food and beverages. This internationally recognized collection consists of indications of maximum level permissible limits of each colour additive in various food categories, guidelines, and other recommendations relating to this class of compounds (CODEX STAN 192-1995 2019).

Specifically, in European Union, the European Food and Safety Authority (EFSA) recognizes the use of four main colourants structurally related to the chlorophylls for use in foodstuff, denominate under the code E140i, E141i, E140ii, and E141ii (EFSA 2008). According to current legislation (Regulation (EC) No 1333/2008), E140i, also known as magnesium-based chlorophyll, comprises extracts obtained from plants, which are more liposoluble, since their composition consists mainly of species of chlorophyll *a*, chlorophyll *b,* and its derivative compounds. While E140ii (also known as sodium or potassium chlorophyllins) has characteristic hydrosoluble, which favours its use in water-soluble foods. In contrast, E141 products are composed of copper complexes of chlorophyll derivatives stable colouring (E141i or Cu-chlorophyll) or chlorophyllin (E141ii or Cu-chlorophyllin). As for your application, are authorized for use in a wide variety of foodstuffs with maximum limit at *quantum satis*, thus shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose (EFSA 2015a, b, c). Currently, as alternative sources to chlorophyll compounds, blends with *Spirulina* or their extract are considered a food ingredient with colouring properties in the European Union, can be added to all food categories without restrictions with the aim of conferring green colour (Scotter 2015; Oplatowska-Stachowiak and Elliott 2015; Lehto et al. 2017).

Table 16.4 Chlorophyll dyes for use in foodstuff authorized according to different regulations

Natural colourantes	Colouring	Description	EU	USA	India	China	Japan	Canada	Australia and New Zealand	Brazil
Liposoluble										
Chlorophyll	Olive green to dark green (depending on the content of coordinated Mg)	Waxy solid	E140i	–	6	–	177	E 140	140	140i
Cu-chlorophyll	Blue-green to dark green (depending on the source material)	Waxy solid	E141i	–	–	CNS 08.153	266	–	141	141i
Hydrosoluble										
Chlorophyllin	Green to blue/black powder	Powder	E140ii	–	–	–	116	–	–	140ii
Cu-chlorophyllin	Dark green to blue/black	Powder	E141ii	73.125	–	CNS 08.009	265	E 141	141	141ii
Na–Fe-chlorophyllin	Green	Powder	–	–	–	–	257	–	–	–

Consequently, the same chlorophyll colouring products approved in the EU are allowed in Japan, but are given denominations with numbers: 177 (Chlorophyll), 116 (Chlorophyllin), 266 (Cu-chlorophyll), and 265 (Cu-chlorophyllin). In addition to these, Japan also allows the use of a fifth natural green dye denominated Na–Fe-chlorophyllin (257), according to Ministry of Health and Welfare of Japan (MHLW). Simultaneously, the report details the target foods allowed for application these dyes, the limitation for use, maximum quantitative values, and other requirements (Japanese Food Additives Regulations 2019).

Table 16.4 Chlorophyll dyes for use in foodstuff authorized according to different regulations.While the Food and Drug Administration (FDA) in the United States authorizes the use of green natural colouring limited to Cu-chlorophyllin (classification 73.125) in mixtures of citrus-based dry beverage in an amount not exceeding 0.2 per cent in the dry mix, the Food Safety and Standards Authority of India only authorizes extracts of chlorophyll (US FDA 2019a; FSSAI 2019). Similarly as the EU, in the USA, *Spirulina* aqueous extract of the biomass of *Arthrospira platensis* (denomination 73, 530) is considered a colour additive. Thus, can be used at levels consistent with good manufacturing practice in colouring confections (including candy and chewing gum), frostings, ice cream and frozen desserts, dessert coatings and toppings, beverage mixes and powders, yoghurts, custards, puddings, cottage cheese, gelatin, breadcrumbs, and ready-to-eat cereals (excluding extruded cereals) (US FDA 2019b).

In China, current regulations are restricted only to chlorophyll copper complexes, probably due to the higher colour instability of these compounds. Thus, only the use of Cu-chlorophyll and Cu-chlorophyllin is regulated in line with China National Center for Food Safety Risk Assessment (CFSA), which designates maximum values and allowable food (CFSA 2019). In Australia and New Zealand, the FSANZ (Food Standards Australia and New Zealand) is the government body responsible for regulating the food additives that may be used as colouring agents including chlorophyll, chlorophyll copper complex, and chlorophyllin copper complex, sodium and potassium salts (FSANZ 2019). In the same way, Health Canada includes the chlorophyll and copper chlorophyllin in the list of colouring agents permitted for use in foodstuffs. The maximum level of use for chlorophyll is in accordance with good manufacturing practices and a limit of 300 ppm for sodium copper chlorophyllin is stipulated (Health Canada 2019).

In respect to Brazilian legislation, Resolution No. 44 of 1977 and its amendments published by Brazilian Health Regulatory Agency (Anvisa) classifies chlorophylls as a colour additive in foods according to the way they are obtained. Thus, in the class of the natural organic dyes of green colouration, authorizes the use only to extracts of chlorophyll. Chlorophyllin, copper chlorophyll, and copper chlorophyllin are authorized but classified as a synthetic organic dye identical to the natural one. Nonetheless, the legislation lacks food information, and maximum values allowed for application since it simply establishes the tolerated use of these compounds (Anvisa 2019). Therefore, these parameters are available in individual regulations for each foodstuff, and usually establish the use of *quantum satis,* similarly to the European Union.

Although these regulatory standards are reviewed continuously, there are still several unknowns to be clarified as to the composition of each chlorophyll-related compound. This is potentially correlated with chlorophyllins and their copper complex, compounds most used by the food industry worldwide, and lack characterization standardized. Likewise, to supply the market for natural green dyes, it is necessary news legislations in accordance to the emergence of alternatives sources to these compounds, strongly including the microalgae, microorganisms in constant exploitation for this purpose. Therefore, safety aspects should be considered for the implementation of microalgae as sources of green dyes.

16.7 Final Considerations

The market of the food colourants is evolving to more natural formulations. Thus, the development of natural alternatives for colourants formulations is necessary. Associated with this, it is indispensable to know the structural characterization of this family of pigments, its natural distribution, and information knowledge about their biological actions. The microalgae have received considerable attention in the last decades, indicating its potential as a renewable source of natural pigments. However, some hurdles must be overcome for these bioprocesses to be successful in the market, such as the selection of appropriate microalgae strains, the care for chlorophylls compounds instability, since natural green colourants are very labile and also it is not easy to reproduce green hues naturally. As well as new legislation will be necessary for the obtaining of natural chlorophylls from microalgal bioprocesses to be established in the natural pigment market. Finally, through considerable advances in research on natural pigments, in the near future, the consumer will face new green authorized food colourants.

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Chapter 17
Carotenoids as Food Additives

Ágnes Farkas, Tímea Bencsik, and József Deli

Abstract Carotenoids are hydrophobic pigments with a tetraterpene skeleton, widely distributed in higher plants, as well as microalgae from both marine and freshwaters. Animals and humans are unable to synthesize them, and they need to obtain them from dietary sources. The most characteristic feature of carotenoids is the long series of conjugated double bonds forming the central part of the molecule, providing their shape, chemical reactivity and light-absorbing properties. The colour, ranging from pale yellow to red, and the antioxidant properties of carotenoids highly depend on these conjugated double bonds and other functional groups in the molecule. Food additives are generally divided into six categories: taste enhancers, preservatives, stabilizers, emulsifiers, antioxidants and colouring agents. This chapter is dedicated to the latter two categories. Carotenoid-containing plants (e.g. saffron stigmas, pepper pericarps) and their extracts have been used as food colourants for centuries. They are generally considered safe; however, there are only a few studies about their stability, biochemistry, bioavailability, pharmacokinetics and toxicology. Although carotenoids are relatively unstable due to their susceptibility to light, heat, oxygen, autoxidation and isomerization, there are several commercially synthesized carotenoids (e.g. β-carotene), which are accepted colour additives. Today, some of the most important carotenoids used in food, feed and cosmetics industries as natural colourants are produced by microalgae in bioreactors (fermentation technology). Some of the naturally occurring carotenoids (e.g. β-carotene, astaxanthin) are widely commercialized, while others (e.g. lutein, canthaxanthin, zeaxanthin, lycopene) are used less frequently. The global carotenoid market is estimated at US$ > 1 billion per year, and it is still constantly growing.

Keywords Food colouring · Antioxidants · Carotenoids · Microalgae

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17.1 Introduction

In the last decades, the increased population and lifestyle changes promoted considerable alterations in the formulation of food products. Additionally, the modification of the eating habits and the nutritional demands caused an important transformation in the food industries, which involved the incorporation of additional food ingredients to food products. The purpose of these compounds (food additives) is to add dietary nutrition (fortifiers), increase the shelf life and/or to improve the physicochemical, sensorial and microbiological properties of the industrialized foods (Damodaran and Parkin 2017; Martins et al. 2019).

A recent review study states that, currently, there are almost 2500 types of food additives used globally, and US FDA lists around 3000 ingredients in the food additive database (Abdel 2015). It also reports that almost 200,000 tons of food additives are used per year, which highlights the fact that Western diet consists of approximately 75% of processed food. The average annual intake of food additives per person is estimated to be 3.6–4.5 kg (Abdel 2015).

Food additives are generally divided into six categories: taste enhancers, preservatives, stabilizers, emulsifiers, antioxidants and colouring agents. This chapter is dedicated to the latter two categories.

Antioxidants are mainly used in food to prevent off-flavours by oxidation of fats, therefore, halting their peroxidation in the initiation or propagation phases. There are five types of antioxidants: the primary antioxidants known as radical scavengers or chain-breaking antioxidants; chelators, that bind to metals and prevent them from initiating radical formation; quenchers, which deactivate high-energy oxidant species; oxygen scavengers, that remove oxygen from systems, avoiding their destabilization; and finally, the antioxidant regenerators, that regenerate other antioxidants when these become radicalized. The main foods where antioxidants are used are meats, oils, fried foods, dressings, dairy products, baked goods and extruded snacks (Baines and Seal 2012; Carocho et al. 2015).

Food items that are rich in nutrients, flavour, aroma and texture are consumed only if the colour exhibited makes its appearance relishing. Consumers are fond of colourful food products and food dishes worldwide. Colour and appearance create the first impression and greatly influence the acceptability of food; hence, the development of food items with attractive colour and appearance is an important goal in the food industries. Colour is added to food to replace the colour lost during processing, to enhance the colour already present, to minimize batch to batch variations and to colour otherwise uncoloured food.

Food colours can be classified as natural colours, nature-identical colours, synthetic colours and inorganic colours. Nowadays, food producers pay more attention to colours and additives of natural origin, since many artificial colours and additives have been shown to impart negative health effects. Based on the chemical structure, natural pigments are grouped as tetrapyrroles (e.g. chlorophyll), tetraterpenoids (e.g. carotenoids), flavonoids (e.g. anthocyanins), anthraquinones (e.g. carmine, lac, etc.) and betains (Arimboor et al. 2015).

From a chemistry perspective, food-colouring agents share the ability to absorb light due to their conjugated double bonds, creating a delocalized electron system. The number of conjugated double bonds is indicative of the wavelength of the absorbed light (Schoefs 2002). Traditional sources of natural food colour additives are essentially plants, plant extracts and, to a lesser extent, other sources such as animals (most importantly, insects), algae, fungi and bacteria (including cyanobacteria) are also used.

Microbial pigments not only add colour to food but also have numerous medicinal properties like antioxidant, antimicrobial, anticancer, immunoregulatory and anti-inflammatory effect, etc.

The question arises, why should we use microbial pigments as a food additive? Consumers' ability to differentiate between the benefits of microbial pigments and hazardous effects of synthetic pigments has greatly boosted the application of microbial pigments as a food additive. According to the current trends, consumers' tendency to interpret the utilization of synthetic pigments as mere contaminants has been augmented. Robust development and advances in technology and genetic engineering techniques have enabled the food industries to produce microbial pigments; this has led to an increase in demand of natural food additives (Nigam and Luke 2016). Currently, the public's interest in synthetically extracted pigments has decreased owing to their toxicity, oncogenicity and teratogenic properties, whereas microbial sources of pigments have gained consideration as safe alternatives. There is a worldwide interest in developing new procedures for obtaining pigments from natural sources.

The long polyene structure of carotenoids is responsible for the characteristic colours and the antioxidant properties of this group of compounds. Due to the long chain of conjugated double bonds, blue and green parts of the visible light spectrum are absorbed. Carotenoids do not only give their colour to blossoms, fruits and vegetables but are also responsible for the strong colours of bird feathers, tropical fishes and shellfishes. By food intake, carotenoids are enriched in various tissues of the animals, like muscles, feathers or beak.

Moreover, carotenoids protect other molecules from unwanted oxidation reactions, by acting as efficient radical quenchers. The electron-rich polyene chain, which can easily be attacked by free radicals and oxidizing reagents, donates electrons and hydrogen atoms to neutralize radicals.

There are more than 750 known carotenoids. Around 60 of them present in food affect human nutrition. In the human plasma, mainly α-carotene (0.03–0.22 μmol/L), β-carotene (0.13–0.53 μmol/L), β-cryptoxanthin (0.15–0.37 μmol/L), lycopene (0.61–1.38 μmol/L), lutein (0.14–0.34 μmol/L) and zeaxanthin (0.03–0.05 μmol/L) could be found (Khachik et al. 1997; Maiani et al. 2009). Other carotenoids and their metabolites like phytoene and phytofluene are generally in lower concentrations (Shmarakov et al. 2013).

Carotenoids play an increasing role in food industries as natural and nature-identical colouring compounds. They are especially suitable for yellow to orange-coloured soft drinks and are also used to colour various other foods. Due to the

improved carotenoid formulation processes, e.g. insertion in emulsions or nanoparticles, carotenoid colourings show high stability with regard to light, acids and heat. Due to the antioxidant properties of carotenoids, the product gets an additional health benefit for the consumer. Carotenoids are also used as animal food additives and as supplements (Westphal and Böhm 2015).

As food additives, carotenoids play an important role in the global food industry. In Europe, carotenoids are the most preferred food-colouring agents. The global market for carotenoids reached $1.5 billion in 2017 and should reach $2.0 billion by 2022, at a compound annual growth rate (CAGR) of 5.7% for the period of 2017–2022 (www.bccresearch.com).

The carotenoid market is segmented by type, source and application. By type, carotenoids are further sub-segmented into β-carotene, astaxanthin, lutein, annatto, canthaxanthin, lycopene, zeaxanthin and others. Among these, β-carotene is estimated to have the fastest growth rate during the forecast period. It is widely used for food colouring and as a natural supplement.

17.2 The Most Important Carotenoids Used in Food, Feed and Cosmetics Industries

17.2.1 Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione, Fig. 17.1) is an orange-red hydrophobic xanthophyll (ketocarotenoid) derived from β-carotene. Altogether, there are 13 conjugated double bonds in the molecule, and it shows the strongest hitherto demonstrated antioxidant effect due to its keto and hydroxy groups at 4,4'- and 3,3'-β-ionone ring positions, respectively (Liu and Osawa 2007). Because a free ester astaxanthin form is exceptionally vulnerable to oxidation, astaxanthin is predominantly esterified with one or two units of fatty acids (monoesters and diesters) or conjugated with proteins providing stability to the molecule (Breithaupt 2004; Higuera-Ciapara et al. 2006; Yang et al. 2015).

(3*S*,3'*S*)-Astaxanthin is widely distributed in nature and largely discovered in the marine environment (Lorenz and Cysewski 2000; Li et al. 2011). It is most commonly synthesized from canthaxanthin or zeaxanthin by photosynthetic microalgae (Mann et al. 2000). It was originally isolated and identified from lobster *Astacus gammarus* in 1937 (Kuhn and Soerensen 1938), but it also confers a pink-red colour of some

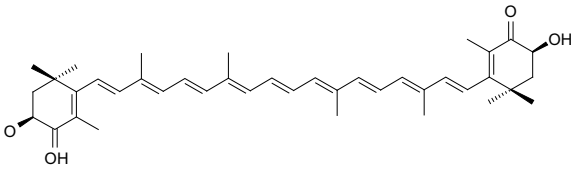

Fig. 17.1 Astaxanthin ((3*S*,3'*S*)-3,3'-Dihydroxy-β,β-carotene-4,4'-dion)

microalgae, yeast, flesh of some fish (e.g. salmon, trout), carapace of many crustaceans (e.g. krill, shrimp, crabs, lobsters, crayfish), shellfish and feathers of some birds (e.g. flamingo, red ibis, quail) (Lorenz and Cysewski 2000; Stewart et al. 2008; Zhu et al. 2009; Mendes-Pinto et al. 2012). These animals are unable to synthesize astaxanthin de novo; astaxanthin is biosynthesized by microalgae or phytoplankton, these are foods for zooplanktons, which in turn are ingested by fish, insects or crustaceans, and finally, astaxanthin is absorbed and deposited in these mentioned animals giving them mostly a pinkish hue (Kitahara 1984; Foss 1987; Nakano et al. 1995; Lorenz and Cysewski 2000; Moriel et al. 2005). But, for example, in lobsters, astaxanthin can be bound to proteins, and the astaxanthin–protein complexes exhibit a blue tint (van Wijk et al. 2005).

(3*R*,3'*R*)-Astaxanthin less commonly occurs in nature. It was isolated as the major carotenoid from the red yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) (Andrewes and Starr 1976), and as a minor carotenoid in a mixture with (3*R*,3'*S*)- and (3*S*,3'*S*)-astaxanthin from eggs of lobster (*Homarus gammarus*) (Rønneberg et al. 1980). Astaxanthin is a strong, deep red-colouring agent (Guerin et al. 2003; Tanaka et al. 2012). It is one of the most commercially valuable carotenoids on the market (Berman et al. 2015), a predicted sales volume of astaxanthin is increasing year by year. Astaxanthin and β-carotene together make-up almost half of the carotenoid market. Currently, the global market is dominated by the synthetic pigment derived from petrochemical precursors (Li et al. 2011), because natural astaxanthin is quite expensive. Synthetic astaxanthin is often a mixture of *R*- and *S*-enantiomers (Capelli et al. 2013) and not suitable as a nutraceutical supplement without further complex and cost-intensive purification steps before application. Additionally, consumers' demand for natural products has raised interest in an environmentally friendly, natural production of astaxanthin by microbial hosts (Lee and Schmidt-Dannert 2002; George et al. 2004; Cutzu et al. 2013; Zhu et al. 2014). Therefore, some biological production systems have also been developed [e.g. the heterobasidious red yeast *Xanthophyllomyces dendrorhous* (formerly termed *Phaffia rhodozyma*), freshwater green microalgae (e.g. *Haematococcus pluvialis*, *Chlorella zofingiensis* and *Chlorococcum* sp.) and bacteria (like the marine *Agrobacterium aurantiacum*)]. Among the exploited sources, *Haematococcus pluvialis* contains the highest level of astaxanthin (>30 g/kg dry biomass), and therefore, it is considered to be the most promising and less expensive natural source of astaxanthin (Lorenz and Cysewski 2000; Dufosse et al. 2005; Higuera-Ciapara et al. 2006; Del Campo et al. 2007). To a lesser extent, astaxanthin can also be extracted from shrimp (*Pandalus borealis*) or krill species (*Euphausia* sp.) (Spolaore et al. 2006; Del Campo et al. 2007; Tanaka et al. 2012).

Within the EU and the USA, astaxanthin (E161j) is considered as a food dye. The FDA considers natural astaxanthin safe and has approved astaxanthin and astaxanthin dimethyldisuccinate, *Haematococcus algae* meal and *X. dendrorhous* as food colourings and additives for specific uses in animal and feeds (e.g. carp, salmonids, red sea bream, shrimps, ornamental fish like cichlids, danios, koi, goldfish, gouramis and tetras—however, it shall not exceed 80 mg/kg) and it is currently used in a hundred-ton scale (Molino et al. 2018; Code of Federal Regulations 2019). According to

Commission Regulation 1288/2004 (EC), additive E161z is astaxanthin produced by *P. rhodozyma* and can be used in salmon and trout feed at a maximum concentration of 100 mg/kg of the complete feed, whereas Commission Regulation (EC) 393/2008 lists E161j as astaxanthin dimethylsuccinate with the same purpose. Astaxanthin can also be produced in *Paracoccus carotinifaciens* and added to feed at a maximum concentration of 100 mg/kg (Commission Regulation 721/2008). It is furthermore an attractive component for pharmaceutical, nutraceutical and cosmetic applications with antioxidant activities (e.g. in human dietary supplements, soft gels, tablets, capsules, syrups, oils or creams) (Iwamoto et al. 2000; Dufosse et al. 2005; Higuera-Ciapara et al. 2006; Zhu et al. 2009; Fassett and Coombes 2011; Li et al. 2011), and to a lesser extent, in poultry feed to produce an advantageous pink flesh colour pigmentation according to the regulatory requirements and consumers' demand (to boost and improve the product quality and price). Moreover, it also presents other physiological benefits such as various improvements in survival, growth performance, reproductive capacity, stress tolerance, disease resistance and immune-related gene expression in aquaculture (Lim et al. 2017). To the best of our knowledge, no notable negative impacts of dietary astaxanthin intake have been published on animal health regardless whether it was of a natural or synthetic origin, and several studies proved its safety in humans as well (Fassett and Coombes 2011). Astaxanthin is fairly stable in coconut, gingelly (sesame), groundnut, mustard, olive, rice bran, sunflower and palm oils when stored at room temperature for four straight months. The stability of astaxanthin in edible oils is attributed to the presence of other compounds such as flavonoids, polyphenols and tocopherols which are known to have stabilizing activity (Ambati 2014). Astaxanthin-enriched caseinate-stabilized emulsions were also found to be relatively stable over a range of light exposures, storage temperatures and salt levels (Liu et al. 2016). The polarity and thereby the water solubility of astaxanthin can be modified either through hemisynthesis or through molecular engineering, e.g. disodium disuccinate astaxanthin (CardaxTM) has water dispersibility of 8.64 mg/mL (Gateau et al. 2017).

17.2.2 Bixin, 9-Cis-Norbixin

Bixin (6-methyl hydrogen (9Z)-6,6'-diapocarotene-6,6'-dioate, Fig. 17.2) is an oil-soluble, yellow-orange to red, lineal diapocarotenoid composed of 25 carbon atoms and 9 double bonds with a carboxylic acid group at one end and a methyl ester

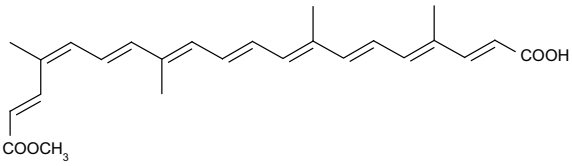

Fig. 17.2 Bixin (6-Methyl hydrogen (9Z)-6,6'-diapocarotene-6,6'-dioate)

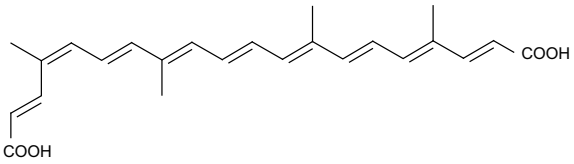

Fig. 17.3 9-*cis*-norbixin ((9Z)-6,6'-Diapocarotene-6,6'-dioic acid)

at the opposite end (Moss et al. 1993; Britton et al. 2004). De-esterification of the methyl ester end produces 9-*cis*-norbixin ((9Z)-6,6'-diapocarotene-6,6'-dioic acid, Fig. 17.3), a water (aqueous alkaline solution-)-soluble analog (Rodriguez-Amaya 2003; Scotter 2009). Both bixin and 9'-*cis*-norbixin belong to the family of xanthophylls (Mercadante et al. 1996). Bixin has two stereoisomers: *cis*- and *trans*-bixin. The pH and solubility affect the colour hue; the greater the solubility in oil, the brighter the colour (Newsome 1986): the orange *cis*-isomer is soluble in most polar organic solvents and largely insoluble in vegetable oils (Mckeown and Mark 1962; Levy and Rivadeneira 2000). It may be readily converted to the red, vegetable oil-soluble all-*trans*-isomer, which is more stable (Scotter 2011). Therefore, water-soluble, oil-soluble and oil/water-dispersible forms of annatto are also available (Newsome 1986).

Annatto (E160b; CI Natural Orange 4) is a yellow to red extract obtained from the seed coat of the fruit of a tropical (American, Caribbean, Indian, and East African) brush: achiote or lipstick trees (*Bixa orellana* L.). The pericarp of the seeds contain 4.5–5.5% pigments, and their major component (70–80%) is bixin (Preston and Rickard 1980; Najar et al. 1988; Rivera-Madrid et al. 2006; Socaciu 2007; Kirk-Othmer 2007; Ulbricht et al. 2012; deMan et al. 2018). The bixin and norbixin contents can vary from less than 1% to over 85% in different forms of preparations (e.g. dry powders, propylene glycol/monoglyceride emulsions, oil solutions, suspensions, aqueous alkaline solutions) (Tennant and O'Callaghan 2005; Kirk-Othmer 2007; Scotter 2011); additionally, bixin concentration differs greatly among the plant variants (Rivera-Madrid et al. 2006). It is used at 0.5–10 ppm resulting in hues ranging from butter yellow to peach in various dairy products like cheese (e.g. Cheddar), margarine, butter, nondairy creamers, salad dressings, ice cream, ice cream cones, sausage casings, bakery goods, savoury snack foods, coated nuts, extruded products, flavoured breakfast cereals, vegetable oils, beverages, smoked fish, spices and dietary supplements (Dendy 1966; Tirimanna 1981; Rao et al. 2002; Kirk-Othmer 2007; Zarringhalami et al. 2009; Scotter 2009; Kang et al. 2010) sometimes in combination with turmeric (Kirk-Othmer 2007). Bixin and/or norbixin are sometimes used as a substitute for saffron (Ulbricht et al. 2012). It is also used in the textile, paintings and cosmetics industries (mainly in suntan lotions) (Giuliano et al. 2003; Kang et al. 2010). Annatto extracts have not only colouring effects, but they also add a slightly sweet and peppery taste to the products (Ulbricht et al. 2012). Annatto is also used as an additive in the feed of laying hens capable of modifying egg yolk colour (Harder et al. 2007).

There are only limited toxicological data on annatto extracts, possibly because they are listed as exempt of certification in the United States (due to they derive from natural sources) (Hallagan et al. 1995), whereas its use is more restricted in the EU (Mortensen 2006). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimated the acceptable daily intake for annatto as 0–2.5 mg/kg body weight/day (JECFA 2012). Annatto extracts did not exhibit any mutagenic, carcinogenic or genotoxic effects in low amounts: 5330 ppm for mice bone marrow cells and 0.1% for rats (Alves de Lima et al. 2003; Hagiwara et al. 2003).

17.2.3 Canthaxanthin

Canthaxanthin (β,β-carotene-4,4'-dione, Fig. 17.4.) is a red-orange diketo-carotenoid that contains 13 double bonds. Canthaxanthin was first isolated from the edible mushroom, *Cantharellus cinnabarinus* (Haxo 1950); since then, it was discovered in some bacteria, cyanobacteria, many green and blue-green algae, fungi, red-feathered birds (such as flamingos, scarlet ibis, summer tanager, white-browed purpletuft, cardinals, robins, orioles, etc.), yolk of bird eggs, fish (like thrush wrasse, golden mullet, annular seabream, wild Pacific salmon, trout, common and koi carp skin, etc.) and crustacean shells (Poole et al. 2000; European Commission 2002; EFSA 2010; Mendes-Pinto et al. 2012; Tanaka et al. 2012; Brulc et al. 2013; Thomas et al. 2014; Chuyen and Eun 2015). The pink-red colour of salmonids is mediated by their ability to combine canthaxanthin with actomyosin acid complexes in their muscle (Brizio et al. 2013), and carotenoid–protein interactions are responsible for a wide range of "red" colours, i.e. in plumage samples (Mendes-Pinto et al. 2012). Furthermore, the Gram-negative bacterium *Paracoccus* sp. and the Gram-positive bacterium *Dietzia* sp. also produce it (Ye et al. 2006; Papp et al. 2013; Hojjati et al. 2014). Among others, the potential algal sources of canthaxanthin are the green alga *Haematococcus pluvialis* (Choubert and Heinrich 1993), and the microalgae *Chlorella zofingiensis* (Hua-Bin et al. 2006) and *Dactylococcus dissociatus* MT1 (Grama et al. 2014). Canthaxanthin can be obtained via total synthesis (Rosenberger et al. 1982) or biosynthesis by microorganisms (Bhosale and Bernstein 2005; Ye et al. 2006; Hu et al. 2013; Papp et al. 2013).

The colour of products prepared by using canthaxanthin (E161g) can vary from very attractive shades of vanilla yellow through pale peach to a strong reddish-orange. Canthaxanthin is classified as a colourant in the United States, and it is under

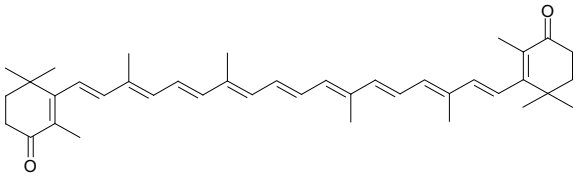

Fig. 17.4 Canthaxanthin (β,β-Carotene-4,4'-dione)

'exempt from certification' category. It is widely used as a drug, food and cosmetic colourant (e.g. for skin tanning), and it gives peach to reddish-orange colour to these products [e.g. dairy products (cheese), confectionary (soft and hard candy), fish and meat products, fruit products, beverages, snacks, beer and wine] (Aberoumand 2011; Tanka et al. 2012; Code of Federal Regulations 2019). It is rather used in water-based (than in fat-based) foods in confectionary (e.g. hard candies, fruit jellies, marzipan, sugar-coated tablets, ice cream, wafer biscuits), bakery products, fruit juices, tomato juices, soups, canned products (like frankfurter sausages, cod roe, prawns, shrimps—where by adjusting the conditions suitably, the carotenoid will adhere to the surface), primarily because of the duplication of colour shade. As a lipophilic compound, canthaxanthin accumulates in food, mostly in fatty tissues, i.e. broiler skin, egg yolks and fish fillets, and in the cuticles of crustaceans (Amaya et al. 2014). By using an appropriate grade of chlorophyll with canthaxanthin, very attractive shades of brown can be achieved for hard candies and marzipan (NIIR Board 2017). It is also widely used in poultry (e.g. broiler, laying hens) as a feed additive to improve the colour shade of yolk and for colouring the skin (Santos-Bocanegra et al. 2004; EFSA 2010; Esatbeyoglu and Rimbach 2017). As canthaxanthin has a high commercial value, its biosynthesis has also been studied extensively. It is biosynthesized from β-carotene with the help of β-carotene ketolase, which adds carbonyl groups to the carbon atoms at the 4 and 4' positions in the β-carotene molecule (Torregrosa-Crespo et al. 2018). Canthaxanthin is more stable (less susceptible to photodegradation) than carotene (Aberoumand 2011). Pectin jellies coloured with β-carotene and canthaxanthin as well as other natural colours showed excellent stability over six months stored in daylight and darkness (NIIR Board 2017). During the oxidation and degradation of carotenoids, the colour intensity decreases or can be lost; therefore, their stabilization (e.g. with antioxidants) may be necessary (Esatbeyoglu and Rimbach 2017).

Large quantities of canthaxanthin have been applied for rats without any undesirable symptoms: a tolerance test was carried out in three consecutive generations, and in a separate study, excessively high doses have been used for almost two years (in the highest dose group each animal consumed a total of about 3 kg of canthaxanthin per kg of body weight, enough for the pigmentation of 1500 ton of chicken feed). The weight development of the animals treated with the highest doses did not differ from the controls, and extensive haematological and histopathological examinations also failed to reveal any evidence of toxicity (Isler et al. 1967). The JECFA (*Joint Expert FAO/WHO Committee on Food Additives*) and SCF (*Scientific Committee on Food*) have defined an ADI (*Acceptable Daily Intake*) for canthaxanthin of 0.03 mg/kg bw that was derived from the NOAEL (*No Adverse Effect Level*) (European Commission 2002; EFSA 2010). The oral LD50 of canthaxanthin in mice is 10,000 mg/kg bw. In addition to its low acute oral toxicity, canthaxanthin is not genotoxic (EFSA 2010). Consequently, dietary canthaxanthin is considered to be safe for humans (FDA 2019; EFSA 2010), but higher doses may lead to macular crystal formation and retinopathy (Esatbeyoglu and Rimbach 2017).

17.2.4 β-Carotene

β-Carotene (Fig. 17.5.), an orange-coloured pigment, belongs to the unoxidized group of carotenoids. Its unique feature is the presence of β-ionone rings at both ends of the molecule. β-Carotene is a highly conjugated carotenoid, lacking functional groups, causing it to be highly lipophilic (Torregrosa-Crespo et al. 2018).

The use of carotenes has been approved as Group II food additives, food colours authorized at *quantum satis* by the European Commission. β-Carotene is used as a food colouring agent with the E number E160 (https://webgate.ec.europa.eu/foods_system/main/). It is a high-valued food additive, with large economic significance, due to its colouring properties and bioactivity. In food industries, β-carotene is used as an orange-red pigment in several products, such as non-alcoholic beverages with a taste of tropical fruits, cheese, pastry and ice cream (Breithaupt 2007, Bogacz-Radomska and Harasym 2018).

This pigment is abundant in green leafy plants such as parsley, spinach and broccoli, certain fruits, e.g. mandarin and peach, as well as several vegetables (carrot, pumpkin) (Del Campo et al. 2007). Originally, β-carotene was extracted from plants, mainly carrots. It is among the minor components of red pepper pigments, the main colouring principle being capsanthin (Breithaupt and Schwack 2000). Today, synthetic β-carotene is the leading product in the market, while β-carotene produced by algae has marginal significance (Zakynthinos and Varzakas 2016). Natural sources of β-carotene include the microalgae *Chlorella zofingiensis*, *Coelastrella striolata*, *Coccomyxa onubensis*, *Dunaliella* sp. and *Spirulina* (*Arthrospira*) sp. The major carotenoid produced by *Chlorella zofingiensis* is canthaxanthin (97%), whereas astaxanthin (0.7% dry weight) and β-carotene (0.9% dry weight) are among the minor carotenoid compounds (Sathasivam and Ki 2018). *Coelastrella striolata* produces mainly canthaxanthin (47.5 mg/g) but also astaxanthin (1.5 mg/g) and β-carotene (7 mg/g) (Abe et al. 2007). *Coccomyxa onubensis* provides lutein at a higher concentration (6.48 mg/g) than β-carotene (2.88 mg/g) (Vaquero et al. 2012). In *D. salina*, a higher portion of total carotenoids is made up by β-carotene (10–13% dry weight), with accompanying zeaxanthin, lutein and α-carotene (El-Baz et al. 2002; Sathasivam and Ki 2018). From the several isomers of β-carotene, 9-*cis* and all-*trans* constitute ca. 80% of the total β-carotene present in *D. bardawil* (Torregrosa-Crespo et al. 2018). The microalga *S. maxima* synthesize a wide range of carotenoids, including astaxanthin, β-carotene (80% of total carotenoids), lutein, β-cryptoxanthin, zeaxanthin, echineone, osciallaxanthin and myxoxanthoyphyll (El-Baky et al. 2003; Sathasivam and Ki 2018). On the industrial scale, β-carotene is obtained from *D. salina* cultivation, this alga being the main commercial source of natural β-carotene of microbiological origin (Bogacz-Radomska and Harasym 2018).

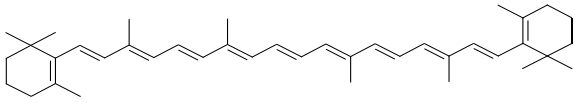

Fig. 17.5 β-Carotene (β,β-Carotene)

β-Carotene is the precursor of vitamin A, which is synthesized from carotenoids via the action of the enzyme β-carotene 15,15'-monooxygenase (Torregrosa-Crespo et al. 2018). In this group of carotenoids, β-carotene possesses the highest bioactivity as vitamin A precursor (Bogacz-Radomska and Harasym 2018).

The anti-cancer activities of carotenoids have been widely demonstrated by a large number of in vitro and in vivo studies. β-Carotene, astaxanthin, canthaxanthin and zeaxanthin were shown to reduce the sizes and numbers of liver neoplasias (Nishino et al. 2002). The dietary intake of carotenoids was reported to reduce the risk of developing colon cancer (Senesse et al. 2005; Ramadas et al. 2010). Administration of a combination of β-carotene, vitamin E and selenium was found to decrease the incidence of mortality due to cancer (Blot et al. 1993). β-Carotene was reported to have anti-angiogenic activity, by reducing the number of tumour-directed capillaries, suppressing the proliferation, migration and tube formation of endothelial cells, as well as affecting serum cytokine levels and inhibiting the activation and nuclear translocation of transcription factors (Guruvayoorappan and Kuttan 2007).

Carotenoids were reported to reduce the risk of type 2 diabetes mellitus in several studies. Higher concentrations of β-carotene in the blood are associated with lower insulin resistance (Ylonen et al. 2003; Sluijs et al. 2015). Also, it was demonstrated that serum concentrations of β-carotene, lutein, lycopene and zeaxanthin were significantly lower in diabetic subjects (Ylonen et al. 2003). In addition, serum levels of β-carotene were found to be important determinants of metabolic syndrome outcome (Suzuki et al. 2011).

As a strong antioxidant, β-carotene can prevent the formation of free radicals that can cause premature ageing of skin cells (Lee et al. 2004).

The lipophilic nature of β-carotene allows its accumulation in the body. Overconsumption of β-carotene may cause carotenosis, a benign condition when the skin turns orange. Chronic intake of high doses of β-carotene supplementation was found to correlate with increased probability of lung cancer in cigarette smokers (Tanvetyanon and Bepler 2008).

17.2.5 Crocin

Crocin can be found in the spice saffron (dried stigmas of the flowers of crocus, *Crocus sativus*, a member of the *Iridaceae* family) or in the fruits of *Gardenia jasminoides* and *G. augusta* together with other crocetin (Fig. 17.6.) glycosides (Scotter 2015). It has an intense bright yellow to orange-red colour. In this carotenoid, the C-20 dicarboxylic acid pigment crocetin (an 8,8′-diapocarotenedioic acid containing

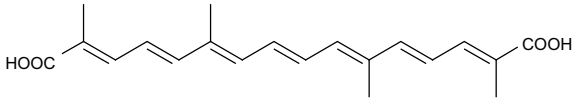

Fig. 17.6 Crocetin (8,8'-Diapocarotene-8,8'-dioic acid)

nine conjugated double bonds) is esterified with two molecules of the disaccharide gentiobiose.

Crocetin glycosides are water-soluble, slightly soluble in absolute alcohol, and insoluble in vegetable oils (Rodriguez-Amaya 2003; Kirk-Othmer 2007). Picrocrocin is a β-D-glucoside of safranal, and this molecule is responsible for the bitter taste of saffron (Alonso et al. 2001). Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), a terpene aldehyde, is the major volatile oil component (60–70%) of the essential oil in the stigma. It is responsible for the distinctive flavour of saffron (it is characterized as floral with a fatty herbaceous undertone), and it is produced by dehydration of picrocrocin during the drying process (Dris and Jain 2004). Protocrocin (a combination of two molecules of picrocrocin and one molecule of crocin) has also been found in saffron (deMan et al. 2018). *Cis*- and *trans*-isomers of crocetin glycosides carrying 1–5 glucose moieties have also been found in saffron (Tarantalis et al. 1994).

Saffron and saffron extracts have a high tinctorial strength: besides crocin, they also contain zeaxanthin and β-carotene (NIIR Board 2017). Saffron is typically used at levels of 1-260 ppm in culinary (e.g. rice dishes, soups, cheese, meat dishes), bakery, sugar confectionery products, alcoholic and non-alcoholic beverages (Maga 1994). The amounts generally needed are extremely low (1–10 mg/kg) except for alcoholic drinks and meats (20–200 mg/kg). It exhibits moderately good resistance to heat (NIIR Board 2017). The production of saffron is very labour-intensive (about 165,000 flowers are needed for 1 kg of saffron stigmas containing about 50 g of the pigment, Kirk-Othmer 2007), therefore the extremely high cost severely limits its use as a food colourant, and it is rather considered as a spice within the EU. In the United States, crocetin is listed as a colour additive exempt from certification. The less expensive *Gardenia* species grow in the Far East, and here they are used to colour sugar and flour confectionery, dairy products, jams, preserves, rice, pasta, ices, noodles, cakes, beans, smoked white fish (such as cod and haddock, where it binds to the flesh) and other dishes. They do not have the same flavour as saffron, only their colour is similar (Maga 1994). Gardenia yellow is a colourant produced by extraction of the fruits of the mentioned *Gardenia* species with ethanol or water; it is an approved food colourant in Japan and China, but not in the United States and the EU (Solymosi et al. 2015).

The LD_{50} value of saffron was 4120 ± 556 mg/kg after oral administration in BALB/c mice and that of crocetin was 1–5 g/kg IP for male Razi mice and Wistar rats. After intraperitoneal administration, the LD_{50} values of safranal were 1.48, 1.88 and 1.50 ml/kg in male mice, female mice and male Wistar rats, respectively (Hosseinzadeh et al. 2010, 2013; Bostan et al. 2017).

To the best of our knowledge, crocin itself has not been found in any microalgae. But some microalgae may contain carotenoid glycosides (e.g. (2′S)-2-hydroxymyxol 2′-α-L-fucoside was found in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*; Nagy et al. 2018), and therefore may be able to be used for microalgal production of carotenoid glycosides, such as crocin.

17.2.6 β-Cryptoxanthin

β-Cryptoxanthin ((3*R*)-β,β-caroten-3-ol, Fig. 17.7) is an orange, yellow-orange 3-hydroxy-β-carotene with 11 conjugated double bonds. It is enzymatically converted from β-carotene in plants, and it is oxidized and isomerized in the presence of light (Li et al. 2015). It occurs in petals of some flowers and several fruits such as peach, nectarine, persimmon, apricot, citrus species [particularly in the Satsuma mandarin (*Citrus unshiu* Marc.) but also in sweet oranges, tangerines, Willowleaf mandarin, Wase satsuma, Hansen mandarin, clementine, caja, etc.], fig, avocados, Brazilian cherry, watermelon, mango, papaya, tree tomato and apple but it can also be found in some vegetables (e.g. broccoli, cabbage, cauliflower, kale, parsley, lettuce, pumpkin, asparagus, onion, carrot, sweet corn, sweet red pepper and hot chilli peppers) (Mangels et al. 1993; Breithaupt and Bamedi 2001; Socaciu 2007; Tanka et al. 2012; Yemis et al. 2012; Wei et al. 2014; Llopis et al. 2019). It can also be produced by microorganisms (Bhosale and Bernstein 2005), and it was also found, e.g. in egg yolk or cow's milk (Heying et al. 2014; Rodriguez-Amaya, 2015). β-Cryptoxanthin is mostly esterified with lauric, myristic and palmitic acids in the flesh of ripened citrus fruits, and only 5–20% remains in free form (Mercadante et al. 2017; Ma et al. 2017; Llopis et al. 2019).

β-Cryptoxanthin (E161c) is a yellow food colouring (Moss et al. 1993). Observational, in vitro, animal and human studies suggest that β-cryptoxanthin has relatively high bioavailability from its common food sources (Burri et al. 2016). In some European countries, the human intake of β-cryptoxanthin from vegetables can be considerable (0.05–1.36 mg/day) depending on the eating habits: it is one of the major xanthophyll carotenoids in the human plasma. It can be used as a biomarker of citrus fruit consumption in Japan, which is higher than in Western populations (Sugiura et al. 2002; Bhosale and Bernstein 2005; Aizawa and Inakuma 2007; Llopis et al. 2019). The European Food Safety Authority considers this carotenoid as safe for the consumers (EFSA 2006), and epidemiological studies on β-cryptoxanthin intake and human health effects generally report no toxic effects (Nomura et al. 1997). It is used as a colouring agent for food products in certain countries (Torregrosa-Crespo et al. 2018). The colour and carotenoid changes of pasteurized orange juices were tested during storage, and a decrease of β-cryptoxanthin was observed only at higher temperatures (Cortés et al. 2006; Wibowo et al. 2015).

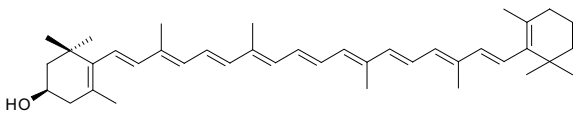

Fig. 17.7 β-Cryptoxanthin ((3*R*)-β,β-Caroten-3-ol)

17.2.7 Lutein

Lutein (β,ε-carotene-3,3'-diol, Fig. 17.8.) is a xanthophyll, with only ten conjugated double bonds, and consequently, a more yellowish-green colour compared to other carotenoids (Torregrosa-Crespo et al. 2018). Lutein is one of the xanthophylls of industrial importance, together with astaxanthin and canthaxanthin (Breithaupt 2007). An increasing interest can be observed in the application of xanthophylls, mainly lutein, in human food, as well as animal feed (Breithaupt 2007). Lutein has been authorized by the European Commission as a food additive in Group III, Food colours with a combined maximum limit, associated with the E number E161b (https://webgate.ec.europa.eu/foods_system/main/).

Lutein is not produced synthetically, due to a lengthy synthesis (Breithaupt 2007). This carotenoid is the main xanthophyll found in the majority of green plants, such as spinach and kale, mainly in the leaves, but also in the petals of flowers, and in yellow carrot (Breithaupt 2007; Torregrosa-Crespo et al. 2018). Other natural sources of lutein include microalgae, which may contain 0.5–1.2% lutein (Lin et al. 2015a, b; Chen et al. 2018). A wide array of microalgae synthesizes lutein, together with astaxanthin (*Auxenochlorella protothecoides*, *Chlorella* sp., *Coelastrella* sp., *Parachlorella kessleri*, *Scenedesmus* sp. and *Vischeria stellata*). Lutein is the major carotenoid produced by microalgae *Ch. protothecoides*, *Galdieria sulphuraria* and *S. almeriensis* (Sathasivam and Ki 2018). *Coccomyxa onubensis* and *Muriellopsis* sp. are also potential sources of lutein at concentrations of 6.48 and 6.51 mg/g, respectively (Del Campo et al. 2000; Vaquero et al. 2012). In *C. pyrenoidosa,* lutein makes up only 0.2–0.4% of dry weight (Wu et al. 2007).

A test on the effects of adding microalgae to bread dough revealed that up to 3.0 g of *C. vulgaris*/100 g of wheat flour, the impact of microalgae was positive on dough rheology and viscoelastic characteristics, with the strengthening of gluten network. However, consumer preferences were negative, due to a strong marine taste (Graca et al. 2018).

Lutein and zeaxanthin are the two major components of the macular pigment of the retina (Torregrosa-Crespo et al. 2018), and maintaining their level in the lens and retina of the eye is thought to be essential in protecting vision in older people (Junghans et al. 2001). Consumption of lutein and zeaxanthin shows a positive correlation with the prevention of age-related macular degeneration (AMD) (Olmedilla et al. 2001). Also, dietary supplementation of lutein alone or lutein combined with other nutrients can improve visual function in patients suffering from AMD (Richer et al. 2004).

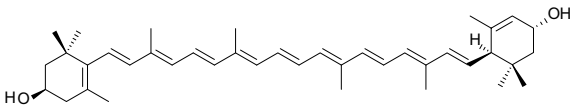

Fig. 17.8 Lutein ((3*R*,3'*R*,6'*R*)-β,ε-Carotene-3,3'-diol)

Lutein is present at a high concentration in the human serum and breast milk. Infant formulas fortified with lutein and β-carotene were found to be safe, well-tolerated and supported the physical growth of infants (Kon et al. 2014).

In addition, as an antioxidant agent, lutein was shown to protect against UV-induced oxidative damage in the epidermal and dermal layers of skin (Lee et al. 2004).

17.2.8 Lycopene

Lycopene (ψ,ψ-carotene, Fig. 17.9.) is a carotenoid with a highly unsaturated hydrocarbon chain, consisting of 11 conjugated and 2 unconjugated double bonds, lacking the terminal β-ionone ring. Due to the presence of double bonds, it can exist in both *cis*- and *trans*-isomeric forms, in nature being present primarily in the all-*trans* isomeric form. Lycopene is highly stable under the conditions of thermal processing and storage (Torregrosa-Crespo et al. 2018).

Lycopene is responsible for the red colour in several fruits and vegetables, such as tomatoes, watermelon, papaya, apricots and guava (Kong et al. 2010; Torregrosa-Crespo et al. 2018). The use of lycopene as a food additive and colouring agent has been approved by the European Commission (https://webgate.ec.europa.eu/foods_system/main/), being associated with the E number E160d when used as a colouring agent for food products. Although lycopene is found abundantly in natural sources, mainly tomatoes, it is not widely used as a food antioxidant (Carocho et al. 2015).

Attempts have been made to interfere with steps of carotenoid biosynthesis in microalgae in order to increase the production of lycopene. An important step of the carotenoid pathway leading to the formation of α- and β-carotenes is the synthesis of the linear lycopene molecule, which is a substrate for the enzymes lycopene beta- and epsilon cyclases, which are responsible for adding beta and epsilon rings, respectively, to the two ends of lycopene (Yildirim et al. 2017). Molecules such as imidazoles, pyridines and nicotine have been used to regulate carotenoid biosynthesis, due to their ability to inhibit cyclization of the lycopene molecule. The effect of nicotine on carotenoid production was studied in the microalgae *Dunaliella bardawil* and *D. salina*, but only trace amounts of lycopene were detected in the final product (Shaish et al. 1990; Fazeli et al. 2009). In the unicellular alga, *Chlorella regularis,* nicotine successfully inhibited the cyclization of lycopene; however, it also severely affected cell growth due to its toxicity (Ishikava and Hiroshi 2004). Triethylamine was found to enhance lycopene accumulation in *D. bardawil*, but it negatively influenced cell growth even at low concentrations (Liang et al. 2016).

Lycopene is known to effectively scavenge singlet oxygen (Di Mascio et al. 1989). The 5-*cis* form is the most stable isomer of lycopene and shows the highest antioxidant

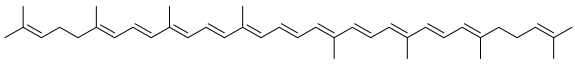

Fig. 17.9 Lycopene (ψ,ψ-Carotene)

activity, followed by the 9-*cis*, 7-*cis*, 13-*cis*, 11-*cis* and all-*trans* isomeric forms (Rao and Rao 2007).

Lycopene was found to exhibit much higher anti-cancer potential than most other carotenoids, significantly reducing tumour cell growth in various tumour cell lines (Nishino et al. 2002; Graydon et al. 2007; Schwarz et al. 2008). The protective effect of lycopene against prostate cancer was demonstrated by Stacewicz-Sapuntzakis and Bowen (2005), as well as Giovanucci (2011).

In addition, lycopene has prophylactic and/or therapeutic effects in various central nervous system disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, cerebal ischemia, epilepsy and depression (Chen et al. 2019).

Supplementing antioxidant micronutrients such as lycopene was reported to reduce skin roughness (Heinrich et al. 2006). Another study demonstrated that individuals with higher levels of antioxidants, e.g. lycopene, showed fewer furrows and wrinkles than those with lower levels of antioxidants (Darvin et al. 2008).

17.2.9 Zeaxanthin

Zeaxanthin (β,β-carotene-3,3'-diol, Fig. 17.10.) is one of the most common carotenoid alcohols present in nature. It is a polyene-like molecule with nine alternating conjugated double and single bonds. The carbon backbone is terminated at each end by an ionone ring, to which a hydroxyl group is attached (Sajilata et al. 2008). Zeaxanthin is isomeric with lutein, differing only in the location of a double bond: in zeaxanthin, all the double bonds are conjugated. As a food colourant, it is associated with the E number E161h (Torregrosa-Crespo et al. 2018).

It is synthesized by several plants (e.g. paprika, corn, saffron, wolfberries) and certain microorganisms (Torregrosa-Crespo et al. 2018). For example, the microalgae *Corallina* sp., *Dunaliella bardawil* and *D. parva*, *Erythrotrichia carnea*, *Jania* sp., *Microcystis aeruginosa*, *Prochloron* sp. and *Pleurochloris commutata* were reported to produce zeaxanthin (Withers et al. 1978; Ben-Amotz et al. 1982; Andrew and Britton 1990, Palermo et al. 1991; Chen et al. 2005). The microalga *Porphyridium cruentum* produces only two carotenoids: zeaxanthin and β-carotene (97.4% and 2.6% of total carotenoids, respectively) (Schubert et al. 2006; de Jesus Raposo et al. 2015; Sathasivam and Ki 2018). Also, the blue-green algae *Cyanophora paradoxa* and *Glaucocystis nostochinearum* synthesize only zeaxanthin and β-carotene (Goodwin 1976). From *Dunaliella salina,* a zeaxanthin-overproducing mutant strain *zea1* was generated, which may be a potential candidate for commercial exploitation. Since the mutant strain is deficient regarding the zeaxanthin-epoxidation step, it lacks

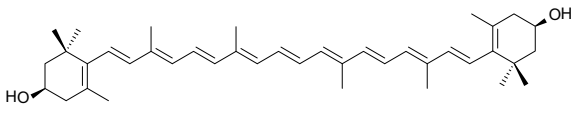

Fig. 17.10 Zeaxanthin ((3*R*,3'*R*)-β,β-Carotene-3,3'-diol)

neoxanthin, violaxanthin and antheraxanthin but accumulates zeaxanthin even under normal growth conditions (low light). Under these conditions, the mutant strain is able to produce 15-fold higher zeaxanthin content (6 mg/g dry weight) compared to the wild type (Jin et al. 2003). Zeaxanthin is one of the major carotenoids produced by the blue-green alga *Spirulina* and is rapidly converted to astaxanthin, thereby enhancing the carapace colour of prawns, when prawns are fed with *Spirulina*-supplemented diet (Liao et al. 1993). Similarly, *Spirulina* increases the yellowness and redness of broiled chicken due to accumulation of zeaxanthin within the flesh (Toyomizu et al. 2001). The green alga *Neospongiococcum* is among FDA-approved alga strains that are generally recognized as safe for feeding poultry to enhance yellow pigmentation (Sajilata et al. 2008). *N. excentricum* can produce up to 0.65% xanthophylls (Liao et al. 1995).

Similarly to lutein, zeaxanthin is present in significant concentrations in human milk (Costa et al. 2015). Furthermore, zeaxanthin is one of the major components of the macular pigment of the retina, and together with lutein perform a critical role in the prevention of AMD (Olmedilla et al. 2001). The above two pigments may also be protective against the age-related increase in lens density and cataract formation (Sajilata et al. 2008).

The safety of zeaxanthin in supplements has been confirmed by the New Dietary Ingredient Notification. The acceptable daily intake of zeaxanthin is 0–2 mg/kg body weight (Sajilata et al. 2008).

17.3 Conclusion

Food additives have been used for flavouring, to extend shelf life, promote safety and attract consumers for ages. The majority of carotenoids used in food, healthcare and cosmetic industries are synthesized chemically, and only a smaller portion is obtained from natural sources such as plants and algae. Currently, however, the demand for products with natural ingredients is growing. Extracting carotenoids from cultivated algae may provide an alternative source of natural carotenoids (Cardoso et al. 2017; Sathasivam and Ki 2018).

Microalgae present a rich source of biologically valuable compounds such as vitamins, proteins, polysaccharides, fatty acids, minerals, photosynthetic pigments (carotenoids, chlorophylls), enzymes and fibre (Mimouni et al. 2012; Matos et al. 2017; Sathasivam and Ki 2018). Consumption of microalgae can provide health benefits, due to their antioxidant, anti-inflammatory, anti-mutagenic and anti-microbial properties (Matos et al. 2017).

Natural compounds are 2–10 times more expensive than synthetic ones and are often not very convenient or easy-to-handle stock commodities. Microalgae constitute a very promising platform for the production of high-value molecules, including natural carotenoids (Gateau et al. 2017).

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Chapter 18
Nutraceutical and Pharmaceutical Applications of Carotenoids

Masaki Honda

Abstract Carotenoids, the most common fat-soluble plant pigments in nature, play important roles in scavenging peroxyl radicals and singlet oxygen in human. Dietary carotenoids are considered to be beneficial for the prevention of various diseases, including certain cancers, cardiovascular disease, and eye disease. Due to such bioactivities of carotenoids, they are often used in nutritional supplements, skin care cosmetics, and pharmaceutical products as well as in a natural color for foods and feeds. In particular, the demands of β-carotene, lycopene, astaxanthin, lutein, zeaxanthin, β-cryptoxanthin, and fucoxanthin are increasing for nutraceutical and pharmaceutical applications in recent years. Although carotenoids exhibit high antioxidant activity in common, the bioactivity varies depending on the structure, e.g., lutein and zeaxanthin are effective for the prevention of eye disease, and fucoxanthin exhibits a strong anti-obesity activity. The objective of this contribution is to review the bioactivities and applications of commercially important carotenoids. Furthermore, the aspects of the recent progress in processing technology of carotenoids for nutraceutical and pharmaceutical applications are also included in this review.

Keywords Carotenoid · Bioactivity · Application · Market size · *E*/*Z*-isomer

18.1 Bioactivity of Carotenoids

Most carotenoids have a 40-carbon basal structure including a system of conjugated double bonds. Carotenoids can be roughly classified into the following two groups: (1) carotenes, which are nonoxygenated molecules such as lycopene, α-carotene, and β-carotene; (2) xanthophylls, which are oxygen-containing molecules such as lutein, astaxanthin, and fucoxanthin (Fig. 18.1), and more than 750 naturally occurring carotenoids have been identified until now (Maoka 2009).

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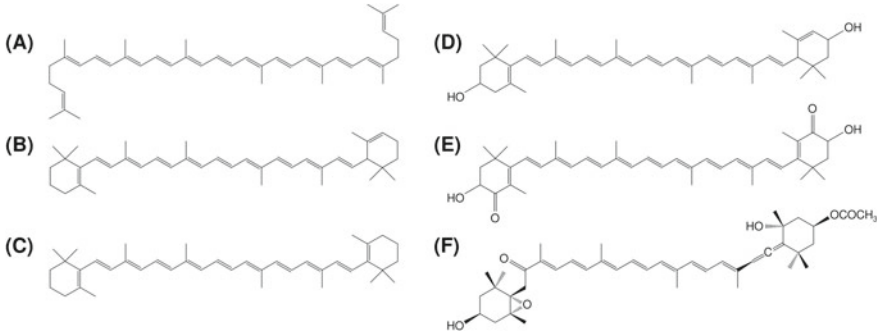

Fig. 18.1 Chemical structures of **a** (all-*E*)-lycopene, **b** (all-*E*)-α-carotene, **c** (all-*E*)-β-carotene, **d** (all-*E*)-lutein, **e** (all-*E*)-astaxanthin, and **f** (all-*E*)-fucoxanthin

Carotenoids show strong antioxidant activity such as peroxyl radical and singlet oxygen scavenging activities and the activity are related to the number of conjugated double bonds in the molecule; carotenoids with more than 9 conjugated double bonds display antioxidant activity (Di Mascio et al. 1989; Müller et al. 2011a). Generally, the antioxidant activity is enhanced with increasing the number of conjugated double bonds. For example, astaxanthin, which contains 13 conjugated double bonds, shows a higher singlet oxygen scavenging activity than that of β-carotene, which contains 11 conjugated double bonds (Di Mascio et al. 1989; Ouchi et al. 2010). In addition, each carotenoid has unique bioactivity caused by the specific structures in the molecule such as conjugated carbonyl and allene groups. Moreover, several carotenoids such as β-carotene and β-cryptoxanthin are very important as provitamin A. Since human and nonhuman animals cannot synthesize carotenoids, the pigments must be supplemented in the diets, and thus the proper understanding of their influence on the body is very important. The typical bioactivities of carotenoids are summarized in Fig. 18.2 and the details are as follows .

18.1.1 Lycopene

Lycopene is an acyclic carotenoid principally responsible for the bright red color of ripe tomatoes and tomato-based products as well as watermelons and gac (*Momordica cochinchinensis* Spreng). Lycopene with 11 conjugated double bonds shows especially powerful antioxidant activity of the carotenoids. Di Mascio et al. (1989) have reported that singlet oxygen scavenging activity of carotenoids, when β-carotene is defined as a reference (=1.0), was higher in following order: lycopene (2.2) > γ-carotene (1.8) > astaxanthin (1.7) > canthaxanthin (1.5) > α-carotene (1.4) > β-carotene (1.0) > zeaxanthin (0.7) > lutein (0.6) > α-tocopherol (0.02). The singlet

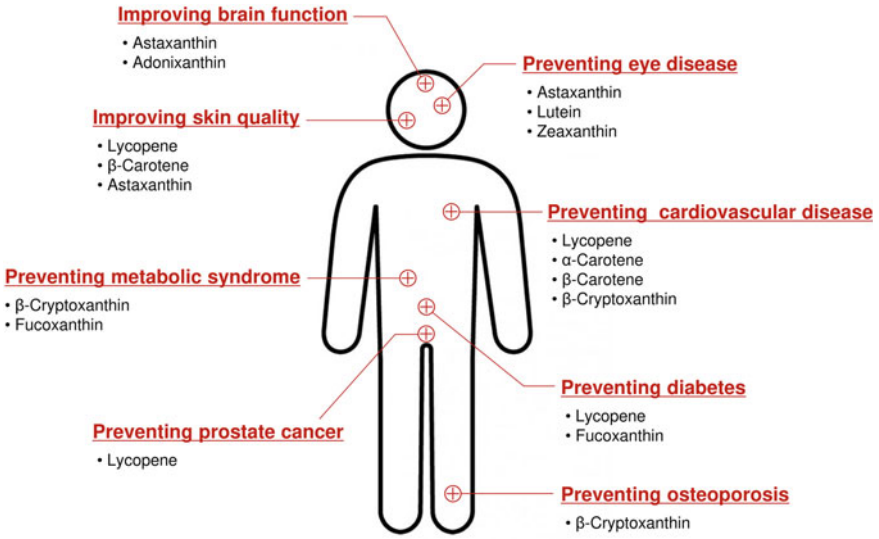

Fig. 18.2 Typical health effects of carotenoids

oxygen scavenging activity of lycopene was one hundred or more times as high as that of α-tocopherol. Ample studies have demonstrated that the daily intake of lycopene-rich foods can significantly reduce risk of cancer and cardiovascular disease (Mordente et al. 2011; Wang et al. 2010). Lycopene is accumulated in various tissues and organs of human, e.g., liver, intestine, and prostate, by eating lycopene-rich foods such as tomatoes. Especially, a large amount of lycopene is accumulated in prostate and ample studies have addressed that higher dietary and circulating lycopene concentrations are inversely associated with prostate cancer risk. For example, Etminan et al. (2004) reported that high intakes of tomato or tomato-based products were associated with approximately 10 to 20% reduction in prostate cancer risk, which was investigated in a meta-analysis of studies published from 1966 to 2003. Experimental and epidemiological studies also demonstrated that lycopene would act as a chemopreventive agent against lung cancer, gastric cancer, and breast cancer (Mein et al. 2008; Wang et al. 2010). Moreover, several studies suggested that oxidized low-density lipoprotein (LDL) is implicated in the pathogenesis of atherosclerosis, and the atherosclerosis could be attenuated by lycopene, which inhibit LDL oxidation (Cheng et al. 2017; Palozza et al. 2010). In fact, some dietary studies have demonstrated that tomato products rich in lycopene increase resistance of LDL to oxidation against human (Silaste et al. 2007; Upritchard et al. 2000). Besides the above, several reports suggested that lycopene may have antidiabetic and anti-obesity activities as well as improvement in skin type (Darvin et al. 2008; Kuhad et al. 2008; Marcotorchino et al. 2012). There are especially many reports on health benefits of lycopene compared to other carotenoids and active empirical research on lycopene bioactivity has been carried out in recent years. Furthermore,

since lycopene is abundant in the tomato, which is one of the most popular and widely consumed vegetables in the world, the carotenoid is easily ingestible. Thus, it is considered that the marketplace is expected to further expand.

18.1.2 β-Carotene

β-Carotene, which has two unsubstituted β-ionone ring half-sites and 11 conjugated double bonds, is a red-orange pigment found in many vegetables such as carrots and spinach. β-Carotene is an important vitamin A source for human and the method for industrial synthesis was established in half a century ago, so that the basic research of the carotenoid has been done since quite a long time ago compared to other carotenoids. Several studies reported that β-carotene would reduce risk of some cancers such as stomach cancer and skin cancer (Blot et al. 1993; Köpcke and Krutmann 2008). On the other hand, some large-scale intervention studies of β-carotene supplementation has not been shown any beneficial effect on cancer prevention or shown an adverse effect on the incidence of lung cancer and gastric cancer with 20–30 mg of β-carotene per day in smokers and workers exposed to asbestos (Albanes et al. 1996; Omenn et al. 1996). Several researchers indicated that one of the largest factors of that would excess intake of β-carotene: the normal intake of carotenoids is approximately 1.5 mg per day for people in the United States (Druesne-Pecollo et al. 2010). In fact, Siems et al. (2005) indicated that, obviously, on certain conditions of high-dose carotenoid supplementation, both the antioxidant and prooxidant reactions arise. Therefore, smokers and workers exposed to asbestos should avoid high-dose β-carotene supplementation. β-Carotene would be effective against erythropoietic protoporphyria. Ample studies have demonstrated that the oral dose of β-carotene relieves the photosensitivity associated with the disease (Mathews-Roth et al. 1974, 1977). In addition, the dietary β-carotene could have an age-related macular degeneration-preventing effect (West et al. 1994). The simultaneous ingestion of β-carotene, zinc, and antioxidants such as vitamin C and vitamin E would further enhance the preventive effect, i.e., trial participants who received a daily high-dose supplement of β-carotene (15 mg), vitamin C (500 mg), vitamin E (400 IU), and zinc (80 mg) had an approximately 25% reduced risk of progression from relatively advanced early age-related macular degeneration stages to late age-related macular degeneration (Age-Related Eye Disease Study Research Group 2001). On the other hand, Tan et al. (2008) indicated that higher β-carotene intake may be associated with a risk increase of age-related macular degeneration. From the above, β-carotene shows various health benefits, especially high provitamin A activity, but it is considered important to be aware of proper intake.

18.1.3 Astaxanthin

A xanthophyll, astaxanthin, is principally responsible for the dark-red color and is present in various marine animals such as salmon, trout, red seabream, and microorganisms such as *Haematococcus pluvialis* and *Paracoccus carotinifaciens* (Ambati et al. 2014). It has a structure composed of 2-β ionone rings and a conjugated polyene chain at the center, with hydroxyl and keto moieties on each ionone ring. Astaxanthin has 13 conjugated double bonds in the molecule, and thus the carotenoid shows very high antioxidant activity (Di Mascio et al. 1989; Ouchi et al. 2010). As with other carotenoids, ample studies indicated that astaxanthin would reduce risk of certain cancers, e.g., lung cancer and colon cancer (Kowshik et al. 2014; Palozza et al. 2009). Several researches have indicated that astaxanthin exerts anti-apoptosis, anti-proliferative, and anti-invasion influences via different molecules and pathways: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription 3 (STAT-3), and peroxisome proliferator-activated receptor gamma (PPARγ) (Zhang and Wang 2015). Astaxanthin is closely involved in eye health. Astaxanthin is capable of passing the blood-brain barrier and will deposit in the retina of mammals (Dhankha et al. 2012). Several studies indicated that the daily astaxanthin intake could improve eye fatigue such as against visual display terminal workers, and it might increase retinal capillary blood flow in eye (Kidd 2011; Nagaki et al. 2005, 2006). In addition, several studies reported that dietary astaxanthin can accumulate in animal brain and indicated that the carotenoid may be useful as a preventive and/or therapeutic agent for Alzheimer type dementia (Che et al. 2018; Manabe et al. 2018; Rahman et al. 2019). For example, Rahman et al. (2019) investigated the role of astaxanthin on hippocampal insulin resistance in rat model of Alzheimer's disease, and revealed that astaxanthin showed neuroprotective and anti-amyloidogenic effects in neurohistology. Moreover, ample studies indicated that astaxanthin would have a skin protecting function. Namely, astaxanthin can prevent skin thickening and reduce collagen reduction against UV-induced skin damage, and topical administration of astaxanthin liposomes containing cationic lipid inhibited melanin production in skin exposed to UV (Ambati et al. 2014; Hama et al. 2012; Yamashita 2013).

18.1.4 Lutein and Zeaxanthin

Lutein and zeaxanthin are xanthophylls, which are principally responsible for yellow-orange color found in vegetables such as artichoke and corn as well as in egg yolk (Perry et al. 2009). Both carotenoids have high singlet oxygen quenching activitiy (Di Mascio et al. 1989) and dietary lutein and zeaxanthin would have a potential role in the prevention and treatment of several cancers such as colorectal cancer and lung cancer (Gallicchio et al. 2008; Männistö et al. 2006). Lutein and zeaxanthin are concentrated in the primate retina, where they form the macular pigment, and thus

multiple studies have focused on their beneficial roles in eye health. Most clinical epidemiological researches supported that lutein and zeaxanthin have reduce the risk of some eye diseases, e.g., age-related macular degeneration, cataract, and retinitis pigmentosa (Ma and Lin 2010; Stringham et al. 2010). The powerful blue-light filtering activity and antioxidant activity of these carotenoids may be responsible for the protective effects (Junghans et al. 2001; Kijlstra et al. 2012). Moreover, lutein and zeaxanthin are present in human skin, and animal studies have provided evidence of significant efficacy against UV-induced skin damage (Roberts et al. 2009).

18.1.5 β-Cryptoxanthin

β-Cryptoxanthin, a major source of vitamin A, is a yellow xanthophyll found abundantly in red chili, papaya, and Satsuma mandarin (*Citrus unshiu* Marc.) (Breithaupt and Bamedi 2001; Takayanagi et al. 2011). β-Cryptoxanthin may be effective for reduction in the risks of lung and oral cancers and stimulate the repair of DNA oxidation damage due to strong antioxidant activity (Leoncini et al. 2015; Lorenzo et al. 2008; Männistö et al. 2004). A number of studies have addressed that the carotenoid would have the preventive role in osteoporosis. β-Cryptoxanthin has stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption, and they induce increase of bone mass (Yamaguchi and Uchiyama 2003, 2004). In addition, the carotenoid has an effect on the gene expression of several proteins which are associated with osteoblastic bone formation and osteoclastic bone resorption (Uchiyama and Yamaguchi 2005; Yamaguchi 2012). Some epidemiological studies also support that the intake of foods rich in β-cryptoxanthin may reduce risks of osteoporosis and osteoarthritis (Sugiura et al. 2008; Wang et al. 2007). Moreover, β-cryptoxanthin has anti-obesity activity (Shirakura et al. 2011; Takayanagi et al. 2011). For example, Takayanagi et al. (2011) reported that oral administration of Satsuma mandarin (*Citrus unshiu* Marc.) rich in β-cryptoxanthin repressed body weight, abdominal adipose tissue weight, and serum lipid concentrations in obese model mouse. They also confirmed by gene expression analysis that the carotenoid can alter cytokine secretion and cell proliferation. In addition, high plasma levels of β-cryptoxanthin were associated with a lower risk of atherosclerosis (Nakamura et al. 2006).

18.1.6 Fucoxanthin

Fucoxanthin, an allenic xanthophyll, is an orange-colored pigment found abundantly in edible brown seaweeds such as kombu (*Laminaria japonica*) and wakame (*Undaria pinnatifida*) (D'Orazio et al. 2012). As with other carotenoids, fucoxanthin had strong anti-cancer and anti-angiogenic activities as well as high antioxidant activity (Hosokawa et al. 2004; Sachindra et al. 2007; Sugawara et al. 2006;

Terasaki et al. 2019). For example, Hosokawa et al. (2004) reported that fucoxanthin significantly reduced the viability of human colon cancer cell lines such as HT-29, Caco-2, and DLD-1 cells. In addition, fucoxanthin has gained much attention for its anti-obesity and anti-diabetic effects due to a unique mechanism. Maeda et al. (2005) reported that fucoxanthin induces uncoupling protein 1 (UCP1) expression in white adipose tissue (WAT), and UCP1 can dissipate energy via fatty acid oxidation and heat production. Moreover, the carotenoid enhanced insulin resistance and improved blood glucose levels via the up-regulation of glucose transporter 4 (GLUT4) in skeletal muscle and downregulation of adipocytokines related to insulin resistance in WAT (Maeda 2015; Maeda et al. 2009; Miyashita et al. 2010). Thus, foods rich in fucoxanthin, such as brown seaweeds, are expected to have a preventive effect on metabolic syndrome.

18.1.7 Other Carotenoids

In addition to the above carotenoids, functional studies of α-carotene, canthaxanthin, and capsanthin are well documented. These carotenoids have powerful antioxidant activity (Di Mascio et al. 1989; Ouchi et al. 2010) and may have anti-cancer activity (Kim et al. 2009; Tanaka et al. 2012) like other carotenoids. For example, epidemiological and animal studies indicated that capsanthin appears to possibly exert a potent inhibitory effect on colon carcinogenesis (Kim et al. 2009; Narisawa et al. 2000). Moreover, multiple studies have reported some other health benefits of α-carotene, canthaxanthin, and capsanthin: high plasma levels of α-carotene were associated with a lower risk of atherosclerosis (D'Odorico et al. 2000); canthaxanthin would have immunomodulatory activity, i.e., the carotenoid enhanced the proliferation and function of immune competent cells (Esatbeyoglu and Rimbach 2017); dietary capsanthin increased in plasma HDL-cholesterol level (Aizawa and Inakuma 2009). In recent years, bioactivities of several rare carotenoids such as adonixanthin and siphonaxanthin are spotlighted. In detail, adonixanthin found abundantly in astaxanthin synthesize bacteria such as *Paracoccus* sp. and *Agrobacterium* sp. have very high antioxidant activity of carotenoids (Maoka et al. 2013). Furthermore, Iwata et al. (2018) reported that dietary adonixanthin may protect against brain damage induced by cerebral hemorrhage, including structural and functional symptoms. Siphonaxanthin, a marine carotenoid from green algae such as *Codium fragile* and *Caulerpa lentillifera* (Sugawara et al. 2014), effectively induced apoptosis in human leukemia (HL-60) cells (Ganesan et al. 2011) and inhibited angiogenesis by downregulating fibroblast growth factor 2 (FGF-2)-mediated intracellular signals in vascular endothelial cells (Ganesan et al. 2013). More than 750 naturally occurring carotenoids had been reported until now, but their functional studies have been carried out only in part. Hence, further progress in this research area is expected in the future.

18.1.8 Z-Isomer of Carotenoids

Since carotenoids contain multiple conjugated double bonds, a large number of geometric isomers are theoretically possible. In fact, *Z*-isomers of carotenoids are widely present in plants, processed foods, and animal bodies. For example, more than 20% of total lycopene is present as the *Z*-isomers in some processed tomato products such as tomato sauce and tomato soup (Honda et al. 2017a), and more than 30% of total astaxanthin is present as the *Z*-isomers in human plasma (Coral-Hinostroza et al. 2004). Recently, ample studies have addressed that *Z*-isomers of carotenoids such as lycopene and astaxanthin showed higher bioavailability and antioxidant activity than the all-*E*-isomers (Cooperstone et al. 2015; Müller et al. 2011; Yang et al. 2017a). For example, a human oral administration test reported by Cooperstone et al. (2015) showed that *Z*-isomer-rich tangerine tomato juice (containing 94% total *Z*-isomer content of lycopene) was over 8 times more bioavailable than an all-*E*-isomer-rich red tomato juice (containing 10% total *Z*-isomer content of lycopene). On the other hand, several studies have reported that *Z*-isomers of β-carotene showed less bioavailability than the all-*E*-isomer (During et al. 2002; Gaziano et al. 1995). In fact, although a great amount of the *Z*-isomers of lycopene (>50% of the total lycopene) and astaxanthin (>30% of the total lycopene) was present in human serum, the total amount of β-carotene *Z*-isomers was less than 10% (Coral-Hinostroza et al. 2004; Stahl et al. 1992). In addition, although there are limited evidences, several carotenoid *Z*-isomers exhibit higher bioactivities such as anti-cancer activity and anti-atherosclerosis effect than the all-*E*-isomers. For example, (9*Z*)-β-carotene from the green halophilic alga *Dunaliella* sp. would have higher anti-atherogenesis and anti-atherosclerosis effects than the all-*E*-isomer (Harai et al. 2008; Relevy et al. 2015). Nakazawa et al. (2009) reported that fucoxanthin *Z*-isomers showed higher anti-cancer activity than the all-*E*-isomer, that is evaluated by measuring the potent inhibitory effects on human promyelocytic leukemia cells (HL-50) and colon cancer cells (Caco-2). These functional changes between all-*E*-isomer and *Z*-isomers of carotenoids may be caused by the change in the physicochemical properties such as solubility and crystallinity (Honda et al. 2018a; Murakami et al. 2017), e.g., Honda et al. (2018a) reported that the solubility in ethanol of astaxanthin containing 63.2% *Z*-isomer was approximately 700 times higher than that of the all-*E*-isomer, and (all-*E*)-astaxanthin was present in the crystal state, while the *Z*-isomers were present in an amorphous state, that was confirmed by differential scanning calorimetry (DSC), powder X-ray diffraction (XRD), and scanning electron microscopy (SEM) analyses. The effects of *Z*-isomerization of carotenoids on the bioavailability, antioxidant activity, and bioactivity are summarized in Table 18.1. To my best knowledge, among the over 750 naturally occurring carotenoids, only approximately 10 carotenoids have been characterized in terms of the effects of *Z*-isomerization. Thus, there is still considerable room for development of this research area.

Table 18.1 Summary of the effects of *Z*-isomerization of carotenoids on the bioavailability, antioxidant activity, and bioactivity

Carotenoids	Bioavailability	Antioxidant activity	Bioactivity
Lycopene	• $E^a < Z^b$ (Sun et al. 2016) • $E < Z$ (Cooperstone et al. 2015) • $E < Z$ (Unlu et al. 2007)	• $E \lesssim Z$ (Müller et al. 2011b) • $E \lesssim Z$ (Böhm et al. 2002)	–
α-Carotene	–	• $E \approx Z$ (Böhm et al. 2002)	–
β-Carotene	• $E > Z$ (Deming et al. 2002) • $E > Z$ (During et al. 2002) • $E > Z$ (Gaziano et al. 1995) • $E > Z$ (Stahl et al. 1995)	• $E \approx Z$ (Böhm et al. 2002) • $E > Z$ (Lavy et al. 1993) • $E < Z$ (Levin et al. 1997)	Anti-atherogenesis activity: • $E < Z$ (Harai et al. 2008) Anti-atherosclerosis activity: • $E < Z$ (Relevy et al. 2015)
Astaxanthin	• $E < Z$ (Yang et al. 2017a) • $E < Z$ (Coral-Hinostroza et al. 2004) • $E \lesssim Z$ (Østerlie et al. 2000)	• $E < Z$ (Yang et al. 2017a) • $E \lesssim Z$ (Yang et al. 2017b) • $E < Z$ (Liu and Osawa 2007)	–
Canthaxanthin	–	• $E < Z$ (Venugopalan et al. 2013)	Pro-apoptotic activity: • $E < Z$ (Venugopalan et al. 2009)
Fucoxanthin	–	• $E \approx Z$ (Zhang et al. 2014) • $E > Z$ (Kawee-ai et al. 2013)	Anti-cancer activity • $E < Z$ (Nakazawa et al. 2009)
Lutein	• $E > Z$ (Yang et al. 2018)[c] • $E < Z$ (Yang et al. 2018)[d]	• $E \lesssim Z$ (Yang et al. 2018)	–
Zeaxanthin	–	• $E \gtrsim Z$ (Böhm et al. 2002)	–

[a]all-*E*-Isomer of carotenoid.
[b]*Z*-Isomer of carotenoid.
[c]Evaluated by a digestion model.
[d]Evaluated in Caco-2 cells

18.2 Nutraceutical and Pharmaceutical Applications of Carotenoids

18.2.1 Market Size

The market size of carotenoids was valued at $1.5 billion in 2014 and is expanding year by year, i.e., compound average growth rate (CAGR) from 2014 to 2019 is estimated at 3.9% (Business Communications Company 2015). The aging of citizens in developed countries is a serious social problem. Since carotenoids have preventive effects against age-related diseases such as age-related macular degeneration and Alzheimer type dementia, this aspect is considered to be boosting the demand for carotenoids. In addition, the demands for natural food colorants and color-restoring feed with carotenoids for farm animals and cultured fish drive the global carotenoids market growth. Lycopene, β-carotene, astaxanthin, lutein, zeaxanthin, canthaxanthin, and capsanthin are the major carotenoids, which are available commercially. The carotenoid market would have several changes in recent years. Namely, the market share for major carotenoids in 2010 was higher in the order of β-carotene (22%) > lutein (20%) > astaxanthin (19%) > canthaxanthin (7%) > lycopene (6%) (Business Communications Company 2011), whereas prospective that in 2021 would be higher in the order of β-carotene (26%) > astaxanthin (25%) > lutein (18%) > canthaxanthin (10%) > lycopene (6%) (Rammuni et al. 2019). The market size of astaxanthin has growth potential and is expected to rise sharply owing to its increasing usage in a antioxidant for health food and an animal and fish feed ingredient. Moreover, the commercially available carotenoids are mainly synthesized chemically, whereas a portion is obtained through natural sources such as fermentation and extraction from plants or algae. Natural carotenoid category is anticipated to have the high growth in the future due to the increase in consumer demand for natural products.

18.2.2 Distribution Form and Applications

Commercially available carotenoids are mainly distributed as the (1) dried ground powder of carotenoid-rich plants, microalgae, and bacteria, (2) oil suspension (oleoresin or the diluted product by vegetable oil), (3) liquid or dried emulsion formulation, and (4) beadlet formulation (high carotenoid concentrated powder). The carotenoid concentration of the dried ground powders and emulsion formulations is around a few weight percent (1–3 wt%). On the other hand, as for the oil suspensions and beadlet formulation, products with various carotenoid concentrations are distributed, which are normally from a few weight percent to 50 wt%. Generally, the prices of carotenoid products are depending on the carotenoid content. The typical applications of each formulation are shown below.

(1) Dried ground powder: farm animal and fish feeds, foods, and extract materials for carotenoids
(2) Oil suspension (oreolesin): supplements (soft capsule) and cosmetics
(3) Liquid or dried emulsion formulation: food and beverage colorings and cosmetics.
(4) Beadlet formulation: foods and supplements (tablet).

18.2.3 Processing

Carotenoids can be obtained from plants, algae, and bacteria, or can be produced by chemical synthesis. Generally, carotenoids are present in the all-*E*-configuration that has high hydrophobicity and high crystallinity. These properties limit their use for food and cosmetic industries, and thus many commercially available carotenoid products are subjected to the emulsification and micronization treatments. However, the efficiencies of carotenoid processings such as extraction, emulsification, and micronization are very low due to their properties of high hydrophobicity and high crystallinity, and that results in price increase and quality deterioration of the carotenoid products. Therefore, many studies have been carried out to improve the carotenoids processing efficiency. For example, as for carotenoids extraction, extraction conditions (type of solvent, temperature, and time) and extraction apparatus are well studied, and as for the emulsification, type of emulsifier as well as emulsification conditions and apparatus are well documented. In recent years, carotenoid processing utilizing a green solvent, supercritical CO_2 (SC-CO_2), which is non-toxic and easily separated from the products, has been extensively examined. However, there are few practical examples for carotenoid processing because most carotenoids are very low solubility in SC-CO_2. Very recently, it was revealed that *Z*-isomers of carotenoids were higher solubility in solvents including SC-CO_2 and lower crystallinity than the all-*E*-isomers (Honda et al. 2018a; Murakami et al. 2017). By utilizing the change in the physicochemical properties via the *Z*-isomerization, several studies successfully improved the efficiency of carotenoid processing using SC-CO_2. For example, Honda et al. (2017b, 2018b) demonstrated that the thermal *Z*-isomerization pretreatment to tomato pulp and gac (*Momordica cochinchinensis* Spreng.) markedly improved the SC-CO_2 extraction efficiency of lycopene. Moreover, when *Z*-isomer-rich carotenoids were used as the raw material for emulsification and micronization processings using SC-CO_2 as a mediator, those efficiencies were notably improved compared with using the all-*E*-isomers (Kodama et al. 2018; Ono et al. 2018), e.g., in emulsification of β-carotene using SC-CO_2 as the organic phase, the encapsulated efficiency of β-carotene *Z*-isomers was 21.2 times higher than that of the all-*E*-isomer (Ono et al. 2018). For the sustainable development of carotenoid products in the future, further development of carotenoids processing with SC-CO_2 is expected and utilizing the change in the physicochemical properties of carotenoids by the *Z*-isomerization may be breakthrough of that realization.

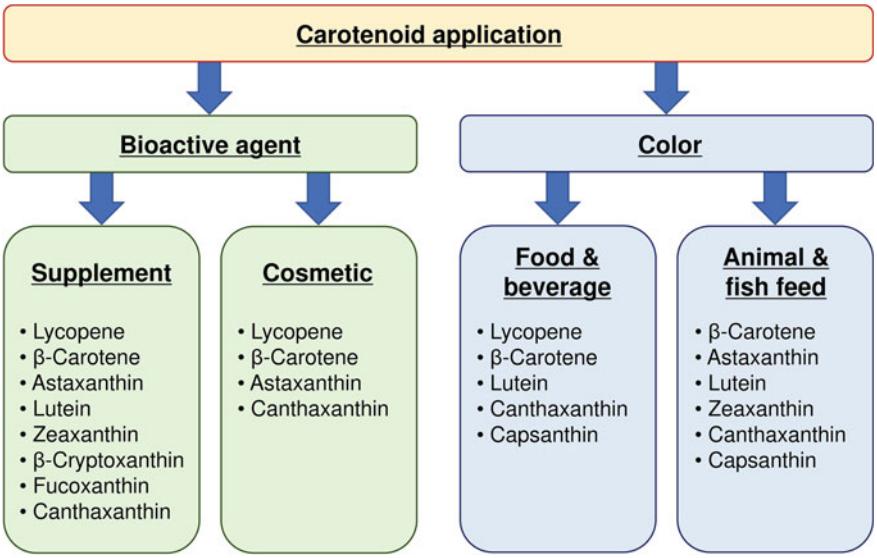

Fig. 18.3 Applications of commercially used carotenoids

18.2.4 Nutraceutical and Pharmaceutical Applications

The major applications of carotenoids are for "bioactive agent" such as nutritional supplements and cosmetics, and "color" for foods and beverages, and animal and fish feeds (Fig. 18.3). Focusing on the nutraceutical and pharmaceutical segments, the primary applications of each carotenoid are summarized below.

18.2.4.1 Lycopene

The main application of lycopene is nutritional supplement appealing high antioxidant activity and reduce risks of prostate cancer and cardiovascular disease: approximately 80% of global lycopene consumption was supplement in recent years. From the early 2000s, the demand for the cosmetic segment is also increasing year by year because in addition to the development of the emulsification technology, its high antioxidant singlet oxygen scavenging activity and skincare effect were demonstrated. Furthermore, the recent demands for natural food colorants drive lycopene market as a colorant agent for a number of food products. Mainly, synthetic lycopene and extracted lycopene from tomatoes are available commercially. The tomato extracts rich in lycopene are also expected to be health benefits from components other than lycopene such as phytoene, phytofluene, and β-carotene. Most lycopene in the market is the all-*E*-form, e.g., the content of lycopene *Z*-isomers, which have higher bioavailability and antioxidant activity than the all-*E*-isomer, in tomato oleoresin sold by LycoRed Ltd. (Lyc-O-Mato®; Beer-Sheva, Israel), which is the biggest

producer of natural lycopene, is around only 10% (Honda et al. 2017a). However, recently, Licofarma S.r.l. (Galatina, Italia) has sold lycopene supplements appealing high *Z*-isomer content. Since the functional study and manufacturing technique development on lycopene *Z*-isomer have been carried out in recent years, further demand for (*Z*)-lycopene is expected to increase.

18.2.4.2 β-Carotene

β-Carotene currently has two major applications, i.e., colorant for food products and supplements. There is almost no food segments that does not use β-carotene as a colorant. For example, the yellowish pigment, β-carotene, is used for beverages such as fruit-based juices and carbonated drinks, margarine, ice cream, and so on. The major concept of β-carotene supplement is based on the understanding that the carotenoid has antioxidant activity and can reduce risk of some cancers. However, several reports indicated that an excessive intake of β-carotene under certain circumstances such as smokers and workers exposed to asbestos may stimulate the development of cancer and the carotenoid act as a prooxidant (Albanes et al. 1996; Omenn et al. 1996). Therefore, it is important to pay attention to its intake. As minor applications, β-carotene is used for a fertility increasing agent in animal feed (Folman et al. 1987) and an anti-wrinkle and natural bronzing agent in cosmetics (Kim et al. 2009). The major source of β-carotene is chemical synthesis, but the shares of algae extraction, fermentation, and palm oil are increasing in recent years. β-Carotene from the green halophilic alga *Dunaliella* sp. contains the 9*Z*-isomer in large quantity (sometimes, around 50% of total β-carotene isomers). Since the isomer would have higher anti-atherogenesis and anti-atherosclerosis effects than the all-*E*-isomer (Harai et al. 2008; Relevy et al. 2015), the demand of supplements using *Dunaliella* sp. as the β-carotene source has been expanding.

18.2.4.3 Astaxanthin

The greater part of the astaxanthin consumption has been the colorant for fish and shrimp. Astaxanthin is widely found in aquatic animals and it has a major role in their body color. However, since the aquatic animals cannot biosynthesize astaxanthin, they need to take the pigment from diets. The consumers check the fish and shrimp quality by pigmentation on them. Hence, the feeding of astaxanthin is very important. As many governments support for promoting aquaculture, the demand of astaxanthin has further growth potential as a pigment coloring agent. Moreover, multiple studies demonstrated that dietary astaxanthin offers numerous health benefits for humans such as brain health improvement and healthy vision, and thus the carotenoid is often used for nutritional supplement. In addition, since astaxanthin may exert its benefits on skin homeostasis, it is gaining popularity and acceptability in cosmetics manufacturers and numerous wellness spa service providers.

18.2.4.4 Lutein and Zeaxanthin

A global market for lutein was established in the segment of eye health as a standalone, occasionally together with zeaxanthin. Thus, the major application of lutein and zeaxanthin is nutritional supplement appealing eye health. The supplements would protect from detrimental effects of computer display light exposure and reduce the risk of age-related macular degeneration. Lutein is also deal as a medicine for eye disease, which is listed on the Australian Register of Therapeutic Goods (ARTG). In addition, helenien (lutein dipalmitate) is widely used as a medicine for the visual disorders (Khan et al. 2014). Helenien is often isolated from the blossom leaves of *Helenium autumnale*. Recently, chronic eye disorder age-related macular degeneration accounted for over 50% of blindness cases in North America, and it should favor the supplements and medicine demands. Lutein and zeaxanthin are also used for feed as a colorant for egg yolks and broiler skin, and marigold is widely used as their source. Feeds containing lutein and zeaxanthin derived from marigold result in a strong yellowish or even orange color hue of the yolks.

18.2.4.5 Canthaxanthin

The major application of canthaxanthin is feeds, e.g., coloring hen's egg yolks and fish pigmentation. Since its lower price compared to other carotenoid sources for feeds such as astaxanthin, the consumption for the industry is expanding year by year. Although the market size is small (around 10% of total canthaxanthin market size), canthaxanthin is used for the nutraceutical and pharmaceutical applications. Namely, the carotenoid is used for supplement appealing antioxidant activity, anti-atherosclerosis activity, and enhancement of fertility and sexual performances. Since canthaxanthin is also promoted as a self-bronzing agent, it is used for some creams and lotions. In addition, as canthaxanthin has a bright red color, it is popular in coloring pharmaceutical tablets. This application is widespread, but the volumes consumed are not many. The market for nutraceutical and pharmaceutical applications of canthaxanthin is expanding year by year especially in North America.

18.2.4.6 Other Carotenoids

Since the bioactivities such as reduction risk of osteoporosis and anti-obesity activity of β-cryptoxanthin and fucoxanthin, respectively, were reported in the middle of the 2000s (Uchiyama and Yamaguchi 2005; Maeda et al. 2005), the development of supplements containing their carotenoids has been actively carried out and their market sizes are growing larger every year especially in Japan. Generally, the natural sources are used for the supplements, i.e., the major source of β-cryptoxanthin is Satsuma mandarin (*Citrus unshiu* Marc.) and that of fucoxanthin is edible brown seaweeds such as kombu (*Laminaria japonica*) and wakame (*Undaria pinnatifida*). In addition, α-carotene and capsanthin also have markets in supplement appealing

antioxidant, anti-cancer, and anti-atherosclerosis activities. However, in many cases, their carotenoids are used together with the other carotenoids such as β-carotene and lutein, and are sold as a multi carotenoid supplement. Capsanthin has relatively big market share of carotenoids (around 10% of carotenoid market in 2010), but the main applications are not for supplements but for food colors and feeds.

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Chapter 19
Use of Microalgae Pigments in Aquaculture

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Abstract Aquaculture sector shows the fastest growth among the global food production industries. However, nutrition and diseases are two important hurdles that need to be addressed to propel the industry forward. Consistent supply of high-quality feed for growing larvae of important aquaculture species is one of the bottlenecks of the local larviculture industry. Microalgal pigments containing carotenoids, phycobiliproteins and chlorophylls are highly nutritious functional foods containing high antioxidant biomolecules associated with fast growth, high immunity, enhanced pigmentation and high productivity. Their high contents of lipids and fatty acids make them valuable alternatives for replacement of fish meals in livestock and fish feeds. Polyunsaturated fatty acids (PUFAs), functional amino acids, vitamins and other biomolecules associated with health are also found in many microalgae species. Major pigments such as astaxanthin, lutein, beta-carotene, chlorophylls, and phycobiliproteins have been proven as valuable sources for the development of functional, nutritional and therapeutic commodities to enhance pigmentation, survival, disease resistance and health of cultured organisms. In spite of their well-known applications, commercial production of pigment supplies such as astaxanthin, fucoxanthin,

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canthaxanthin, β-carotene and phycobiliproteins are still limited by production technologies. More research and innovation for cost-effective pigments production and applications in improving growth and health in aquatic organisms are important for a sustainable aquaculture industry.

Keywords Microalgae · Pigments · Carotenoids · Astaxanthin · Fucoxanthin · Phycobiliproteins · Aquaculture

19.1 Aquaculture and Microalgae—The Current Status

Aquaculture is one of the main elements of sustainable development goals 14 (SDG14) that emphasizes on conservation and sustainable use aquatic resources for sustainable development. Aquaculture together with fisheries forms the core for the food security, nutrition and health of the global population. Fish accounted 17% of the animal protein consumed by the global population. In fact, with the stagnation of capture fisheries, aquaculture continues to grow at an impressive pace exceeding those of other food production sectors. Global aquaculture production including plants in 2016 was 110.2 million tonnes valued at USD243.5 billion (FAO 2018). Although its annual growth rates declined from 9.5% (1990s) to 10.5% (1980s) to only 5.8% between 2001 and 2016, aquaculture annual growth rate is still the highest among the global major food production sectors. The demand for aquaculture products keeps growing as reflected in the fish consumption pattern that grew from 9.0 kg per capita in 1961 to 20.5 kg in 2017 (FAO 2018).

However, aquaculture industry is still faced with serious problems related to nutritional health such as poor growth and survival in hatchery and grow-out ponds, vulnerability to diseases, and poor production. Feeds in aquaculture is the highest item of the production cost, but in some cases are still substandard and do not improve the culture production. In fact, the use of fish meal in aquaculture is one of the unsustainable practices with high carbon footprint. Thus, it is very important to improve the aquaculture nutrition using highly nutritious feed, high in health-promoting substances such as polyunsaturated fatty acids (PUFAs) and carotenoids, and yet its production is environmentally friendly.

Microalgae are a valuable source of highly nutritive polyunsaturated fatty acids (PUFA) such as docosahexaenoic (DHA), and eicosapentaenoic (EPA) and other omega-3 fatty acids which are high in antioxidants and suitable for immune enhancement in humans and animals (Natrah et al. 2007; Park et al. 2018). Microalgae produce a variety of biomolecules including pigments such as chlorophylls, carotenoids, phycobiliproteins and phenolic compounds (Koller et al. 2014). In fact, most species of microalgae have high protein contents that can be valuable sources of protein concentrates, hydrolysates and bioactive peptides. These protein-based products, together with highly nutritive fatty acids and pigments, make microalgae valuable sources for the development of functional, nutritional, therapeutic and cosmeceutical commodities (Soto-Sierra et al. 2018; Farahin et al. 2019).

In addition, microalgae culture that consumes CO_2 and nutrients improves the environment by decreasing wastes, thus preventing eutrophication in water bodies and help to decrease atmospheric carbon content.

Cultivation of microalgae is gaining more attention worldwide as it is environmentally friendly and sustainable. However, large scale production of microalgae biomass is still limited and costly. Microalgae farming such as *Spirulina* spp., *Chlorella* spp. and *Haematococcus pluvialis* on small scale like backyard operation to large scale production can be found in approximately 11 countries in 2016 (FAO 2018). For microalgal pigments, commercial production and extraction of carotenoids are limited to a few species of microalgae. For example, β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis* are extracted for use as food colourants (Ju et al. 2011), while *Muriellopsis* has been used commercially to produce lutein due to its high lutein content and high growth rate (Lin et al. 2015; Wang et al. 2018a). Efforts are still on to economically and sustainably optimize large scale microalgae biomass and bio-compound production (Yusoff et al. 2019). In addition to culture, microalgae large scale harvesting such as centrifugation, fractionation, flocculation and coagulation, filtration and ultrasonic separation is also costly and energy intensive. Xiao et al. (2019) suggested that microfiltration technique was highly efficient in microalgae harvesting.

In addition to the common commercially important microalgae (*Spirulina* sp., *Chlorella* spp., *Haematococcus* spp., and *Dunaleilla* sp.), other potential species include those brown/golden microalgae such as *Chaetoceros* spp., *Thalassiosira* spp., *Skeletonema* spp., and other diatom species that contain fucoxanthin (Foo et al. 2015, 2017). Park et al. (2018) proposed a new microalga *Schizochytrium* sp. as another potentially important commercial species due to its high levels of carotenoids and fatty acids suitable for feed additives and other health products. In addition, Deli et al. (2014) also reported that *Euglena sanguinea* and *Nostoc* sp. have shown high contents of carotenoids.

Compared to terrestrial plants, microalgae have a number of advantages including faster growth rate (five to ten times higher), unlimited by land area and climate (controlled culture conditions) and year-round production (Heo et al. 2018; Yusoff et al. 2019). In addition, some carotenoids such as fucoxanthin and loroxanthin are lacking in terrestrial plants (Foo et al. 2015; Wang et al. 2018a). Thus, microalgae have many species that can be commercially developed to provide adequate nutritional sources for aquaculture industry.

19.2 Microalgae Have Many Uses

Microalgal cells in general are highly nutritious containing biomolecules associated with fast growth and high immunity such as vitamins, antioxidants and anti-inflammatory, in addition to basic food components such as proteins, carbohydrates, lipids and trace elements (Goh et al. 2014; Yaakob et al. 2014). Feller et al. (2018) reported that microalgae are potent source for functional foods due to their ability

to synthesize polyunsaturated fatty acids and carotenoids and phenolic compounds with high antioxidative properties. In fact, most microalgae have characteristics that are required to be natural supplements in human (Brizio et al. 2013) and animal feeds including aquaculture. Their high contents of lipids and fatty acids make them valuable alternatives for replacement of fish meals in livestock and fish feeds. The much needed polyunsaturated fatty acids (PUFAs) and beta-1,3-glucan, and other biomolecules associated with health are also found in many microalgae species (Foo et al. 2019). In addition, microalgae have valuable pigments such as astaxanthin, lutein, beta-carotene, chlorophylls and phycobiliproteins (Begum et al. 2016; Foo et al. 2017).

The demand for natural and healthier food is increasing worldwide. Commercially, industries related to food and beverages, nutraceuticals, pharmaceuticals, cosmeceuticals and aquaculture use algal pigments such as beta-carotene, astaxanthin, fucoxanthin, lutein, chlorophylls, phytocyanin and phycoerythrin. Due to these valuable health-related properties, global algae products market account for USD3.4 billion in 2017 and is expected to reach USD 6.09 billion by 2026, growing at a CAGR (compound annual growth rate) of 6.7% (Globe Newswire 2019). The natural food colour business in 2016 was USD1.3 billion with 6.8% annual growth rate, and is expected to reach USD1.77 billion by 2021 (Kannaujiya et al. 2017).

As human dietary supplements, pigments such as carotenoids and phycobiliproteins function as anticancer, therapeutic treatment against pathogenic bacteria, booster of immunity and prevention of cardiovascular diseases. Lycopene has been reported to have the capacity to prevent prostate cancer (van Breemen et al. 2012). In addition, microalgae pigments are also used in poultry and livestock industries (Higuera-Ciapara et al. 2006). In fact, microalgae are not only used as food and health products, but also as the feedstocks for biofuels (Medipally et al. 2015; Rastogi et al. 2018; Rizwan et al. 2018). Due to their fast growth, microalgae have been noted to be more efficient producers of biofuels compared to land plants, especially those microalgae species with enhanced lipid production when grown using waste-waters and flue-gas (Mohamed Ramli et al. 2017; Foo et al. 2019).

19.3 Microalgae Pigments

Microalgae pigments are chemical compounds with distinct light spectrum and wavelength which are involved in the photosynthesis as light energy absorbers. These pigments are known to contain health-promoting properties such as anti-inflammatory agents, immune enhancers, antioxidants and vitamin precursors. Thus, numerous studies have been conducted on the potential of microalgae-derived pigments for commercial application in pharmaceutical, cosmetics, health supplement and feed industries. There are three major groups of pigments in microalgae; chlorophylls (all higher plants and photosynthetic algae and bacteria); carotenoids (some higher plants and most algae) and phycobiliproteins which are exclusive to cyanobacteria and some red algae. In microalgae, pigments can be grouped into three

classes: phycobiliproteins (up to 8% dry weight), chlorophylls (0.5–1.0% dry weight) and carotenoids (0.1–0.2% dry weight, and up to 14% dry weight for β-carotene) (Gouveia and Empis 2003; de Jesus Raposo et al. 2013; Markou and Nerantzis 2013; Barra et al. 2014).

Microalgae pigments are often associated with antioxidant activities whereby they delay the oxidation of protein, carbohydrates, lipids, DNA, cell membrane and other cellular components. This is because microalgal cells contain vitamin A, vitamin C, vitamin E, β-carotene, lycopene, polyphenols, flavonoids and carotenoids (Table 19.1). Da Silva Vaz et al. (2016) reported that several genera of microalgae such as *Spirulina, Botryococcus, Chlorella, Dunaliella, Haematococcus* and *Nostoc* have been grown for bioactive compounds. Some species of microalgae such as *Isochrysis galbana, Tetraselmis suecica* and *Pavlova salina* showed high carotenoid contents and have the potential to be commercially produced (Ahmed et al. 2014). Some microalgae produce high biomass because they can grow by more than one mode. Liu et al. (2014) reported that *Chlorella zofingiensis* showed better growth (phototrophically, heterotrophically and mixotrophically) than *H. pluvialis* for mass production of astaxanthin.

With an estimated microalgae species in the range of 200,000 to several million species (Norton et al. 1996), the search for high-value microalgae species will continue. Safafar et al. (2016) demonstrated that *Nannochloropsis salina* can be used to produce high-value compounds such as carotenoids, eicosapentaenoic acid (EPA), tocopherol and proteins by optimizing the photobioreactor and culture media. Other microalgae species such as *Dunaliella* sp., *Tetraselmis* sp. and *Nannochloropsis gaditana* also have high carotenoid and phenolic contents and have the potential to be developed as new sources of antioxidants (Maadane et al. 2015). Some microalgae species have many types of pigments within a single cell. For example, a freshwater alga, *Oedogonium intermedium* showed high oxidant activities by seven different carotenoids; neoxanthin, 9′-cis neoxanthin, loroxanthin, violaxanthin, lutein, α-carotene and β-carotene (Wang et al. 2018a). Pigments in microalgae can be diverse, and some are found only in a specific group of microalgae that they can be used as markers. Paliwal et al. (2016) studied carotenoid contents from 57 species of Chlorophyta and Cyanobacteria and suggested that lutein and violaxanthin can be used as markers for Chlorophyta, and myxoxanthophyll and echinenone as markers for Cyanobacteria.

19.4 Carotenoids

Carotenoids are lipophilic isoprenoid compounds with long conjugated double bonds, chemical characteristics which are responsible for health-related properties such as antioxidant activities, protection from eye diseases, anti-inflammatory, prevention of age-related macular degeneration, rheumatoid arthritis, cardiovascular problems and anticancer activities (Ahmed et al. 2014; Heo et al. 2018). van Breemen et al. (2012) reported that approximately 50 carotenoids are precursors of vitamin A, which is

Table 19.1 Uses of microalgae as sources of biomolecules

Compounds	Microalgae species	Uses	Effects	References
Total carotenoids: Astaxanthin, lutein, zeaxanthin, canthaxanthin, chlorophylls a and b	*Chlorella vulgaris*	Carotenogenic microalgal diet in gilthead sea-bream, *Sparus aurata*	Pigment enhancement	Gouveia et al. (2002), Batista et al. (2013)
Carotenes and xanthophylls	*Spirulina platensis*	Dietary pigments in ornamental koi, *Cyprinus carpio*	Enhanced colouration, increased carotenoid content in fish, increased growth and feeding efficiencies	Sun et al. (2012)
Carotenoids:	*Spirulina* sp., *Chlorella vulgaris*	Alternative microalgae diets for fairy shrimp *Branchinella thailandensis*	Enhanced growth, survival and reproduction	Chaoruangrit et al. (2018)
Lutein, zeaxanthin, β-carotene Chlorophyll a	*Spirulina maxima*	Functional foods	Improved health	Batista et al. (2013)
Astaxanthin	*Haematococcus*, *Chlorella zofingiensis*	Natural antioxidant	Food and health industries	Liu et al. (2014), Yaakob et al. (2014)
Astaxanthin, canthaxanthin and echinenone	*Haematococcus pluvialis*	Application in aquaculture, pharmaceutical and cosmeceutical industries Dietary supplementation for Chinese mitten crab, *Eriocheir sinensis*	Antioxidant capacity and colouration	Hagen et al. (2001), Liu et al. (2014), Yaakob et al. (2014), Long et al. (2017)

(continued)

Table 19.1 (continued)

Compounds	Microalgae species	Uses	Effects	References
Astaxanthin, lutein, zeaxanthin, canthaxanthin, Chlorophylls a and b	*Haematococcus pluvialis*	Feed supplements in rainbow trout, *Oncorhyncus mykiss*	Increased blood astaxanthin	Barbosa et al. (1999), Batista et al. (2013)
Carotenoids: Astaxanthin	*Haematococcus pluvialis*	Dietary supplement in *Litopenaeus vannamei*	Pigment enhancement	Ju et al. (2011)
Carotenoids: Astaxanthin	*Haematococcus pluvialis*	Pigment enhancement in salmon and rainbow trout	Enhanced body colouration	Brizio et al. (2013)
Carotenoids: Astaxanthin	*Haematococcus pluvialis*	Growth, antioxidant responses and skin pigmentation in olive flounder, *Paralichthys olivaceus*	Increase total carotenoid contents, antioxidant activities and pigmentation	Pham et al. (2014)
Carotenoids: Astaxanthin	*Haematococcus pluvialis* (biomass)	Dietary feeds of large yellow croaker *Pseudosciaena crocea*	Enhanced growth performance, antioxidant status and immune response	Li et al. (2014)
Total microalgae biomass: Carotenoids	*Haematococcus pluvialis*	Dietary supplementation with microalgae biomass for *Litopenaeus vannamei*	Improved immune capacity, low salinity tolerance	Xie et al. (2018)
Carotenoids: mainly fucoxanthin	*Phaeodactylum tricornutum*	Feed additives	Enhanced health due to high antioxidant properties	Feller et al. (2018)
Carotenoids: mainly zeaxanthin	*Nannochloropsis oculata*	Feed additives	Enhanced health	Feller et al. (2018)
Phycobiliproteins: Phycoerythrin	*Porphyridium cruentum*	Feed additives	Enhanced health	Feller et al. (2018)

(continued)

Table 19.1 (continued)

Compounds	Microalgae species	Uses	Effects	References
Carotenoids: violaxanthin, astaxanthin, lutein, zeaxanthin, β-carotene and α-carotene	*Chlorococcum humicola*	Rich in bioactives	Improved growth and health	Sivathanu and Palaniswamy (2012)
Carotenoids: Fucoxanthin, lutein, zeaxanthin, β-carotene Chlorophylls a and c	*Isochrysis galbana, Tetraselmis suecica, Dunaleilla salina and Pavlova salina*	High antioxidant activities; functional foods	Food and health industries	Ahmed et al. (2014), Batista et al. (2013)
Fucoxanthin	A diatom, *Chaetoceros calcitrans*	Nutraceuticals and food industries	Improve human health, anticancer	Foo et al. (2019)
Fucoxanthin, lutein, zeaxanthin, β-carotene Chlorophylls a and c	*Diacronema vlkianum*	Functional foods	Improve health	Batista et al. (2013)
Lutein	*Chlorella vulgaris, Scenedesmus, Muriellopsis*	Natural colouration of foods, drugs and cosmetics, feed additives	Improve human and animal health	Del Campo et al. (2000), Ahmed et al. (2014)
Lutein	*Chlorella sorokiniana* (mixotrophic growth)	Feed additives	Reduce risk of age-macular degeneration; reduce cardiovascular diseases, cancer and degenerative human diseases	Chen et al. (2017)
Lutein	*Desmodesmus* sp.	Growth and lutein productivity	Food additives and pharmaceutical products	Xie et al. (2017)
Lutein	*Scenedesmus obliquus*	Nutritional and pharmaceutical applications	Prevent cardiovascular diseases, cancer and age-related diseases	Ho et al. (2014)

(continued)

Table 19.1 (continued)

Compounds	Microalgae species	Uses	Effects	References
Lutein	Freshwater microalga *Muriellopsis* sp., *Parachlorella* sp.	Enrichment of live feed, a rotifer, *Brachionus* sp.	Provide carotenoid lutein for fish larvae	van Bergeijk et al. (2013), Heo et al. (2018)
β-carotene	*Dunaliella salina*	Colouration	Pigment enhancement	Boonyaratpalin et al. (2001)
β-carotene	*Dunaleilla salina*	Supplemented diets in severum fish (ornamental fish), *Heros severus*	Enhanced hemato-immunological responses.	Alishahi et al. (2014)
Carotenoids: Astaxanthin, Canthaxanthin β-carotene, Echinenone	*Schizochytrium* sp.	Food additives and supplements due to high antioxidant and omega-3 fatty acid contents	–	Park et al. (2018)
Carotenoids: β-carotene, Lutein, violaxanthin, neoxanthin,	*Dunaliella*	Food additives	–	Deli et al. (2014)
Carotenoid: Echinenone	*Nostoc* sp.	Food additives	–	Deli et al. (2014)
Carotenoids: Diatoxanthin, lutein, β-carotene	*Euglena sanguinea*	Food additives	–	Deli et al. (2014)
Carotenoids: Neoxanthin, 9'-cis neoxanthin, loroxanthin, violaxanthin, lutein, α-carotene, β-carotene	A freshwater alga *Oedogonium intermedium*	Important source of bioactive ingredients, especially loroxanthin, which is lacking in terrestrial plants	Application in functional foods, nutraceutical products (supplements for diabetes, anti-obesity and anti-inflammation)	Wang et al. (2018a)
Total biomass: Proteins, lipids, fatty acids and carotenoids.	*Isochrysis galbana, Nannochloropsis gaditana, Scenedesmus almeriensis*	Partial fish meal replacement in aquafeeds	Improved growth	Camacho-Rodríguez et al. (2018)

essential for vision and embryological development in human and animals. Thus carotenoids, which are best known for their yellow, orange and red pigments, are important micronutrients, used as functional bioactives, feed additives, natural food ingredients and health supplements (Bonilla-Ahumada et al. 2018; Yusoff et al. 2019). Carotenoid global market was approximately USD1.24 billion in 2016 and projected to reach USD1.53 billion by 2021 (Heo et al. 2018).

Carotenoids can be synthesized by most photosynthetic organisms (for light harvesting and photoprotection) and some non-photosynthetic organisms such as bacteria and fungi. Rodriguez-Concepcion et al. (2018) summarized that carotenoids can be found in 691 organisms and categorized them as carotenes (formed exclusively by carbon and hydrogen atoms) and xanthophylls (contain oxygenated radicals) based on their chemical composition. Xanthophylls are further classified into those with hydroxyl (lutein and zeaxanthin), epoxide (violaxanthin, neoxanthin) or carbonyl (canthaxanthin, capsaxanthin) groups. Other oxygenated groups in natural carotenoids include carboxylic, acetate, lactone and sulphate groups.

Like the culture of microalgae biomass which is controlled by the physicochemical factors of the culture media, the molecular contents of microalgae cells are also related to the surrounding environmental factors. Thus, microalgae and their biomolecule productivity can be enhanced by manipulating the growth factors such as light, temperature, pH, nutrients, aeration and mixing (Sun et al. 2015; Yusoff et al. 2019). Benavente-Valdés et al. (2016) manipulated different factors such as nutrient sources (nitrogen, phosphorus, carbon) and physical factors (light intensity, salinity and electromagnetic fields) to optimize the biomass and pigment yields in microalgae culture. Similarly, Hagen et al. (2001) studied the effects of light, nitrate, acetate and phosphate on the growth of *H. pluvialis* and concluded that more detail studies should be carried out to determine the pathways of the pigment biosynthesis. Rearte et al. (2018) demonstrated that lipids and carotenoid contents of a microalga, *Golenkinia* sp. could be induced by increased salinity. Other carotenoids, such as lutein productivity also could be optimized by regulating light intensity and carbon source (Ho et al. 2014; Heo et al. 2018). Thus, biotic stresses are important tools for metabolites in microalgae. Temperature, nutrient starvation, salinity and light affect PUFAs, nitrogen and light stress stimulate phycobiliproteins, while salinity, light and nutrients influence carotenoids (Sun et al. 2015; Adenan et al. 2016; Benavente-Valdés et al. 2016; Imaizumi et al. 2016; Rearte et al. 2018).

19.4.1 Astaxanthin

Astaxanthin (3, 30-dihydroxy-b,b-carotene-4,40 dione) is a red ketocarotenoid from the xanthophyll group with high antioxidant property and thus demand a high commercial market. Some plants, microalgae, bacteria and fungi are known to synthesize astaxanthin. Astaxanthin has superior health-related properties compared to other carotenoids such as β-carotene, lutein, lycopene, canthaxanthin and vitamin E, and thus a sought-after pigment globally as it is becoming a popular dietary

supply for humans, live-stocks and aquaculture. Commercially, astaxanthin is mainly extracted from *H. pluvialis* and *Chlorella zofingiensis*, and cost approximately USD1900–2000/kg (Yaakob et al. 2014), and it is one of the most available microalgal pigment worldwide. Subramanian et al. (2013) reported that astaxanthin has effective bio-functionalities approximately 10 times higher than those reported for other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene due to its high contents of antioxidants.

Major sources of astaxanthin include artificial synthesis, extraction from shell animals and production from natural organism such as bacteria, yeast and algae (He et al. 2007). A basidiomycetous fungi, *Xanthophyllomyces dendrorhous*, is the only known fungus that is able to produce astaxanthin as its major carotenoid (Libkind et al. 2012). Charest et al. (2001) demonstrated that astaxanthin can be extracted from crawfish shells using supercritical CO_2 with ethanol. In crustaceans, astaxanthin binds to a specific protein called crustacyanin (CRCN), and it is the interaction between astaxanthin and CRCN that produces diverse range of colours in the cultured animals (Wade et al. 2012).

Under natural aquatic environment, microalgae biosynthesize astaxanthin, and transported along the food chain to zooplankton and larvae of various species of aquatic animals, which are eventually ingested by fish and other top predators (Ashour et al. 2018). In aquaculture farms, access to astaxanthin contained natural foods is limited and thus astaxanthin has to be included in the feeds (Higuera-Ciapara et al. 2006). Domínguez et al. (2005) demonstrated that a live feed rotifer, *Brachionus plicatilis*, can accumulate astaxanthin from *Hematococcus pluvialis* and transfer this biomolecule to fish larvae.

A green alga, *Haematococcus pluvialis*, is the highest producer of astaxanthin to date, exceeding 4% of its dry weight (Boussiba and Vonshak 1991). He et al. (2007) illustrated that stresses applied in the culture which interfere with cell division such as high light intensity and nutrient (iron, sulfur and phosphate) starvation could enhance astaxanthin accumulation. However, there are variations of astaxanthin productivity among different species and strains. Allewaert et al. (2017) reported that different species and strains of *H. pluvialis* and *H. rubicundus* had different astaxanthin production rates.

Although the natural astaxanthin is stronger and safer than the artificial astaxanthin, the supply is still limited. The outdoor mass production is more cost effective, but there are limitations such as high temperature and the short life span of outdoor photobioreactors. Wan et al. (2014) reported that microalgae produced under controlled temperature regimes had biomass and astaxanthin production of more than five times compared to those produced using conventional approaches. In addition to temperature, Zhang et al. (2016) reported that photorespiration played critical roles in astaxanthin production mainly through enhancement of PGA (glycerate-3-phosphate) that is related to the astaxanthin accumulation in *H. pluvialis.*

19.4.2 Fucoxanthin

Fucoxanthin is one of the microalgal pigments that can only be found in marine environment, including Phaeophyta (brown seaweeds) and Bacillariophyceae (diatoms). Like any other carotenoids, fucoxanthin has strong antioxidant activities and other health-related properties such as anti-inflammatory, anticholinesterase, anticancer and anti-obesity (Viera et al. 2018). In fact, fucoxanthin scavenges free radicals better than other pigments such as β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin. Fucoxanthin has attracted great interest due to its high potential and promising application in human and animal health (Foo et al. 2019), including aquaculture.

19.4.3 Beta-Carotene and Other Carotenoids

Besides astaxanthin and fucoxanthin, β-carotene, lutein, zeaxanthin, and canthaxanthin (Table 19.2) are also important in human health and other health-related activities. β-carotene is one of the carotenoid pigments, that acts as a precursor to vitamin A. β-carotene is commercially produced from *Dunaliella salina,* mainly in China, India, Australia, Israel and USA. Lutein is a yellow xanthophyll pigment commonly found in terrestrial plants and is well known for its potential to reduce age-related muscular degeneration (Heo et al. 2018). Microalgae were reported to have lower lutein productivity compared to conventional sources from terrestrial plants. However, Heo et al. (2018) suggested that new microalgae species have the potential to produce high lutein and other carotenoid concentrations and should be explored.

19.5 Phycobiliproteins

Phycobiliproteins are a major component of the photosynthetic membrane in cyanobacteria, red algae, cryptomonads and glaucophytes (Schirmer et al. 1985), which possess brilliant colours and have highly fluorescent light-harvesting proteins accessory. Phycobiliproteins are water-soluble and form particles (phycobilisomes) on the surface of thylakoids, unlike chlorophylls and carotenoids (Jian-Feng et al. 2006. Normally, phycobiliproteins make up 24% of the total cellular proteins (dry mass) (Glazer 1985; Grossman et al. 1993; Cai et al. 2001). Nonetheless, the content of phycobiliproteins can be boosted up to 40% under low light intensity (Glazer 1994).

Phycobiliproteins are classified based on their colour and absorption properties. Phycobiliproteins can efficiently absorb sunlight at λ 480–660 nm (Sun et al. 2003) due to its various spectral properties as the bilins have individual absorption spectra

Table 19.2 Use of pigments in aquaculture

Pigment type	Cultured animals/plants	Advantages/effects	References
Astaxanthin	*Penaeus monodon*	Correction of colouration	Menasveta et al. (1993)
Astaxanthin	Rainbow trout, *Oncorhyncus mykiss*	Increased blood astaxanthin	Barbosa et al. (1999)
Carotenoids	Gilthead sea-bream, *Sparus aurata*	Increased pigmentation	Gouveia et al. (2002)
Carotenoids: Astaxanthin, canthaxanthin	Salmonids and crustaceans	Pink colour characteristics	Higuera-Ciapara et al. (2006)
Carotenoids: Astaxanthin, β-carotene, canthaxanthin, lutein, zeaxanthin	Channel catfish, *Ictalurus punctatus*	Accumulation of pigments in the flesh	Li et al. (2007)
Carotenoids: Astaxanthin, canthaxanthin	Rainbow trout, *Oncorhyncus mykiss*	Enhanced accumulation of carotenoids for human health	Brizio et al. (2013)
Astaxanthin	Spinecheek anemonefish, *Premnas biaculeatus*	Dermal colouration and chromatophore physiology	Ho et al. (2013)
Astaxanthin and Xanthophylls	Yellow croaker, *Larimichthys croceus*	Growth and skin pigmentation	Yi et al. (2014)
Astaxanthin	Olive flounder, *Paralichthys olivaceus*	Increased total carotenoid, antioxidant activities and skin pigmentation	Pham et al. (2014)
Carotenoids: Astaxanthin, xanthophylls and canthaxanthin	Discus fish (*Symphysodon aequifasciatus axelrodi*)	Pigmentation enhancement	Liu et al. (2016)
Carotenoids	Crayfish: *Procambarus clarkii, Pacifastacus leniusculus, Astacus* spp., *Cherax* spp.	Broodstock reproduction enhancement	Harlıoğlu and Farhadi (2017)
Carotenoids	Pearl oysters, *Pinctada fucata*	Enhanced tolerance to temperature stress	Meng et al. (2017)
Astaxanthin	Chinese mitten crab: *Eriocheir sinensis*	Colouration, ovarian development and anti-oxidant capacity	Long et al. (2017)

(continued)

Table 19.2 (continued)

Pigment type	Cultured animals/plants	Advantages/effects	References
Astaxanthin	Chinese mitten crab *Eriocheir sinensis*	Enhanced carapace astaxanthin content Alleviated oxidative damage due to high pH stress	Wang et al. (2018b)
Astaxanthin	*Penaeus monodon*	Improved colour, growth, carotenoid digestibility and the accumulation of specific carotenoid esters	Wade et al. (2017a)
Carotenoids: Astaxanthin, β-carotene	Crustaceans: *Penaeus monodon, Litopenaeus vannamei, Marsupenaeus japonicus*	Improved pigmentation of shrimps	Boonyaratpalin et al. (2001), Wade et al. (2017b)
Astaxanthin	Rotifers, *Brachionus manjavacas*	Enhanced nutritional contents of live feeds	Johnston et al. (2018)
Phycobiliproteins: Phycocyanin	Guppy fish *(Poecilia reticulata)*	Increase in the final weight, length, specific growth rate, and protein efficiency ratio; enhanced skin colour	Biabani Asrami et al. (2019)
Phycobiliproteins: Phycocyanin	Asian seabass, *Lates calcarifer* larvae	Reduced cannibalism, improved survival and disease resistance	Gora et al. (2019)
Phycobiliproteins: Phycocyanin	*Cyprinus carpio*	Improved survival and non-specific immune response	Muchtar et al. (2019)

(Reis et al. 1998). According to Bryant et al. (1979), major classes of phycobiliproteins in red microalgae and cyanobacteria are allophycocyanin (APC, bluish green), phycoerythrin (PE, red), phycocyanin (PC, blue) and phycoerythrocyanin (PEC, orange). The maximum absorbance of phycoerythrin is λ_{Amax} 565–575 nm, phycoerythrocyanin λ_{Amax} 577 nm, phycocyanins λ_{Amax} 615–640 nm and allophycocyanin λ_{Amax} 650–655 nm whereas they emit light at 660 nm, 637 nm, 577 nm and 607 nm, respectively (Begum et al. 2016).

Allophycocyanin, a core-constructing phycobiliproteins, can be found in all phycobilisome-containing organisms, red algae, glaucophytes and cyanobacteria which grow in natural environment. The content of allophycocyanin is higher with respect to total phycobiliprotein content in cyanobacteria when compared with red algae (Chakdar and Pabbi 2017).

Most glaucophytes, red algae, some cryptophytes and cyanobacterial species that grow in natural environment contain high amounts of phycocyanin. The phycocyanins can be grouped into (i) C-phycocyanin (C-PC, λ_{max} ~ 615–620 nm), (ii) phycoerythrocyanin (PEC, λ_{max} ~ 575 nm) and (iii) R-phycocyanin (R-PC, λ_{max} ~ 615 nm) (Chakdar and Pabbi 2017). These blue- or blue- purple-coloured phycobiliproteins are able to absorb light from 580 to 630 nm (Chakdar and Pabbi 2017).

Phycobilins are a group of photoreceptor pigments having similar components to the porphyrin without a metallic atom and can only be found in Rhodophyta and cyanobacteria. These phycobilins play an important role in pharmaceutical, food and textile industries (Udayan et al. 2017).

The most abundant phycobiliprotein in red algae and some cyanobacteria is phycoerythrin. It is a major component of light-harvesting complex and can be categorized according to its absorption bands in the green region of the visible spectrum (480–570 nm) and by intensive fluorescence emissions (575–580 nm). Thus, these red-coloured proteins are grouped according to their absorption spectra: (i) B-phycoerythrin (B-PE) (λ_{max} ~ 540–560 nm, (ii) R-phycoerythrin (R-PE) (λ_{max} ~ 565, 545 and 495 nm) and (iii) C-phycoerythrin (C-PE) (λ_{max} ~ 563, 543 and 492 nm) (Chakdar and Pabbi 2017). Among the three types, R-PE is the predominant natural form.

Phycoerythrocyanin is a cyanobacterial phycobiliprotein which has similar structures to phycocyanin but similar functions to phycoerythrin (Bryant 1982).

19.6 Structure of Phycobiliproteins

Phycobiliproteins or tetrapyrrole biliproteins are attached on the thylakoid membranes and are present as phycobilisomes. They lie adjacent to the photosynthetic reaction centre of the PSII in cyanobacteria and red algae. The colour of phycobiliproteins comes from the covalent bond of prosthetic groups (phycobilins) (Scheer 1981) attached to different cysteine residues of biliproteins (Sidler 1994). The phycobilins contain one to four open chain tetrapyrrole chromophore bearing A, B, C and D pyrrole ring (Grossman et al. 1993; Padyana et al. 2001) having the ability to capture and transfer the solar photonic energy to the photosystem for use during photosynthesis.

The core of phycobiliproteins is composed of allophycocyanin from which arise six rods of varying length consisting of phycocyanins (proximal) and phycoerythrins (distal) (Fig. 19.1). The core units of phycobiliproteins are made up of α- (12–20 kDa) and ß- (15–22 kDa) chains (Sinha et al. 1995; Kannaujiya et al. 2014) in cyanobacteria, whereas in some red algae, γ chain was found. The formation of functional phycobiliprotein core assembly involves coloured phycobiliproteins (85%) and colourless linker polypeptides (15%) (Parsiegla et al. 2012). The biosynthesis and stabilization of the phycobiliproteins are achieved by different types of structural elements which are also known as colourless linker polypeptides that help

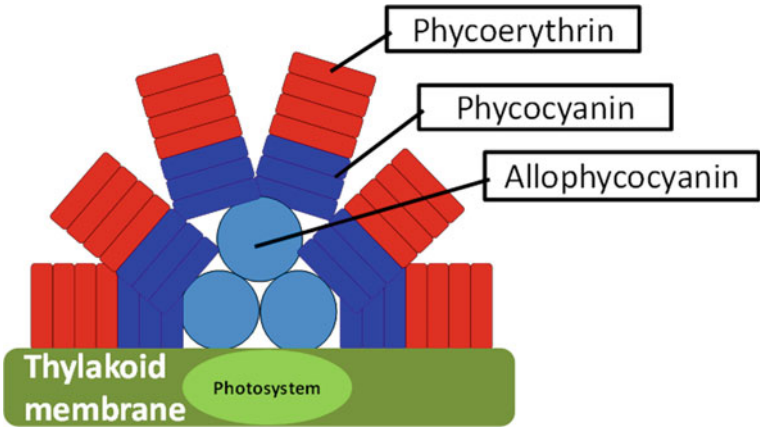

Fig. 19.1 Schematic representation of phycobilisome

stabilize the phycobilisome complex (Ana et al. 2009). Approximately 20% of allophycocyanin and phycocyanin are found in the core and periphery region of the photosynthetic membrane, respectively (Singh and Montgomery 2013). Phycoerythrin can be found at the distal region of the phycobiliproteins and functions in adaptation during fluctuations from photosynthetically active radiation (Rodríguez et al. 1991).

19.7 Pigments in Aquaculture

Nutrition is one of the major determining factors in the success of aquaculture industry, as it is closely related to growth, survival and health of the cultured organisms. In aquaculture, microalgae are used as live feeds, supplements, water quality bioremediation (Khatoon et al. 2007), growth promoter and animal colour enhancer (Begum et al. 2016). The colour of microalgae is influenced by the pigments which are a part of the cell's photosynthetic system and naturally contain high-value compounds such as polyunsaturated acids, fatty acids, chlorophylls, phycobiliproteins and carotenoids.

Microalgae pigments such as carotenoids with high contents of omega-3 fatty acids and high antioxidants have contributed significantly to the advancement of aquaculture industry (Viera et al. 2018). Microalgae are frequently used in hatchery to enrich zooplankton such as copepods and *Artemia* as live feeds, which could be used to increase growth and survival of fish and shrimp larvae. El-Sheekh et al. (2016) reported that there were significant increases in protein, carbohydrate, ascorbic acid and β-carotene in *Artemia* enriched with *Nannochloropsis oculata*. Li et al. (2014) showed that the large yellow croaker, *Pseudosciaena crocea*, fed with *Haematococcus pluvialis* biomass had significantly higher growth compared with those fed

with the astaxanthin extract, indicating that the microalgae biomass might have more biomolecules contributing to the growth compared to the pure astaxanthin alone (Table 19.2). In fact, whole microalgae biomass also improved immune capacity and enhanced low salinity tolerance in *Penaeus vannamei* (Xie et al. 2018).

19.7.1 Carotenoids in Aquaculture

Nutritious and balanced diets can influence the immunity and disease resistance in cultured animals and reduce disease-related economic loss. Dietary supplementation with key amino acids, polyunsaturated fatty acids, vitamins and carotenoids were critical in modulating immune response and productivity of the aquaculture industry (Khatoon et al. 2007; Pohlenz and Gatlin 2014; Begum et al. 2016; Ahmad et al. 2019).

Carotenoids encompass approximately 1200 pigments categorized into two major groups (carotenes and xanthophylls) and they are known as one of the most important sets of compounds in improving growth and health of many organisms (Lockwood et al. 2003; Paliwal et al. 2016; Heo et al. 2018; Wang et al. 2018b). Carotenoids are synthesized by higher plants, mosses, bacteria, fungi and algae. However, there are some pigments, such as fucoxanthin, which are only found in certain microalgae (Foo et al. 2015, 2017). Most animals, with a few exceptions, do not produce carotenoids de novo, but take them in their diets.

In aquaculture, carotenoids function in pigment development, antioxidants and vitamin enrichment, growth and reproduction improvement, cellular protection from photo-damage and health enhancement (Table 19.2). Due to their antioxidant and free radical scavenging properties, the best known function of carotenoids in aquaculture besides increased growth and enhanced immunity (Lim et al. 2018) is pigmentation (Menasveta et al. 1993; Wade et al. 2017a). Colours play a major role in improving perceived quality, acceptability and willingness to pay for aquaculture products. Higuera-Ciapara et al. (2006) noted that orange-reddish colour is one of the most important factors in the perceived quality of salmonids and crustaceans. Since the colour of fish skin is an important factor in determining the retail value of a commodity, Pavlidis et al. (2006) developed a new index, the Entire Colour Index (ECI) to express the actual colour of the fish or invertebrates, which is influenced by hue and chroma as a combined variable as these factors should not be considered separately. Sun et al. (2012) reported that carotenoids showed better effects on skin pigmentation compared to xanthophylls.

Colours in organisms are mainly determined by genetic inheritance, but the pigmentation can be greatly influenced by environmental factors and occurrence of diseases. Carotenoids, which are dependent on diets and body conditions, are responsible for many yellow, orange and red hues in animals including fish and aquatic invertebrates (Sefc et al. 2014). Wade et al. (2017b) reported that 30–50 mg/kg body weight of carotenoids is required to achieve optimal colours in penaeid shrimps. The enhancement of colouration in fish can be due to the proliferation of chromatophores

induced by the presence of carotenoids (Liu et al. 2016). Yi et al. (2014) demonstrated that astaxanthin and other xanthophylls were useful in improving growth and colour in yellow croaker. In ornamental fish industry, colouration is one of the product main attributes. Ho et al. (2013) and Liu et al. (2016) demonstrated the effects of astaxanthin on the pigmentation in Spinecheek anemonefish and discus fish, respectively (Table 19.2). However, the effects on pigmentation could vary with the pigment sources. Sun et al. (2012) reported that diet supplementation with a photosynthetic bacterium, *Rhodopseudanonas palustris*, did not significantly enhance the colouration of the Japanese ornamental koi, *Cyprinus carpio*, compared to a blue-green algae, *Spirulina platensis*.

In channel catfish, on the other hand, yellowish-reddish pigments are undesirable because any deviation from the normal white flesh the fish reduces its marketability (Li et al. 2007). However, the accumulation of carotenoids in catfish can benefit the human health which can satisfy the emerging markets of healthy functional foods. According to Barbosa et al. (1999), astaxanthin and canthaxanthin are responsible for the typical red colour in salmonids similar to the wild fish, and thus these two pigments are commonly used in the diet of farmed salmonids for pigmentation enhancement (Table 19.2).

Carotenoids are also used to improve aquaculture production by enhancing the broodstock quality and improvement of live feeds (Table 19.2). In crayfish, carotenoids improved the broodstock reproduction (Harlıoğlu and Farhadi 2017). Live feeds such as rotifers and microcrustaceans, like other aquatic consumers do not contain endogenous carotenoids, but can be enriched through their diets. Carotenoid lutein was used to enrich live feeds such as rotifers (van Bergeijk et al. 2013). Johnston et al. (2018) noted that the rotifer population supplemented with astaxanthin was more productive and had higher nutritional contents for healthy development of larval fish compared to rotifers without the supplement. Chaoruangrit et al. (2018) reported that microalgal diets improved growth, survival, reproduction and carotenoid contents in a fairy shrimp, *Branchinella thailandensis*. High carotenoid contents in cultured animals such as pearl oysters can also increase their resistance to environmental stressors such as high temperature (Table 19.2), suggesting that antioxidant system in the carotenoids participated in maintaining the oxidation and reduction balance when the animals were exposed to high temperature stress (Meng et al. 2017). In addition, Nadukooru and Yallapragada (2015) reported that carotenoids can also be used as a sensitive indicator of sublethal cadmium toxicity in *Penaeus monodon* post larvae, indicating the multi bio-functionalities of carotenoids in aquaculture.

Different sources of pigments may affect aquaculture organisms differently. Many studies showed that astaxanthin was more effective in improving growth and health of many cultured organisms (Table 19.2). However, Boonyaratpalin et al. (2001) reported by supplementing tiger shrimp diet with β-carotene from *Dunaliella salina* resulted in no significant differences in terms of growth rate, survival rate, feed conversion ratio and immune response compared to those using the more expensive astaxanthin.

In feed industries, many synthetic commercial antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone

(TBHQ) and ethoxyquin have been used to retard oxidation and peroxidation. However, these synthetic compounds have shown potential health hazard and efforts are ongoing to find natural alternatives (Aklakur 2018). Barbosa et al. (1999) reported that there was higher blood astaxanthin concentration in fish fed with supplements microalgal biomass (containing astaxanthin) compared to synthetic astaxanthin.

19.7.2 Phycobilliproteins in Aquaculture

The bright colour phycobiliproteins are non-toxic in nature and have anti-inflammatory, anticancer, antioxidant, hepatoprotective, immunomodulatory, neuro-protective and nephroprotective effects after being purified (Pandey et al. 2013; Sonani et al. 2016). Hence, phycobiliproteins have the potential to be commercialized and be applied in various industries such as biomedicine, pharmaceuticals, biotechnology, food products and colourants. Also, phycoerythrin has important fluorescent properties and it is widely used as a fluorescent probe.

Fatty acids and pigments including phycobiliproteins from microalgae have the highest demand in the market (Minhas et al. 2016). In the 1980s, *Haematococcus* sp. and *Dunaliella* sp. were cultivated for its pigments particularly astaxanthin and carotene for use as additives in food and feed. In the 1990s, microalgae were cultivated for the production of PUFAs such as eicosapentaenoic acid and docosahexaenoic acid for use in aquaculture feed and enrichment of nutritional products. The top two commercially produced phycobiliproteins are phycoerythrin from *Porphyridium* spp. and phycocyanin from *Spirulina* spp. (Plaza et al. 2009; Rodriguez-Sanchez et al. 2012; Borowitzka 2013).

There are very limited studies on the effects of phycobiliproteins in aquaculture. According to Prasanna et al. (2007), Abd El Baky et al. (2015) and Liu et al. (2016), phycocyanin from blue-green algae can be used as a practical food. For instance, *Spirulina platensis* was often used as a dietary supplement in animals (Vonshak and Tomaselli 2002) as it contained high amounts of vitamins, minerals, antioxidant activity and phycocyanin (Estrada et al. 2001). Of late, there is an increasing study using *Spirulina* as a feed supplement in the farming of fish, shrimp and ornamental fish. Biabani Asrami et al. (2019) reported a significant increase in the final weight, final length, specific growth rate, weight growth rate, length growth rate, average daily growth rate and protein efficiency ratio in guppy fish (*Poecilia reticulata*) fed with 0.15% phycocyanin extracted from *Spirulina platensis*. Phycocyanin added diet also significantly increased the total pigments in guppy tissue and enhanced the skin colour. Also, protease activity and digestive enzymes in the treatment fish were significantly higher ($p < 0.05$).

Gora et al. (2019) in their studies on Asian seabass *Lates calcarifer* larvae fed dietary supplementation showed that cannibalism (%) was significantly reduced in the treatments fed phycocyanin (8.57 ± 0.3) pigmented diets compared to the control group. Bacterial challenge with pathogenic *Vibrio alginolyticus* revealed the highest

resistance in astaxanthin and phycocyanin fed larvae. The study validated that astaxanthin and phycocyanin at 1% dietary levels improved survival and disease resistance in Asian seabass larvae but did not affect growth performance.

In another study by Muchtar et al. (2019), phycocyanin extracted from *Spirulina platensis* and administered in the feed at a dose of 150 mg/kg feed improved survival and non-specific immune response of common carp *Cyprinus carpio* when challenged with *Aeromonas hydrophila.* Spirulina was also reported to be able to increase immune responses in sturgeon (*Huso huso*) against *Streptococcus iniae,* Nile tilapia (*Oreochromis niloticus*) against *Pseudomonas fluorescence,* and carp (*Cyprinus carpio*) against *Aeromonas hydrophila* (Watanuki et al. 2006; Adel et al. 2016; Mahmoud et al. 2018). This may be due to the nutritional composition, bioactive compounds and pigments such as phycocyanin (17.2%) and chlorophyll (1.2%) present in *Spirulina* (Gershwin and Belay 2007). In mice, phycocyanin showed antioxidative properties, and was able to prevent the formation of malondialdehyde in the liver, kidney and pancreas (Ou et al. 2012). Phycocyanin was also reported to protect against renal kidney failure (Iijima et al. 1982). The role of phycobiliproteins for fish health requires further investigations.

19.8 Photobioreactor Performances in Carotenoid Production

The development of the efficient system for mass production of carotenoids represents one of major issue in microalgal biotechnology due to great potentials of microalgae as high valued commercial products in nutraceutical, cosmeceutical and pharmaceutical industries (Gong and Bassi 2016). The types of carotenoids produced by microalgae depend on the species and strains, and the carotenoids concentration and production rate in microalgae biomass greatly rely on types of photobioreactor and its operational conditions (García-Malea et al. 2009; Ma et al. 2019; Prieto et al. 2011). Many kinds of carotenoids are produced by microalgae, however only a few number of carotenoids including astaxanthin, lutein and β-carotene have been produced on commercial scale.

Table 19.3 shows the types of photobioreactor, the light intensity, the biomass production rate and the astaxanthin content in previous studies on *Haematococcus* spp. It has been observed that *Haematococcus* cells easily turn to cyst and accumulate astaxanthin under the stress conditions including high light intensity and low nutrient concentrations (Zhang et al. 2018). Generally, production process of astaxanthin can be divided into two stages: a green stage for cell growth without stress and a red stage for astaxanthin accumulation with stress condition (Boussiba and Vonshak 1991; Choi et al. 2011). In the green stage, higher growth rate can be maintained because of the non-stress condition for cell growth (Boussiba and Vonshak 1991). It has been mentioned that *Haematococcus* genus has relatively low growth rate compared with other green algae, while some of *Haematococcus* strains have high growth rates

Table 19.3 Astaxanthin productivity under different types of reactor and culture conditions in previous studies

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Haematococcus pluvialis	Astaxanthin	AGM system	Outdoor	–	11.0	29.00	319	Olaizola (2000)
H. pluvialis, CCAP 34/8	Astaxanthin	Bubble column	Indoor	500	14.7	2.13	31	García-Malea et al. (2009)
	Astaxanthin	Bubble column	Indoor	500	22.2	0.06	1	
	Astaxanthin	Bubble column	Indoor	500	24.7	0.08	2	
	Astaxanthin	Bubble column	Indoor	500	25.5	0.08	2	
	Astaxanthin	Bubble column	Indoor	750	18.4	2.61	48	
	Astaxanthin	Bubble column	Indoor	750	29.7	0.14	4	
	Astaxanthin	Bubble column	Indoor	750	25.5	0.08	2	
	Astaxanthin	Bubble column	Indoor	750	27.2	0.08	2	
	Astaxanthin	Bubble column	Indoor	1000	17.6	2.80	49	

(continued)

Table 19.3 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
	Astaxanthin	Bubble column	Indoor	1000	29.7	0.28	8	
	Astaxanthin	Bubble column	Indoor	1000	29.7	0.11	3	
	Astaxanthin	Bubble column	Indoor	1000	28.5	0.11	3	
	Astaxanthin	Bubble column	Indoor	1250	18.0	3.11	56	
	Astaxanthin	Bubble column	Indoor	1250	30.6	0.25	8	
	Astaxanthin	Bubble column	Indoor	1250	33.1	0.11	4	
	Astaxanthin	Bubble column	Indoor	1250	31.4	0.11	4	
	Astaxanthin	Tubular	Outdoor	–	12.5	12.64	158	
	Astaxanthin	Tubular	Outdoor	–	12.5	13.44	168	
	Astaxanthin	Tubular	Outdoor	–	14.7	6.43	95	
H. pluvialis	Astaxanthin	Flat plate	Indoor	423	3.0	53.20	160	Poonkum et al. (2015)
	Astaxanthin	Flat plate	Indoor	423	3.2	52.70	166	
	Astaxanthin	Flat plate	Indoor	423	3.1	52.00	159	
	Astaxanthin	Flat plate	Outdoor	–	2.7	44.70	121	

(continued)

Table 19.3 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
	Astaxanthin	Flat plate	Outdoor	–	–	–	103	
	Astaxanthin	Flat plate	Outdoor	–	–	–	89	
	Astaxanthin	Flat plate	Outdoor	–	2.8	42.80	121	
	Astaxanthin	Flat plate	Outdoor	–	2.7	42.10	115	
H. pluvialis	Astaxanthin	Bubble column	Indoor	50	3.4	14.01	48	Zhang et al. (2018)
	Astaxanthin	Bubble column	Indoor	100	4.5	20.41	92	
	Astaxanthin	Bubble column	Indoor	200	4.7	27.30	128	
	Astaxanthin	Bubble column	Indoor	400	5.1	37.90	195	
	Astaxanthin	Bubble column	Indoor	50	3.8	6.43	24	

(continued)

Table 19.3 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
	Astaxanthin	Bubble column	Indoor	100	5.5	8.66	48	
	Astaxanthin	Bubble column	Indoor	200	7.1	16.21	115	
	Astaxanthin	Bubble column	Indoor	400	8.6	20.82	179	
H. pluvialis	Astaxanthin	Bubble column	Indoor	650	–	10.31	–	Guler et al. (2020)
	Astaxanthin	Bubble column	Indoor	470	2.4	30.23	71	
	Astaxanthin	Bubble column	Indoor	620	3.9	48.88	188	

(García-Malea et al. 2009). García-Malea et al. (2009) have reported that a high aerial biomass production rate of 33.1 g-ds/m^2/day on a *Haematococcus* sp. was obtained in a high light intensity of 1250 μE m^{-2} s^{-1}. At that time a low astaxanthin content of 0.11 mg/g-ds was achieved, meaning that the cells in the condition were under the green stage even in the strong light condition. According to previous reports on *Haematococcus* spp., the astaxanthin content in the dry biomass of *Haematococcus* cell can be increased up to 5% in the red stage (Bubrick 1991; Harker et al. 1996). This indicated that the content of the pigment obtained in *Haematococcus* was quite high compared with the other carotenoids such as β-carotene and lutein in other microalgae. Although astaxanthin can be accumulated in a green microalga *Chlorella zofingiensis*, the content is much lower than that of *Haematococcus* sp. (Del Campo et al. 2004). Thus, *Haematococcus* sp. is usually utilized for the commercial scale production of astaxanthin from microalgae.

The production of lutein on microalgae has also been carried out throughout the two step processes, same as the production of astaxanthin. The candidates for astaxanthin production are only *Haematococcus* species, whereas the candidates for lutein production have not been restricted to a particular species yet: *Chlamydomonas* sp., *Chlorella zofingiensis*, *Chlorococcum citriforme*, *Dunaliella salina, Muriellopsis* sp., *Neospongiococcus gelatinosum, Scenedesmus almeriensis* (Table 19.4). Though the lutein content in previous studies is much less than the content of astaxanthin, the areal production rate of lutein has reached the same level as astaxanthin production rates. However, the lutein producer was a terrestrial plant marigold (Lin et al. 2015). The high biomass production rates of 50–60 g-dw m^{-2} day^{-1} obtained by microalgae had been reported only in a small scale reactor (Del Campo et al. 2000; Sánchez et al. 2008). If improvement of biotechnology in the production, especially in the cost, can be achieved in commercial scale, the lutein production may be developed using microalgae. In addition to the improvement in large scale process, enhancement of the cellular lutein content should be also important due to its low content compared with the content of astaxanthin in *Haematococcus* species (Table 19.4).

Because fucoxanthin is the main carotenoid for brown algae, a large number of species including *Chaetoceros gracilis*, *Isochrysis galbana* and *Phaeodactylum tricornutum* have been observed as potential candidates for fucoxanthin production (Table 19.5). Fucoxanthin has been known as a primary carotenoid of microalgae, while both astaxanthin and lutein are categorized as secondary carotenoids. In general primary carotenoids are naturally accumulated in the cell without any stress, meaning that the fucoxanthin production can be operated in one step. To avoid inefficient accumulation of fucoxanthin under high light intensity, many researches have operated at relatively low light intensity of below 200 μE m^{-2} s^{-1}. However, the biomass production rate is limited by the low light condition, which is necessary for effective fucoxanthin accumulation, resulting in relatively low production rates of fucoxanthin (McClure et al. 2018).

Furthermore, microalgae can accumulate other carotenoids such as β-carotene, canthaxanthin and zeaxanthin in the cell (Table 19.6). It has been reported that β-carotene can be accumulated up to 100 mg g-dw^{-1} by a green microalga, *Dunaliella salina*, especially under the combination of high salinity, nutrient deficiency and high

Table 19.4 Lutein productivity under different types of reactor and culture conditions in previous studies

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Chlamydomonas sp. JSC4	Lutein	Bubble column	Indoor	150	18.3	3.00	55	Ma et al. (2019)
	Lutein	Bubble column	Indoor	250	29.7	2.92	86	
	Lutein	Bubble column	Indoor	375	37.8	3.28	112	
	Lutein	Bubble column	Indoor	500	46.6	3.10	145	
	Lutein	Bubble column	Indoor	625	54.2	3.18	172	
	Lutein	Bubble column	Indoor	750	61.8	2.74	170	
	Lutein	Bubble column	Indoor	1100	58.5	2.62	156	
Chlorella zofingiensis	Lutein	Bubble column	Indoor	460	21.3	2.82	60	Del Campo et al. (2000)
Chlorococcum citriforme	Lutein	Bubble column	Indoor	460	59.9	7.44	445	Del Campo et al. (2000)
Dunaliella salina UTEX 2538	Lutein	Air lift tubular	Outdoor	–	2.0	–	–	García-González et al. (2005)
Muriellopsis sp.	Lutein	Bubble column	Indoor	460	3.5	5.55	19	Del Campo et al. (2000)

(continued)

Table 19.4 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Muriellopsis sp.	Lutein	Air lift tubular	Outdoor	666	16.3	4.63	75	Del Campo et al. (2001)
	Lutein	Air lift tubular	Outdoor	666	14.6	4.36	64	
	Lutein	Air lift tubular	Outdoor	666	23.5	3.83	90	
	Lutein	Air lift tubular	Outdoor	1174	24.2	4.31	104	
	Lutein	Air lift tubular	Outdoor	1174	37.0	4.41	163	
	Lutein	Air lift tubular	Outdoor	1174	40.6	4.28	174	
	Lutein	Air lift tubular	Outdoor	1174	36.0	3.92	141	
	Lutein	Air lift tubular	Outdoor	1174	36.9	3.91	144	
	Lutein	Air lift tubular	Outdoor	1338	40.8	4.42	180	
Neospongiococcus gelatinosum	Lutein	Bubble column	Indoor	460	67.5	4.40	297	Del Campo et al. (2000)
Scenedesmus almeriensis	Lutein	Bubble column	Indoor	1625	58.0	5.48	318	Sánchez et al. (2008)

Table 19.5 Fucoxanthin productivity under different types of reactor and culture conditions in previous studies

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Chaetoceros calcitrans	Fucoxanthin	Annular	–	150	–	5.25	–	Foo et al. (2015)
Chaetoceros gracilis	Fucoxanthin	Cylinder	–	–	–	2.24	–	Kim et al. (2012)
C. gracilis	Fucoxanthin	Glass tube	Indoor	50	–	–	1.1	Tokushima et al. (2016)
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.5	
	Fucoxanthin	Glass tube	Indoor	50	–	–	1.2	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.6	
	Fucoxanthin	Glass tube	Indoor	50	–	–	1.2	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.5	
	Fucoxanthin	Glass tube	Indoor	50	–	–	1.1	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.4	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.3	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.1	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.8	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.3	
	Fucoxanthin	Glass tube	Indoor	50	–	–	1.6	
	Fucoxanthin	Glass tube	Indoor	50	–	–	0.7	
	Fucoxanthin	Glass tube	Indoor	100	–	–	1.5	
	Fucoxanthin	Glass tube	Indoor	100	–	–	0.6	

(continued)

Table 19.5 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
C. gracilis	–	Cylinder	Indoor	430	0.48	–	–	Pérez et al. (2017)
	–	Cylinder	Indoor	430	0.40	–	–	
	–	Cylinder	Outdoor	1294	1.27	–	–	
	–	Cylinder	Outdoor	1294	1.13	–	–	
Chaetoceros muelleri var. subsalsum	–	Vertical flat plate-glass reactor	Outdoor	–	5.80	–	–	Zhang and Richmond (2003)
	–	Vertical flat plate-glass reactor	Outdoor	–	8.20	–	–	
	–	Vertical flat plate-glass reactor	Outdoor	–	14.00	–	–	
	–	Vertical flat plate-glass reactor	Outdoor	–	9.20	–	–	
Isochrysis galbana	–	Bubble column	Outdoor	–	1.60	–	–	Hu and Richmond (1994)
I. galbana	–	Tubular	Outdoor	–	6.68	–	–	Grima et al. (1994)
	–	Tubular	Outdoor	–	7.64	–	–	
	–	Tubular	Outdoor	–	7.64	–	–	

(continued)

Table 19.5 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Isochrysis sp. F&M-M36	Fucoxanthin	–	–	–	–	17	–	Crupi et al. (2013)
I. galbana CCMP1324	Fucoxanthin	Bubble column	Indoor	32	–	18.23	–	Kim et al. (2012)
I. galbana KMMCC-12	Fucoxanthin	Bubble column	Indoor	32	–	6.04	–	
I. galbana CCMP355	Fucoxanthin	Bubble column	Indoor	60	5.58	9.8	54.6	Sun et al. (2019)
I. galbana CCMP462	Fucoxanthin	Bubble column	Indoor	60	7.18	10.2	73.2	
I. galbana CCMP463	Fucoxanthin	–	Indoor	60	8.46	11.8	99.9	
I. galbana CCMP715	Fucoxanthin	–	Indoor	60	7.90	13.9	109.7	
I. galbana CCMP1244	Fucoxanthin	–	Indoor	60	9.14	12.6	115.2	
I. galbana CCMP1324	Fucoxanthin	–	Indoor	60	8.95	14.5	137.8	
I. galbana CCMP1611	Fucoxanthin	–	Indoor	60	6.09	8.9	54.2	
I. galbana UTEX987	Fucoxanthin	–	Indoor	60	3.58	10.6	37.9	
I. galbana 1292	Fucoxanthin	–	Indoor	60	2.08	12.1	25.2	
I. galbana 2307	Fucoxanthin	–	Indoor	60	8.05	13.4	107.9	
I. galbana CCAP927/1	Fucoxanthin	–	Indoor	60	5.38	15.8	85.0	
I. galbana CCAP927/12	Fucoxanthin	–	Indoor	60	6.64	12.2	81.0	

(continued)

Table 19.5 (continued)

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
I. galbana CCAP927/14	Fucoxanthin	–	Indoor	60	8.00	13.1	104.8	
I. galbana CS-177	Fucoxanthin	–	Indoor	60	10.02	9.4	94.2	
Phaeodactylum tricornutum CS-29	Fucoxanthin	Flat plate	Indoor	100	0.75	42.8	32.0	McClure et al. (2018)
	Fucoxanthin	Flat plate	Indoor	150	1.55	23.2	36.0	
	Fucoxanthin	Flat plate	Indoor	210	2.12	10.4	22.0	
	Fucoxanthin	Flat plate	Indoor	150	2.28	10.1	23.0	
	Fucoxanthin	Flat plate	Indoor	150	1.54	21.1	32.5	
	Fucoxanthin	Flat plate	Indoor	150	1.31	26.7	35.0	
	Fucoxanthin	Flat plate	Indoor	150	1.82	59.2	108.0	

Table 19.6 Productivities of β-carotene, zeaxanthin and canthaxanthin under different types of reactor and culture conditions in previous studies

Microalgae	Pigment	Type of reactor	Indoor or outdoor	Light intensity	Aerial biomass production (g-DW m^{-2} day^{-1})	Contents (mg-Pigment/g-DW)	Aerial pigment production (mg-Pigment m^{-2} day^{-1})	References
Dunaliella salina	β-carotene	Open pond	Outdoor	–	–	–	200	Ben-Amotz (1999)
D. salina	β-carotene	Tubular	Outdoor	–	2	100	100	García-González et al. (2005)
Dictyococcus cinnabarinus	Canthaxanthin	–	Indoor	–	–	1.1	–	Nelis and De Leenheer (1991)
Chlorella zofingiensis	Canthaxanthin	Bubble column	Indoor	50	2.01	0.8	1.6	Pelah et al. (2004)
	Canthaxanthin	Bubble column	Indoor	50	1.96	8.5	16.7	
	Canthaxanthin	Bubble column	Indoor	300	2.14	2.97	6.3	
	Canthaxanthin	Bubble column	Indoor	300	2.18	3.66	8.0	
D. salina	Zeaxanthin	Flat plate	Indoor	100	–	0.23	–	Jin et al. (2003)
Dunaliella salina zea1 (mutant)	Zeaxanthin	Flat plate	Indoor	100	–	5.9	–	
Chlorella ellipsoidea	Zeaxanthin	–	Indoor	0	–	0.608	–	Koo et al. (2012)

light intensity (García-González et al. 2005). It can be suggested that relatively high production rate of β-carotene is obtained in *D. salina*. On the other hand, the contents of canthaxanthin and zeaxanthin have not been achieved at a high level, requiring improvement of biotechnology for the production methods (Jin et al. 2003; Pelah et al. 2004).

19.9 Conclusions

Due to the stagnation of capture fisheries and increasing demand for fishery products, aquaculture grows at the fastest rate compared to other global food producing sectors. However, aquaculture industry is facing various threats related to poor nutrition, slow growth and rampant diseases. One of the means to improve productivity in aquaculture industry is to enhance health through therapeutic nutrition such as utilizing microalgae pigments as feed supplements which contain high carotenoids, phycobiliproteins, antioxidants and polyunsaturated fatty acids.

Microalgae are a valuable source of highly nutritive polyunsaturated fatty acids (PUFA), protein hydrolysates and bioactive peptides and a variety of biomolecules including pigments such as chlorophylls, carotenoids, phycobiliproteins and phenolic compounds acids which are high in antioxidants and suitable for immune enhancement in humans and animals. These growth and health enhancement properties of microalgae are conducive for the development of functional, nutritional and therapeutic commodities.

Microalgae pigments including chlorophylls, carotenoids and phycobiliproteins also exhibit protective effects against several health disorders in humans and animals in addition to their effects of growth and survival that they have wide applications in food, nutraceutical, pharmaceutical, cosmeceutical and aquaculture industries. In aquaculture, microalgal pigments are now becoming popular since synthetic pigments are associated with harmful effects. The potential use of natural products for valuable biomolecules has led the search of microalgae as a source of antioxidants, natural colours and food additives, which are important aspects in aquaculture industry.

Carotenoids, consisting mainly of carotenes and xanthophylls are important nutritive pigments for many aquatic animals, but most animals cannot synthesize carotenoids de novo and depend on dietary supplements for the growth, health and pigmentation enhancement. Major carotenoid pigments such as astaxanthin, fucoxanthin, canthaxanthin and β-carotenes have been proven effective in aquaculture industry to enhance skin pigmentation, growth, survival, health and productivity. Similarly, phycobiliproteins are also important in reducing cannibalism, improve survival and enhance immune response in cultured animals. However, the commercial production of microalgal pigments is still limited to a few species. In addition, cost-effective technologies for large scale production of microalgae pigments are still inadequate to meet the growing demands of these valuable products. Thus, more research and novel approaches in microalgal pigment production and utilization are

needed to improve growth, health and productivity of aquatic organisms that are critical for the sustainability of aquaculture industry.

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Chapter 20
Carotenoids in Aviculture

Peter F. Surai and Ivan I. Kochish

Abstract Carotenoids are the most numerous and widespread group of pigments in nature. Today, the carotenoid family is known to include more than 1100 natural compounds. Carotenoids play important roles in aviculture, including egg yolk and skin pigmentation as well as supplements to improve breeder performance. Only xanthophylls with high efficacy of transfer from the feed to the egg yolk found their way on the commercial poultry feed market. In fact, in commercial egg production desired egg yolk colour intensity is achieved by using a combination of various (usually, yellow and red) dietary carotenoids. Furthermore, canthaxanthin is proven to be an effective feed additive for poultry breeder nutrition. Among many important biological functions of carotenoids, there participation in building an effective antioxidant defence network could be of vital importance. Indeed, biological value of direct AO activity of carotenoids associated with scavenging ROS is probably not very high. It seems likely that indirect effects of carotenoids on the antioxidant defences via upregulation of Nrf2 and downregulation of NF-κB are a driving force of their beneficial effect in avian species in general and in poultry production in particular. Carotenoid protective effects in chicken/avian embryonic development deserve more attention and warrant further investigation.

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Abbreviations

AO	Antioxidant
Apo-EE	β-apo-8′-caroitenoic acid ethyl ester
AX	Astaxanthin
CoQ	Coenzyme Q
CX	Canthaxanthin
NF-κB	Nuclear factor-kappa B
HO	Heme oxygenase
HDL	High density lipoproteins
LPL	Lipoprotein lipase
MDA	Malondialdehyde
Nrf2	Nuclear factor-erythroid-2-related factor 2
SOD	Superoxide Dismutase
TAC	Total antioxidant capacity
YSM	Yolk Sac Membrane

20.1 Introduction

Carotenoids are the most numerous and widespread group of pigments in nature and have had a long and interesting history. Studies on these pigments were started at the beginning of the nineteenth century, when the crystalline yellow pigment carotene was first isolated in 1831 by Wackenroder from carrots, and the yellow pigments of autumn leaves were named as xanthophylls by Berzelius in 1837 (Tee 1992; Karnaukhov 1990). A hundred years later, the number of characterised naturally occurring carotenoids rose from 15 in 1933 to about 80 in 1948 and increased sharply to about 300 (Ong and Tee 1992) and over 750 pigments (Maoka 2009) over the next 60 years. Today, the carotenoid family is known to include 1117 natural carotenoids from 683 source organisms (Yabuzaki 2017).

In nature, carotenoids are responsible for a variety of bright colours in senescent leaves, flowers (narcissus, marigold), fruits (pineapple, citrus fruits, paprika), vegetables (carrots, tomatoes), insects (ladybird), bird plumage (flamingo, cock of rock, ibis, canary) and marine animals (crustaceans, salmon) (Pfander 1992). These pigments provide different colours from light yellow to dark red and, when complexed with proteins, can produce green and blue colourations (Ong and Tee 1992). Carotenoids are exclusively responsible for egg yolk colour and are thought to play specific roles in avian embryonic development (Surai 2002, 2012a, b; Surai et al. 2001a, b).

20.2 Chemical Structure and Properties

All carotenoids may be derived from the acylic $C_{40}H_{56}$ structure having a long central chain of conjugated double bonds (Pfander 1992) and they can be described by the general formula $C_{40}H_{56}O_n$, where n is 0–6; hydrocarbons ($n = 0$) are called carotenes, whereas oxygenated carotenoids (n = 1–6) are termed xanthophylls (Castenmiller and West 1998). Carotenoids are based upon the same C40 isoprenoid skeleton, which is modified by cyclisation, addition, elimination, rearrangement and substitution (Rice-Evans et al. 1997).

20.3 Molecular Mechanisms of Carotenoid Action in Animals

It is well known that carotenes are precursors of vitamin A and this function has been well defined. However, less than 10% of known carotenoids can be converted to vitamin A. Clearly defined roles for non-provitamin carotenoids have still to be definitively established (Thurnham and Northrop-Clewes 1999), but evidence is emerging for several important functions. These include:

- antioxidant activities (Surai et al. 2001a, b; Surai 2002, 2012a, b; Surai et al. 2016; Xavier and Pérez-Gálvez 2016; Bahonar et al. 2017; Weaver et al. 2018; Ribeiro et al. 2018);
- the promotion of cell differentiation (McDevitt et al. 2005; Wójcik et al. 2008; Nagao 2009; Bolhassani et al. 2014; Takahashi et al. 2018; Xie et al. 2019);
- regulation of cell proliferation (Zhang et al. 2011a, b Liu et al. 2013; Barzegari and Pavon-Djavid 2014; Bolhassani et al. 2014; Gupta et al. 2016; Li et al. 2018a, b; Dutta et al. 2018);
- regulation of intracellular communication via gap junctions (Stahl et al. 2002; Livny et al. 2002; Stahl and Sies 2005; Leone et al. 2010; Kim et al. 2016);
- regulation of cellular activity/levels of the detoxifying enzymes (De Flora et al. 1999; Stahl et al. 2002; Rühl et al. 2004; Ben-Dor et al. 2005);
- enhancement of immune function (Moller et al. 2000; Sun et al. 2014; Babin et al. Babin et al. 2010, 2015; Hosseini-Vashan et al. 2016; George et al. 2017; Milani et al. 2017; Toti et al. 2018);
- Nrf2 upregulation (Zhao et al. 2017; Li et al. 2018b; Yu et al. 2018; Chang et al. 2018; Yang et al. 2018; Xie et al. 2018; Zhu et al. 2018; Hu et al. 2018; Lin et al. 2018; Niu et al. 2018; Xue et al. 2019);
- NF-κB downregulation (Sahin et al. 2017; Zhao et al. 2017; Shi et al. 2018; Barros et al. 2018; Sung and Kim 2018; Chang et al. 2018; Icel et al. 2019; Li et al. 2019);
- natural colorants providing coloration to birds, reptiles, amphibians, fish and various invertebrates (Surai 2002);
- cell membrane stabilisers in molluscs (Surai 2002).

These functions are considered to be responsible for the various health-promoting properties of carotenoids. However, the physiological requirement for carotenoids in avian species is not yet established and more work in this field is needed to understand how carotenoids fulfil any essential nutritional function in poultry.

20.4 Carotenoid Absorption and Bioavailability

Carotenoid absorption and bioavailability have received considerable attention for the last few years (Nagao 2011; Kotake-Nara and Nagao 2011; Reboul 2013; Perveen et al. 2015; Cervantes-Paz et al. 2016). However, most of these publications were mainly concerned with humans. Analyses of data associated with avian species are presented below. Birds are considered to be good oxycarotenoid absorbers although some birds, for example, the flamingo, preferentially absorb carotenes (Hudon 1994). The efficiency of carotenoid assimilation from the feed depends on many factors and absorption in the intestine is a major determinant of this process. In general, carotenoid absorption can be divided into four stages: digestion of the food matrix, formation of lipid-mixed micelles, uptake of carotenoids into the intestinal mucosal cells and delivery to the plasma via the lymph system (Williams et al. 1998). Since the lymphatic system of the bird is not developed, carotenoids are probably delivered directly to the liver and other tissues by portomicrons, i.e. the lipid-rich lipoproteins released from the intestinal cells into the portal vein. Carotenoid ester hydrolysis by pancreatic esterases is an important step in their absorption. For example, it has been shown that the dietary lutein diester was hydrolysed mainly to lutein, which was absorbed through the intestinal wall into the blood stream and, in the serum, about 96% of the total lutein occurred as the free alcohol and the remainder was in the form of the monoester (Tyczkowski and Hamilton 1986a, b). As a result, hens fed lutein diester accumulated lutein in their yolks (Philip et al. 1976). When hens were fed zeaxanthin diester, the egg yolk contained only free zeaxanthin (Kuhn and Brockman 1932). It is possible to improve egg yolk pigmentation by prior saponification of marigold meal xanthophyll (Coon and Couch 1976). Furthermore, in broilers free lutein is absorbed almost two-fold faster compared to lutein diester (Tyczkowski and Hamilton 1986a, b). It is interesting to note that, in the chicken, the contents of the jejunum and large intestine contained a mixture of lutein diester, lutein monoester and lutein, but in the serum lutein was found mainly (90%) in the non-esterified form (Tyczkowski and Hamilton 1986a, b). Similarly, in the chicken liver lutein monoester and diester comprise about 20 and 2% of total lutein, respectively (Hamilton 1992). In contrast, in the toe web about half of the total lutein occurred as the diester and the remainder occurred as about equal amounts of free lutein and lutein monoester (Tyczkowski and Hamilton 1986a, b). Using a chicken model and β-apo-8^{I}-caroitenoic acid ethyl ester (Apo-EE) as a reference, it was shown that the relative biological availability of xanthophyll from various sources to be (%): Apo-EE 100, corn gluten meal 64.2 (47.7–89.1), dehydrated alfalfa 45.9 (34.6–65.4), marigold meal 29.1, marigold extracts 62.9 and coastal Bermugrass

22.2 (18.8–27.9) (Middendorf et al. 1980). In this experiment, statistically significant differences among samples within and among generic sources were obtained. Furthermore, xanthophyll (lutein + zeaxanthin) concentrations in different feed vary substantially (mg/kg): lucerne meal (40–260), grass meal (140–500), yellow maize (8–40), plate maize (10–50) and maize gluten meal (60–360) (Belyavin and Marangos 1989).

The main dietary sources of carotenoids in poultry nutrition are maize, maize products, lucerne, alfalfa meal (organic egg production) and commercial concentrated forms including marigold and paprika (Nys 2000). Most of the mentioned feedstuffs provide mainly lutein and zeaxanthin plus β-carotene (alfalfa meal) and β-cryptoxanthin (maize and maize gluten). Our data indicate that a major difference between the American maize-based diet for breeders and the European wheat-barley based diet is in the level of carotenoids (lutein + zeaxanthin), which is more than twice higher in the maize-based diet (Surai and Sparks 2001). This difference is due to a low carotenoid content of wheat and barley although both feeds contain lutein and zeaxanthin as major carotenoids. The main factors affecting carotenoid transfer to the egg yolk are shown in Table 20.1.

After ingestion of feed, carotenoids are released from the matrix by digestive enzymes. The liberated carotenoid compounds together with other feed lipids are emulsified in the presence of bile salts to form oil droplets. Carotenoids can be spontaneously transferred from lipid droplets to mixed micelles. Because of their large size, the droplets are not able to gain proximity to the absorptive surfaces of the small intestine for carotenoid transfer to enterocytes (Combs 1996).

Instead, as a result of lipase action, the droplets are degraded to form mixed micelles. The hydrophobic cores of the micelles contain non-polar, nonamphiphilic lipids, such as cholesterol and oxycarotenoids, which partition between the oil and micellar phases. Carotenoid absorption from the intestinal tract is associated with the same intraluminal, membrane and intracellular events as have been described for dietary lipids (Krogdahl 1985) although some specific features relating to the site of absorption of various carotenoids in birds have been described (Tyczkowski and Hamilton 1986b). This process includes emulsification, solubilisation, diffusion across the unstirred water layer, permeation through the membrane of the enterocytes, incorporation into lipoprotein particles and release into the circulation via lymphatic pathways in mammals (Cohn 1997), or the portal system in birds. Carotenoid absorption is a very delicate and sensitive process and an impairment of any of these stages can decrease carotenoid assimilation from the diet. In birds, lipid digestion starts in the gizzard where bile salts and pancreatic lipases are responsible for about 30% of dietary triglyceride hydrolysis (Sklan et al. 1978). Emulsification of dietary fat is accelerated greatly as the chyme moves into the small intestine and is mixed with bile and pancreatic secretion. The jejunum is the major site of lipid absorption in birds, although some absorption occurs in the ileum (Hurwitz et al. 1973). It has been shown that lutein is absorbed in the duodenum and jejunum (Hamilton 1992) but absorption of zeacarotene takes place mainly in the ileum (Tyczkowski and Hamilton 1986a, b). Carotenoid absorption takes place by unsaturable, passive diffusion down a concentration gradient similar to other nonpolar lipids (Hamilton 1992), although some

Table 20.1 The effectors of carotenoid accumulation in the egg (Adapted from Surai 2002; Baker and Günther 2004)

Dietary factors	*Bird physiology*
Dose and type of product	Feed intake
Duration of carotenoid feeding	Age of bird—stage in laying cycle
Carotenoid content/composition of basic feed	Breed—genetics
Dietary fat composition, content and quality	Temperature and humidity
Presence of emulsifiers.	*Bird health*
Feed antioxidants (vitamin E, ethoxyquin)	Bile quantities in the digestive tract
Competing substances (high vitamin A., nitrate)	Digestive tract integrity
Feed calcium level	Enzymic digestion efficiency
Mycotoxins (reduce accumulation)	Diseases
Gossypol (causing browning)	Parasites
Piperazine (induces brown/green colour)	*Feed production*
Nicarbazine (induces brown/green colour)	Mixing standards
Aufusarin (induces brown colour)	Abrasion/aggressiveness of carrier
Drugs	Pelleting and extrusion conditions
Zn and vitamin B12	Storage duration and conditions

stereospecificity for R-carotenoids has been shown (Hencken 1992; Schiedt et al. 1985). It is shown that carotenoids are absorbed in the free alcohol form by passive diffusion across the brush border membranes of the intestinal mucosal epithelium (Cohn 1997) and the process is thought to be concentration dependent (Bieri and Farrell 1976).

Bile salts and phospholipids take part in the emulsification of dietary triglycerides and other fat-soluble nutrients, including carotenoids. Pancreatic lipase acts on the triglycerides at the water-lipid interface. Free fatty acids and monoglycerides formed during hydrolysis of triglycerides, in the presence of bile salts, spontaneously form very small particles, called mixed micelles. It is believed that solubilisation of water-insoluble materials such as carotenoids is a critical step in their digestion and absorption. Because of the very small size of mixed micelles, they are dispersed in the aqueous environment of the intestinal lumen and can diffuse into the glycoprotein layer surrounding the microvilli or brush border of mucosal cells, where they come into contact with the cell membranes (Tee 1992). The micelles migrate to and merge

with the membranes of intestinal epithelial cells and so the carotenoids within the lipid micelles come in contact with the intestine epithelial cell membranes and their transport to the plasma membrane and/or cytosol of the cell takes place in concert with the transport of fatty acids, monoglycerides and other lipids (Erdman et al. 1993). It is known that the passage of lipids through the brush border membrane is passive, and absorption rates depend on the fatty acid chain length and degree of saturation (Krogdahl 1985). It is becoming increasingly evident that the micelles formed from dietary lipids serve as a delivery system for carotenoids to reach the absorptive surface of the gut. The micelles facilitate fat absorption by providing a high concentration of lipids in the unstirred water layer adjacent to the mucosal cells and disruption of the micelles is necessary before absorption takes place (Krogdahl 1985). The feed matrix is thought to be an important determinant for the absorption of carotenoids and therefore carotenoid absorption varies substantially among different foods and their bioavailabilities can be improved by disruption of the food matrix (van het Hof et al. 1999). The amount and type of feed determine bile secretion, while bile salts and fat determine micelle formation (van Vliet 1996). The pH in the intestinal lumen may also affect absorption via an effect on the surface charges of both the micellar particles and the luminal cell membrane, with less diffusion resistance at lower pH values (Hollander 1981).

The efficiency of dispersion of carotenoids is known to be affected by the presence or absence of other components in the diet, as well as by the general nutritional status of the animal. It has been summarised that 9 major factors determine carotenoid bioavailability: species of carotenoids, molecular linkage, amount of carotenoids consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors and nutrient interactions (Castenmiller and West 1998). In the chicken, absorption and accumulation of major carotenoids is comparatively low. In broilers, 94% of astaxanthin and about 83% of zeaxanthin supplied by the diet were excreted and only 0.4% of the astaxanthin and 1.7% of the zeaxanthin were deposited in the skin (Hencken 1992). Cryptoxanthin in particular is very poorly absorbed in birds (Quackenbush 1970; Tyczkowski and Hamilton 1986b). In laying hens, carotenoid deposition in egg yolk is also comparatively low with canthaxanthin being deposited at 30–45% efficiency, zeaxanthin 25% and astaxanthin 14% (Hencken 1992) and carotenoid excretion was about 70% for astaxanthin and zeaxanthin and 45–50% for canthaxanthin. Earlier it had been shown that 43% (Nakaue et al. 1966) or 56% (Marusich and Bauernfeind 1981) of the ingested zeaxanthin was deposited in egg yolks. In the same experiment, the levels of deposition of cryptoxanthin, lutein and violaxanthin were 21, 9 and 0%, respectively (Nakaue et al. 1966). Astaxanthin transfer from the yeast Phaffia rhodozyma, added to the diet of the quail, to the egg yolk comprised only 4% of the ingested carotenoid; from marigold extract carotenoid transfer to the egg yolk was even lower (3%) (Johnson et al. 1980). About 16% of capsanthin was transferred from the diet (as a saponified oleoresin) into the egg yolk (Hamilton et al. 1990) but when crystalline capsanthin was used only 6.2% of this carotenoid was transferred to the egg yolk (Marusich et al. 1960). In accordance with other observations the efficiency of the deposition of capsanthin

from various capsicum products varied from 1 to 6% (see Hamilton et al. 1990). Therefore, only a small to moderate percentage of the carotenoid ingested with feed will be taken up by the intestinal cells. Furthermore, in chickens, when the mucosal cells are sloughed off into the lumen of the gastrointestinal tract, carotenoids which were not incorporated into portomicrons are lost (Williams et al. 1998).

In poultry, lipoproteins synthesised in the intestinal wall are drained from the intestines by portal blood. Thus, in the absence of a developed intestinal lymphatic system in birds, absorbed lipids enter the portal system as large lipoproteins (called portomicrons) with a diameter of about 150 nm (Annison 1983, Walzem 1996). They consist of triglycerides, free and esterified cholesterol, phospholipids and apoproteins, and fat-soluble vitamins including carotenoids. Very little is known about the cellular events that regulate or mediate the incorporation of carotenoids into portomicrons. This process could be facilitated by cytosolic binding proteins, but these have not been reported in the intestinal mucosa. Thus, carotenoids together with other lipids are transported as portomicrons directly via the portal system to the liver prior to entry into the general circulation. In contrast, in mammals, chylomicrons are delivered into the plasma via the lymphatics and circulate through extrahepatic tissues before passing through the liver. In the capillaries of certain extrahepatic tissues, chylomicrons are hydrolysed by lipoprotein lipase (LPL), with subsequent transfer of some carotenoids to the peripheral tissues and to other lipoproteins. In birds, portomicrons partly undergo some transformation in the liver, but, probably, they are largely released into the circulation unchanged or with minor changes. During their transport in the circulation, portomicrons also undergo catabolism by LPL. This enzyme is synthesised in several chicken tissues, but the largest amounts are associated with skeletal muscle and adipose tissue (Stevens 1996). LPL is secreted into the capillaries where it is bound on the luminal side of the endothelial cells. Lipoproteins are substrates for endothelial bound LPL which catalyses intravascular triglyceride hydrolysis. During portomicron catabolism by LPL, the size of the portomicron triglyceride core is reduced and excess surface is created. This excess surface is transferred to HDL (Traber 1996) and a portion of the carotenoid content is also transferred to HDL. Because HDL readily transfers vitamin E and probably carotenoids to other lipoproteins (Traber et al. 1992), carotenoids are distributed between all of the circulating lipoproteins. Portomicron remnants are cleared from circulation by the liver and in this way carotenoids are delivered to this organ. The factors controlling carotenoid uptake by various avian tissues and their transfer to egg yolk are not known and this subject warrants further investigation. For example, androgens modulate hepatic lycopene metabolism (Boileau et al. 2000). Although a lot of different xanthophylls with biological properties are found in nature, only a few are of industrial importance (mainly astaxanthin, canthaxanthin and lutein).

20.5 Carotenoids in Poultry Nutrition

20.5.1 Egg Yolk Pigmentation

Colour is an important factor affecting consumer choice of foods through sensory evaluation. In many countries of the world, golden colour of yolks is preferred because yolk colouration has been traditionally associated with good health. There are three major carotenoids commercially used in poultry production, including

- Lutein
- Zeaxanthin
- Canthaxanthin

In addition, citranaxanthin, apo-carotene-ester and capsanthin are used to maintain attractive egg colouration. Main sources of feed-derived carotenoids include yellow corn, corn gluten and distillers' grains providing a mixture of lutein and zeaxanthin; as well as alfalfa and its concentrate, providing mainly lutein. Nature-identical or nature-extracted yellow carotenoids are obtained from marigold (lutein) or red carotenoids from paprika (capsanthin and capsorubin with some lutein and zeaxanthin). Furthermore, apo–ester, canthaxanthin and citranaxanthin are the main commercial sources of nature-identical carotenoids for poultry industry. It seems likely that apo-carotene-ester and canthaxanthin (CX) are most effectively deposited in egg yolk yellow (about 55%) and red (about 40%) carotenoids (Surai 2002). In our experiments CX was added to the diet of the broiler breeder birds at 0, 3, 6, 12, 24 mg/kg and its concentration in the egg yolk was shown to be 0, 7.48, 14.75, 28.89 and 56.99 μg/g, respectively (Surai et al. 2003). Interestingly, those egg yolks from breeders supplemented with two highest doses of canthaxanthin were deeply red in colour, reflecting low level of yellow carotenoids provided by basic diet. Indeed, in order to have an optimal (orange) colouration of the egg yolk various combinations of yellow and red carotenoids are commercially used. Generally, commercial technology of egg yolk colouration has been well developed and are widely used. Since organic egg production systems do not allow synthetic carotenoids to the feed alfalfa meal and its extract found their way into organic egg production. Interestingly, alfalfa meal was widely used 30–50 years ago in poultry diets and special technologies of its stabilisation by antioxidants to prevent carotenoid oxidation during meal storage have been developed and effectively used in countries of former USSR (Zhedek et al. 1996). With organic production demand for natural sources of colouring agents for egg yolk those old technologies can be used. Indeed, "All new existed in the past".

20.5.2 Skin Pigmentation

It is well known that skin colour plays an important role in consumer acceptance of broilers and in some countries such as Mexico consumers demand chickens with an extreme yellowness of the skin (Ornelas 1997; Pena et al. 2004). It is important to mention that skin of broilers grown on the typical commercial diets is characterised by low yellowish colour, but it can be manipulated by usage of dietary carotenoid pigments which are deposited in the skin and fat (Perez-Vendrell et al. 2001; Castaneda et al. 2005). There is a range of natural and synthetic sources of pigments which can be used in commercial broiler feed. In particular, feed ingredients with high carotenoid content include yellow corn, corn gluten meal, dehydrated alfalfa meal, algae meals, marigold meals and concentrated extracts from marigold meals or alfalfa meal (Marusich and Bauernfeind 1981). Among the synthetic pigments, canthaxanthin, apo-ester, and others have been developed and successfully used commercially. It is necessary to underline that the effectiveness of a particular pigmenting substance/feed ingredient for poultry products (skin) colouration depends on the chemical nature, level and availability of the xanthophylls in the source (Delgado-Vargas 1997).

20.5.3 Poultry Breeder Nutrition

Natural carotenoids have been included into the category of antioxidants (Surai 2002, 2012a, b). In fact, carotenoids are working in the body as an integral part of the antioxidant system, providing recycling other antioxidants including vitamin E and affecting various transcription factors including Nrf2 and NF-κB.

Poultry diet formulations differ across the world but maize-based diets rich in carotenoids are common in the USA (Ensminger 1980) and wheat-barley diets comparatively low in carotenoids, are widely used in Europe (Hocking and Bernard 1997). Maize is a good source of zeaxanthin and lutein but wheat and other feed ingredients are poor sources of carotenoids, especially zeaxanthin (Marusich and Bauernfeind 1981). The carotenoid concentrations and composition in the egg yolk depend on their feed content (Surai and Speake 1998). We conducted a comparative study of the effect of maize-based diet (M) and wheat-based diet (W) on the egg composition and chicken embryonic development (Surai and Sparks 2001). Both major carotenoids of the egg yolk lutein and zeaxanthin were significantly enhanced in the egg yolk of the M-group. In fact, the zeaxanthin concentration in the egg yolk was enhanced compared with lutein, reflecting the differences in the carotenoid composition of the feed. Carotenoids were shown to be transferred from egg yolk to embryonic tissue more effectively in the M-group than in the W-group. For example, the difference in total carotenoids in the egg yolk between the 2 groups was 2-fold but in the liver of the newly hatched chicks in the M-group the carotenoid concentration was more than 3-fold higher than in the W-group. Similar differences were observed

in other tissues including YSM and adipose tissue. On the other hand, in such peripheral tissues as heart and kidney the accumulation of carotenoids was proportional to their concentration in the egg yolk, while in the other tissues (lung and muscle) it was lower. It is also interesting that there was no discrimination between 2 major carotenoids, lutein and zeaxanthin, during their transfer to the peripheral tissues as was shown for citranaxanthin, canthaxanthin and carotenoic acid earlier (Surai and Speake 1998). For example, in the chick tissues from the M-group the proportions of lutein were quite stable comprising 30–37%. Because lutein proportions in the residual yolk and bile of the chicks from the M-group were substantially lower than in the liver and peripheral tissues it seems likely that preferential absorption from the yolk and delivery of this pigment to the developing tissues took place. Indeed, the mechanisms of carotenoid absorption and distribution in the developing chickens need further investigation. The liver can also serve as a carotenoid reserve during the 1st days of post hatch development when the carotenoid concentration in this tissue is progressively decreased (Surai et al. 1998). Therefore, as with vitamin E (Surai 1999), the increased carotenoid concentration in the liver of the newly hatched chick could be beneficial for maintaining its antioxidant system (Surai et al. 2016). Whether the increased carotenoid status of newly hatched chicks from the parent stock receiving the maize-based diet would be beneficial for chicken growth and development needs further investigation.

Dietary carotenoids determine carotenoid concentration in the egg yolk. For example, in our study, eggs from carotenoid-supplemented layers contained up to 22 times more carotenoids than controls (Karadas et al. 2005a). The concentration of carotenoids in the livers of chicks hatched from the enriched eggs was also 29-fold greater than in the control chicks. During embryonic development part of accumulated egg yolk carotenoids are used for metabolic needs including antioxidant defences (Surai 2002). Female quail fed diets supplemented with natural carotenoids produced eggs with an increase in the yolk concentrations of lutein, zeaxanthin, lycopene and β-carotene (Karadas et al. 2006b). Interestingly, similar to lutein and zeaxanthin, lycopene was shown also to be transferred from the feed to the egg yolk and further to the liver of the developing embryo. Elevated carotenoid concentration in the egg yolk and in the liver of newly hatched quail remains significantly increased during the first week post hatch (Karadas et al. 2006a). In addition, retinyl oleate and retinyl palmitate concentrations in egg yolk and the liver of day-old quail chick was shown to be significantly increased as a result of carotenoid supplementation of the maternal diet (Karadas et al. 2005a).

Carotenoids are characterised by health-promoting properties, including immune system modulation (Moller et al. 2000). For example, dietary lutein supplementation reduced parameters of inflammation in the liver and spleen of laying-type chicks (Meriwether et al. 2010). Carotenoids reserves in the body are limited (Surai 2002). During embryonic development carotenoids are shown to be actively transferred from egg yolk to the developing embryo with the chick liver at hatching time achieving maximal carotenoid concentration (Surai et al. 1996). Similar to vitamin E, carotenoid accumulation in the embryonic liver is considered to be an adaptive mechanism to deal with hatch-related stress conditions and after hatching

carotenoid concentration in the chick liver dramatically decreases (Surai et al. 1998). Interestingly, chicks obtained from carotenoid-depleted eggs are not able to effectively assimilate carotenoids from the diet to the same extent as those hatched from carotenoid-rich eggs (Koutsos et al. 2003). Increased dietary carotenoid supplementation of the maternal diet was associated with elevated vitamin E concentrations in the egg yolk, embryonic tissues and their increased resistance to oxidative stress (Surai and Speake 1998; Surai et al. 2003). A combination of increased concentrations of lycopene and lutein in the egg yolk was associated with elevated concentrations of CoQ in the liver of the newly hatched quail (Karadas et al. 2006a).

20.5.4 Specific Roles of Canthaxanthin (CX) in Poultry Breeder Nutrition

Among more than 1100 known carotenoids, CX has a special place as a carotenoid with proven antioxidant and other biologically relevant functions. A great body of evidence indicates that CX possesses high antioxidant activity that was shown in various in vitro model systems as well as in animal experiments in vivo. To address some important features of CX activity in breeders a study was conducted at the Scottish Agricultural College (SAC) (Surai et al. 2003) using 320 female broiler breeder birds. Four treatments were compared, a low carotenoid control diet containing <2 mg/kg total xanthophylls, and then test diets supplemented with either 3, 6, 12 and 24 mg/kg of a commercial CX product, (CAROPHYLL® Red 10% (Roche Vitamins (UK) Ltd, Heanor, UK). The results of the trial showed that inclusion of CX into the maternal diet caused a significant dose-dependent accumulation in the egg yolk. As a result, egg yolk colour gradually changed from pale yellow in the control group to dark red in the laying hens fed CX at 24 mg/kg. These results are in agreement with previous observations from a similar trial using a carotenoid mixture (Surai and Speake 1998). The most important finding of this study was related to a positive effect of CX on vitamin E in the developing chicks. First of all, CX improved assimilation of γ-tocopherol from the diet and its transfer to the egg yolk. As a result, an increased γ-tocopherol concentration was observed in the liver of the embryo at d 16 of the development. However, at later stages of the development, these differences disappeared, probably due to a tocopherol-binding protein (Surai and Speake 1998). Secondly, increased CX concentration in the egg yolk and embryonic tissues was associated with higher α-tocopherol concentrations in the liver and plasma of 1-d-old chicks. This could be a reflection of antioxidant interactions during embryonic development. Indeed, if the CX that accumulated in tissues scavenged free radicals, fewer radicals would be available to be scavenged by vitamin E. This could spare some vitamin E and lead to its increased concentrations. There is also a possibility that CX may take part in vitamin E recycling leading to increased vitamin E concentration. In fact, vitamin E recycling by β-carotene has been shown (Bohm et al. 1997). The synergistic effect of CX on vitamin E was seen in 7-d-old chicks, when

vitamin E concentrations in the liver and plasma were significantly elevated as a result of maternal diet supplementation with CX. In fact, α-tocopherol concentration in the chick liver decreased more than 10-times in the first week. In such an extreme situation, increased CX concentration in the chick liver slowed down the depletion of vitamin E from the liver, which could be explained by the same mechanisms of CX/vitamin E interactions.

As a result of enrichment of the chick liver with CX and vitamin E due to the maternal dietary supplementation, tissue susceptibility to lipid peroxidation significantly decreased in 1-d-old and 7-d-old chicks. The protective effect CX against lipid peroxidation may be mediated via its effect on antioxidant enzymes (Palozza et al. 2000). The enhancement of the antioxidant system in the developing chick as a result of supplementing the maternal diet presents great opportunities for poultry producers. Postnatal development of the chicken is a crucial time for the maturation of major physiological systems, including the immune system, as well as a time of high risk of peroxidation (Surai 2002). Therefore, increased supplementation of the maternal diet with carotenoids, in particular CX, could help maintain antioxidant system efficiency and increase chick viability.

The idea of exploiting the beneficial effects of dietary CX supplementation in breeder diets was further developed by Zhang et al. (2011a, b). In this study, 270 Chinese Three-Yellow breeder hens were randomly divided into two groups consisting of 135 birds each (five replicates of 27). The breeder hens were fed either a basal diet or one supplemented with 6 mg of CX/kg for 24 weeks. At the end of the experiment, all hatching eggs laid over five consecutive days from each group were collected and incubated. For each breeder group, 100 newly hatched chicks (five replicates of 20) were reared under environmentally controlled conditions for 21 d. The results from this trial showed that CX supplementation gave a significant increase in the yolk colorimetric score on the Roche Yolk Color Scale ($P < 0.001$), confirming an effective CX transfer from the diet to the egg yolk. Egg yolk enrichment with CX was associated with a significant improvement of the antioxidant status of the egg yolk ($P < 0.05$), whereby malondialdehyde (MDA) content of the egg yolk decreased from 139.83 nmol/g down to 86.92 nmol/g ($P = 0.023$). At the same time total antioxidant capacity (TAC) of the egg yolk increased from 1.87 U/g up to 3.16 U/g ($P < 0.001$). These important improvements in the antioxidant status of yolk could be due to CX transfer or improvements of vitamin E status (Surai et al. 2003). One important finding was the improvement of the serum TAC in the breeder hen ($P < 0.029$). This could be an important element in an improvement of protection of laying birds from commercially relevant stresses, including fluctuations in temperature and feed quality (Surai 2002).

The chicks that hatched from eggs laid by breeder hens fed the CX diet demonstrated a higher pigmentation colorimetric score of their shank skin ($P < 0.05$), ascribed to CX accumulation (Surai et al. 2003) and the antioxidant capacity of the newly hatched chicks was significantly increased ($P < 0.05$). There was a significant decrease in MDA (from 4.28 down to 2.61 nmol/ml, $P < 0.001$) in the plasma of day-old chicks, thought to be a result of increased CX and vitamin E concentration in plasma (Surai et al. 2003). It is of great importance to see increased SOD activity

(from 98.4 to 144.7 U/ml, $P = 0.031$) in plasma, and these findings are in agreement with previous observations indicating a stimulatory effect of CX on SOD (Palozza et al. 2000). It is interesting to underline that maternal supplementation with CX has a positive effect on TAC of newly hatched chicks, which increased by 33% (from 13.8 to 18.3 U/ml, $P = 0.052$).

In a field trial, Robert et al. (2008) studied the effect of CX in ROSS breeders on the antioxidant status of their progeny. They observed that the antioxidant status of sera was significantly higher and the TBARS level was significantly lower in 1-day old chicks from hens fed 6 ppm CX in the breeder feed. According to these authors, the results indicate that maternal supplementation with CX (6 ppm) enhances antioxidant capability and depresses oxidative stress in chicks. Similar to finding from previous work (Surai et al. 2003), maternal effects of CX supplementation has been seen in postnatal chicken development. Indeed, shank pigmentation score (a reflection of CX accumulation) and MDA in plasma were significantly lower in 7-day-old chicks hatched from the CX-enriched eggs. The experimental chicks also showed a lower mortality (0 vs. 4%) during the first 21 d post hatch. These findings support the hypothesis that CX supplementation of the maternal diet enhances the protective capacity of tissues against oxidative stress in vivo, which might be beneficial for poultry producers.

The next step in examining the beneficial effects of CX in the maternal diet was provided by an experiment conducted in Brazil in the Poultry Science Laboratory of the Department of Zootechnics at the Federal University of Santa Maria (UFSM); Rosa et al. 2012). Three hundred and sixty female pullets and 36 roosters were placed in an open-sided house allocated into 12 pens. At 42 weeks of age, the breeder hens and roosters were distributed into two experimental groups with similar body weight and uniformity, and from weeks 46 to 66, one group received 6 ppm of CX, supplemented in the diet, and the other group received the diet without addition of CX (control diet). Those fed with CX had significantly better egg-laying rates during certain periods, but overall there was no difference between the two treatment groups. Two weeks after the start of the supplementation the pigmentation measured with the colour fan was 9.33 in the control group and 14.67 in the CX group ($P < 0.0001$) and this difference in yolk colouration remained stable throughout the experiment. These results are in agreement with previous observations (Surai et al. 2003; Zhang et al. 2011a, b). In concurrence with previous observations, a reduction of TBARS was observed in yolks from stored hatching eggs produced by breeders fed CX. The reduction in TBARS was observed in eggs submitted for analysis on the same day they were produced ($P = 0.0214$) and in eggs stored for four ($P < 0.0002$), eight ($P < 0.0003$) and twelve days ($P < 0.0001$).

The most important finding of this study was the positive effect of CX supplementation of the maternal diet on fertility, hatchability and embryonic mortality. In fact, CX in comparison to the control improved fertility (92.1 vs. 91.0%, $P < 0.02$) and hatchability (93.7 vs. 91.3%, $P = 0.0003$) and reduced embryonic mortality (3.7 vs. 5.5%, $P < 0.003$). As a result, hatching rate was significantly (86.2 vs. 83.0%, $P = 0.0001$) improved. As to the different stages of embryo mortality, CX was most efficacious for its prevention in the first 48 h (1.04 vs. 1.8%, $P = 0.008$) as well as

between day 15 and 21 of incubation (1.44 vs. 2.07, $P = 0.017$). It can be postulated that CX supplementation improved breeder fertility ($P = 0.0171$) by improving survival and storage of spermatozoa within the reproductive tract of the hen. Indeed, according to Bréque et al. (2003), long-term storage of spermatozoa is supported by a complex antioxidant defence system present within the oviduct that protects spermatozoa against lipid peroxidation. CX may play an important role in that system. As for the positive effect of CX on hatchability, this can be explained by its participation in antioxidant defences during embryonic development (Surai 2002). It is interesting to mention that another carotenoid with a similar structure astaxanthin (AX) fed to breeders was shown to be transferred to the egg yolk and had a significant protective effect against decreasing hatchability due to egg storage at 21 °C (Saito and Kita 2011). The hatchability of eggs stored at 21 °C was significantly lower than that at 10 °C, and lowered hatchability was restored by maternal intake of AX. Elevated levels of dietary AX from 5 to 20 ppm gradually restrained the decrease in hatchability at 21 °C. And it is interesting to note an additional benefit to use CX supplementation of the breeder diet as an opportunity to identify low-producing breeders by observing high colouration of shanks (Pinchasov et al. 1992). Indeed, there is a distribution of carotenoids between different tissues in the body and if egg production rate is decreasing more CX is available for shank colouration.

Increased CX concentration in the egg yolk was shown to be associated with an increased resistance to oxidative stress (Surai et al. 2003). Recently it has been shown that the egg yolks coming from broiler breeders fed with the sorghum diet supplemented with CX were characterised by the best antioxidant status and the CX supplementation in diets at 64 wk of age of broiler breeders reduced the offspring mortality and improved their viability (Rosa et al. 2017). Furthermore, the CX supplementation (6 mg/kg) in broiler breeder diets improved the productive and reproductive performance (laying % and hatchable eggs) in the second part of reproduction (Bonilla et al. 2017). Interestingly, maternal canthaxanthin at 6 mg/kg increased chick E. coli bactericidal capacity and d 1 oxidative burst and antioxidant capacity (Johnson-Dahl et al. 2017).

Dietary inclusion of CX contributed to a higher resistance of the vitelline membrane of fresh egg yolks (Damaziak et al. 2018). Recently, a meta-analysis was conducted to determine the effect of the CX dietary supplementation on production performance in layer hens. The analysis included 576 performance measurements from 34 trials conducted from 1997 to 2012. Significant dose-dependent increases were found in egg yolk mass (+0.53% per ppm of CX inclusion in the feed, $P < 0.001$), egg mass (+0.47% per ppm, $P = 0.0132$), egg weight (+0.17% per ppm, $P = 0.046$) and in feed intake (+0.32% per ppm, $P = 0.0054$). The deposition of CX in the egg yolk was 2.25 ppm per ppm of CX in the feed ($P < 0.001$). The authors concluded that dietary CX improved egg yolk pigmentation, increased AO defence, enhanced reproduction, and immune-modulation, as well as affected egg mass (Umar Faruk et al. 2018). There are data available on the positive effects of a combination of CX and 25-hydroxycholecalciferol on duck breeders (Ren et al. 2016a, b).

Table 20.2 Carotenoid concentrations (μg/g) in egg yolk from gull, moorhen and coot. Adapted from Surai et al. 2001a, b, c, d

Carotenoids	Gull ($n = 20$)	Moorhen ($n = 10$)	Coot ($n = 10$)
Total	71.6	47.5	131.0
Lutein (L)	14.2	14.9	45.7
Zeaxanthin (Z)	5.1	8.1	17.4
Canthaxanthin (Cnth)	12.0	nd	Nd
B-cryptoxanthin (β-Crpt)	2.4	5.2	26.2
Echinenone (Echn)	9.2	1.2	0.4
β-carotene (β-Car)	18.8	13.8	33.4

20.5.5 Lessons from Wild Birds

Our research related to carotenoid content and composition in egg yolk from various avian species in wild was an important milestone in better understanding the biological and evolutionary role of natural carotenoids. First of all, many avian species in wild were shown to be characterised by comparatively high (5–10 times higher than commercial eggs) carotenoid concentrations (Speake et al. 1999; Surai et al. 2001a; Surai 2002; Ewen et al. 2006; Garamszegi et al. 2007). The same is true for embryonic tissues. For example, depending on carotenoid concentration in the livers various species could be placed in the following descending order: free-living pheasant > free-range guinea fowl > free-range hen > intensively housed hen > wild mallard > housed duck > free-range duck (Karadas et al. 2005b). Secondly, the most striking feature of carotenoid composition of the yolk in three avian species in wild, namely gull, moorhen and coot, was an extremely high proportion of β-carotene (Table 20.2).

In all three species proportion of β-carotene and lutein were two major carotenoids in egg yolk with comparable concentrations. It is not clear at present if accumulation of β-carotene in egg yolk was related to its low conversion to vitamin A, or there are other mechanisms of such phenomenon (Surai et al. 2001a, b, c, d). In newly hatched gull, coot and moorhen the highest carotenoid concentration was found in the liver which was 5–10-fold higher than that in other tissues. In accordance with the carotenoid concentration in the liver the species could be placed in the following descending order: coot > moorhen > gull (Surai et al. 2001a, b, c, d).

Similar to egg yolk, the striking feature of the carotenoid composition of the liver is the very high proportion of β-carotene which comprised 57.7, 37.7 and 36.7% of total carotenoids in the moorhen, coot and gull, respectively. In contrast the proportion of lutein in the liver was very low and much less than that in the initial yolk: 3.3, 8.4 and 7.1% of total carotenoids in the moorhen, coot and gull, respectively. In the same tissue, the proportion of zeaxanthin was also low comprising 5.3, 7.8 and 2.1% in the moorhen, coot and gull, respectively.

The carotenoid concentration in the tissues of newly hatched moorhens was comparable with those in chickens hatched from the eggs produced by hens fed on a maize-based diet (Surai and Sparks 2001). In general, the data indicate that carotenoid concentration in the liver and peripheral tissues of the newly hatched wild birds were comparable with those in chickens obtained from carotenoid-enriched eggs.

Increased lutein concentration in egg yolk was found to have positive effect on offspring survival in the great tit (Marri and Richner 2014). Furthermore, a direct positive relationship between enhanced maternal investment of carotenoids and the ability to cope with a specific and costly parasite in young birds was shown (Ewen et al. 2009). In fact, maternal effects mediated by carotenoids may be an important factor in development and phenotypic plasticity in traits associated with nestling fitness, such as immune response and ability to metabolise and use antioxidants, and ultimately participate in the evolution of phenotypic traits (Biard et al. 2007). Carotenoid availability was suggested to modulate the trade-off between reproduction and resistance to oxidative stress in zebra finches (Bertrand et al. 2006). Indeed, maternally derived carotenoids affected offspring survival, sex ratio and sexual attractiveness in a colourful songbird (McGraw et al. 2005). In the blue tit, nestlings from eggs laid by carotenoid-supplemented females were characterised by longer tarsi, had faster development of the immune system and grew brighter yellow feathers than nestlings from control females (Biard et al. 2005). Carotenoid availability has also been demonstrated to limit egg-laying capacity in gulls (Blount 2004; Blount et al. 2004). In fact, maternal yolk carotenoids can have a major effect in promoting a fundamental component of immunity that predicts offspring survival and suggests that adaptive early maternal effects can be mediated by transmission of antioxidants to egg (Saino et al. 2003). In red-legged partridges, plasma carotenoids at the end of laying period were strongly correlated with the number of eggs laid (Bortolotti et al. 2003). Maternal carotenoid status affected the incorporation of dietary carotenoids into immune tissues of growing chickens (Gallus gallus domesticus) (Koutsos et al. 2003). In a supplemental feeding study of lesser black-backed gulls, Larus fuscus, carotenoid-supplemented females were found to have increased integument pigmentation, elevated plasma concentrations of carotenoids and enhanced antioxidant activity (Blount et al. 2002). It was suggested that maternal investment in yolk carotenoids promotes the evolution of carotenoid-based ornaments and proven that maternal effects mediated by yolk carotenoids play an important role in the evolution of carotenoid-based signals as a response to sexual selection, likely based on organisational effects of carotenoids during embryo development (Biard et al. 2009). Across avian species, there were positive relationships between embryonic growth rate and total yolk levels of carotenoids or vitamin E. Moreover, the eggs of the faster developing species have been shown to have higher carotenoids and vitamin E status (Deeming and Pike 2013). There are two main strategies for carotenoid investment in wild birds. It seems likely that females invest carotenoids into the egg to provide maximum antioxidant defences at hatching time. At the same time male birds often invest carotenoids into plumage. In fact, it was shown that carotenoid-dependent bright colouration of plumage in males in various avian species in wild is indicative of their health, in particular effective immunity (Blount et al. 2003).

20.6 Conclusions and Future Prospects

Among many important biological functions of carotenoids, their participation in building an effective antioxidant defence network could be of vital importance. Indeed, biological value of direct AO activity of carotenoids associated with scavenging ROS is probably not very high (Costantini and Møller 2008). It seems likely that indirect effects of carotenoids on the antioxidant defences via upregulation of Nrf2 (Zhao et al. 2017; Li et al. 2018b; Yu et al. 2018; Chang et al. 2018; Yang et al. 2018; Xie et al. 2018; Zhu et al. 2018; Hu et al. 2018; Lin et al. 2018; Niu et al. 2018; Xue et al. 2019) and downregulation of NF-κB (Sahin et al. 2017; Zhao et al. 2017; Shi et al. 2018; Barros et al. 2018; Sung and Kim 2018; Chang et al. 2018; Icel et al. 2019; Li et al. 2019) are a driving force of their beneficial effect in avian species in general and in poultry production in particular. In fact, it is proven that non-provitamin A carotenoids (lutein, CX, astaxanthin and lycopene) are important regulators of cell-mediated and humoral immune response in animals and humans (Moller et al. 2000; Chew and Park 2004). Recent studies on the role of carotenoids in gene regulation and apoptosis have substantially advanced our understanding of the possible mechanisms by which carotenoids regulate immune functions. For example, a number of differentially expressed genes in chicken muscles as a result of carotenoid dietary supplementation at 100 mg/kg have been identified (Tarique et al. 2014). In particular, in CX and lutein supplemented chickens there were 54 (32 upregulated and 22 downregulated) and 23 (15 upregulated and 8 downregulated) altered known genes, respectively. In fact, the differently expressed genes are shown to be involved in important biological processes, including pigmentation, growth, molecular mechanisms, fat metabolism, cell proliferation, immune response, lipid metabolism and protein synthesis and degradation (Tarique et al. 2014). Carotenoid protective effects in chicken/avian embryonic development deserve more attention and warrant further investigation. Advances in CX usage in poultry breeder nutrition are an example of successful carotenoid application in aviculture.

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Chapter 21
Carotenoids as Anti-obesity Supplements

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Abstract The aim of this review is to summarize the current knowledge regarding the biological impact of carotenoids and their metabolites on obesity management. The relationship between carotenoids and obesity management will be envisioned in clinical studies. Adipose tissue is a key organ in obesity etiology and the main storage site for carotenoids, thus we will first describe the carotenoids metabolism in adipocyte and adipose tissue and present the effect of carotenoids on biological processes that may be linked to obesity management in vitro and in preclinical studies. Beside adipose tissue, it is also now well established that the brain is strongly involved in obesity process, thus a part will be dedicated to the potential effect of carotenoids on obesity management via their biological effects on the brain.

Keywords Adipocytes · Adipose tissue · Brain · Carotenoids · Obesity

21.1 Obesity, Comorbidities, Adipose Tissue, and Brain Dysfunctions

Obesity and overweight are defined by the WHO as abnormal or excessive fat accumulation that presents a risk to health (WHO 2012). Such risk is mainly related to several comorbidities strongly linked to obesity such as metabolic inflammation, insulin resistance, liver steatosis, hypertension, dyslipidemia, certain types of cancers, depression, etc. The WHO considers that around 39% of adult population were overweight, and about 13% of the world's adult population were obese in 2016 (WHO 2012). This is considered as a huge burden in the near future not only for western countries but also for low- and middle-income countries that begin to face obesity and overweight epidemy.

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The excess of fat mass occurring during obesity is characterized by an expansion of adipose tissue mediated by hypertrophia and/or hyperplasia of adipocytes (Arner et al. 2010), which is linked to the complex and tightly regulated adipogenesis. This process has been intensely studied, and the temporal sequences as well as transcriptional regulators involved have been identified. Among them, the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and the CCAAT-enhancer-binding protein (CEBPs) families are considered as transcriptional regulators of adipogenesis (Farmer 2006). Through this mechanism the adipose tissue is able to participate in energy homeostasis, allowing the storage of excess energy as triglycerides (lipogenesis) and the release of energy as fatty acids (lipolysis). This balance is tightly regulated, and dysregulation may result in body weight gain or loss.

In addition, adipose tissue is also regarded as an endocrine tissue producing not only free fatty acids but also a large variety of hormones, cytokines, chemokines, miRNA, as well as adipokines and growth factors, acting on many physiological processes. Indeed, adipose tissue secretes approximately 50 biologically active proteins acting in an autocrine, paracrine, and/or endocrine fashion. Leptin (Friedman 2014) and adiponectin (Ruhl and Landrier 2015) are among the most studied. Both adipocytes and cells belonging to the stromal vascular fraction of adipose tissue, especially macrophages, are able to produce and secrete adipokines. Obesity triggers a chronic low-grade inflammation which is associated to abnormal secretion of cytokines (Gregor and Hotamisligil 2011), chemokines (Tourniaire et al. 2013), miRNA (Karkeni et al. 2016, 2017), acute phase proteins, and other mediators of the immune response together with the activation of inflammatory signaling pathways (Gregor and Hotamisligil 2011; Olefsky and Glass 2010). Adipose tissue is a major contributor to the chronic inflammatory response. The regulation of molecules secreted by adipose tissue is multifactorial and is linked to several physiopathological disorders, including (i) increased levels of circulating free fatty acids, (ii) hypoxia of hypertrophied adipose tissue, (iii) systemic and local oxidative stress, (iv) endoplasmic reticulum stress, and/or (v) the production of inflammatory cytokines. All these types of stress converge toward signaling pathways involving c-Jun amino-terminal kinase (JNK) and IκB kinase β (IKKβ) (Gregor and Hotamisligil 2011; Olefsky and Glass 2010). A large part of this inflammatory state is mediated by the increased number of infiltrated macrophages that occurs during the expansion of adipose tissue (Weisberg et al. 2003). Such infiltration has been positively correlated with adiposity, adipocyte size, and insulin resistance (Bourlier and Bouloumie 2009). Indeed, macrophages interfere with adipocyte function through the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which can lead to insulin resistance, modify adipokine secretion, and lead to an excess of free fatty acid secretion through increased lipolysis and diminished lipogenesis (Cornier et al. 2008), participating thus to the establishment of obesity-associated disorders, such as insulin resistance.

Beside this effect of inflammatory state on adipocyte and adipose tissue function, it has also been that metabolic inflammation is associated to neuro-inflammation.

Inflammation at the central level is largely suspected to be involved in obesity aetiologia via modulation of energy homeostasis (both at food intake and energy expenditure level) (Guillemot-Legris and Muccioli 2017). Indeed, the control of energy homeostasis is finely tuned by nervous and endocrine mechanisms that cooperate to maintain the balance between caloric intake and energy expenditure (Derghal et al. 2017; Morton et al. 2006). In this respect, the central nervous system (CNS) continuously monitors modifications in hormones (insulin, leptin, and ghrelin) or metabolic parameters (i.e., blood glucose or free fatty acids levels) and elicits adaptive responses like food intake (Derghal et al. 2017; Morton et al. 2006). Among the brain regions involved in this regulation, the hypothalamus plays a pivotal role through specific neuronal networks (Derghal et al. 2017; Morton et al. 2006; Berthoud 2002; Schneeberger et al. 2014). Particularly, leptin is crucial to maintain both normal body weight and feeding behavior by action in the different regions of the hypothalamus as arcuate, paraventricular, or ventromedial nuclei as well as the lateral hypothalamus. More particularly, this peripheral signal is detected by hypothalamic arcuate neurons expressing the anorexigenic peptide POMC or the orexigenic peptides Neuropeptide Y (NPY)/Agouti-related peptide (AgRP). These neurons project to melanocortin 3 and 4 receptor-expressing neurons located in hypothalamus and other brain structures (Morton et al. 2006; Mounien et al. 2005). Together these neurons are called the melanocortin pathway and regulate feeding behavior, energy expenditure, and glucose homeostasis through activation of the autonomic nervous system and higher brain structures (Derghal et al. 2017; Morton et al. 2006; Berthoud 2002). A defect in the communication between brain and peripheral organs can affect fat gain and then lead to metabolic syndrome.

Obesity leads to increased inflammatory factors and immune cells in peripheral tissues and in brain regions that are essential for maintaining energy balance (Guillemot-Legris and Muccioli 2017). In fact, the production of inflammatory cytokines by adipose tissue and the accumulation of astrocytes and microglia (the resident immune cells of the brain) in the hypothalamus can interfere with leptin signaling and then can contribute to hyperphagia as well as many other obesity-related diseases (Derghal et al. 2017; Morton et al. 2006). In this context, the endocrine function of the adipose tissue is essential to maintain a normal weight and the regulation of energy homeostasis.

Several strategies have been proposed to fight obesity, including pharmacological approaches, limitation of fat and sugar consumption, promotion of physical activity, and consumption of fruits and vegetables. Indeed plant-based food is classically associated with weight management not only due to its macronutrient composition, but also to the presence of micronutrients, such as carotenoids. Those molecules correspond to a large family of C40 lipophilic pigments produced by plants, fungi, and bacteria (von Lintig 2010). Carotenoids can be divided into two groups according to their chemical structure: carotenes, which are hydrocarbons, and xanthophylls, which also contain oxygen and therefore are less apolar than carotenes. More than 600 different molecules have been identified, of which 50 can be found in human diet, and of which only about 10 are present in significant amounts in human plasma (Paetau et al. 1998). Carotenoids containing an unsubstituted β-ionone ring are

termed provitamin A as they can be cleaved by animals to release retinal, which can be subsequently converted to retinol (von Lintig 2010).

21.2 Carotenoids and Obesity Relationship in Human Studies

21.2.1 Observational Studies

Obesity has been associated in many epidemiological and observational studies with low circulating concentrations of carotenoids (Kimmons et al. 2006; Garcia et al. 2009). A strong inverse correlation between BMI and all measured carotenoids in plasma, except lycopene, has been highlighted in the CARDIA study (Andersen et al. 2006). In addition, many obesity-associated disorders, such as low-grade inflammation or insulin resistance, are also strongly inversely associated with serum carotenoid concentrations (Calder et al. 2011; Beydoun et al. 2011, 2019).

21.2.2 Intervention Studies

Several trials have been conducted to study the putative role of carotenoids on obesity management. Most of these studies used mixtures of carotenoids and vitamins included within natural matrices such as fruit juices or plant extracts (reviewed by Bonet et al. 2015), making the interpretation related to the specific contribution of carotenoids difficult. To our knowledge, only two randomized clinical trials, performed in double-blind and placebo-controlled, investigated the effect of pure carotenoids or xanthophylls supplementation. Indeed Canas et al. (2017) reported a decrease in BMI *z*-score, waist to height ratio, and subcutaneous adipose tissue in children submitted for 6 months to a mixture of carotenoids (β-carotene, α-carotene, lutein, zeaxanthin, lycopene, astaxanthin, γ-tocopherol). Such beneficial effects were strongly associated with an increase in plasma β-carotene concentration in children (Canas et al. 2017). Another study, using a mixture of paprika xanthophylls and carotenoid, administrated for 12 weeks to healthy overweight volunteers. Such supplementation reduced visceral fat area, subcutaneous fat area, and total fat area, as well as BMI in treated group compared to placebo group (Kakutani et al. 2018).

These data suggest that carotenoid may have an impact on adipocyte/adipose tissue biology, that may in turn result in a modification of several parameters linked to obesity and/or associated comorbidities. This assumption is strongly supported by the fact that carotenoids are stored, metabolized, and bioactive in adipocytes and in adipose tissue.

21.3 Carotenoids and Adipocyte/Adipose Tissue Metabolism

21.3.1 Carotenoids Are Stored in Adipocytes and Adipose Tissue

It is established since a long time that carotenoids are stored in adipose tissue notably (Peirce 1954; Virtanen et al. 1996; Parker 1989; Chung et al. 2009; Landrier et al. 2012). Lycopene and β-carotene are the predominant carotenoids in human adipose tissue (Parker 1988, 1989). More precisely, Chung et al. identified lycopene as the most present carotenoid in adipose tissue (more than 1/2), followed by β-carotene (approx. 1/3 of total carotenoids), lutein + zeaxanthin, β-cryptoxanthin, and α-carotene (Chung et al. 2009).

Total carotenoid concentration appears to be site-specific, with abdomen concentration being higher than in the buttocks or thigh (Chung et al. 2009). Adipose tissue concentrations of carotenoids are similar between men and women (Chung et al. 2009). Interestingly, most of the carotenoids are inversely correlated to fat mass and to both general and central adiposity (Chung et al. 2009; Wallstrom et al. 2001), suggesting that during obesity carotenoids are sequestered in adipose tissue. Conversely, weight loss is associated to an increase of lutein and zeaxanthin serum concentration (Kirby et al. 2011). In the case of β-carotene, it is somehow noteworthy that, even if its adipose tissue (?) concentration is lower in obese people, the total pool of β-carotene is similar in obese and nonobese when taking into account the total fat mass (Osth et al. 2014).

Factors governing adipose tissue carotenoids uptake, distribution, and turnover are poorly understood, but we recently reported that carotenoid uptake by adipose tissue was independent of the carotenoid physicochemical properties (Sy et al. 2012), suggesting the involvement of putative transporters or facilitators. In agreement, we demonstrated the involvement of CD36 in lycopene and lutein uptake by adipose tissue and adipocytes (Moussa et al. 2011). In addition, we also showed that lycopene was not only stored in lipid droplets in adipocytes, but is also present in plasma and nuclear membranes (Gouranton et al. 2008).

Adipose tissue carotenoid content is usually considered as a good long-term indicator of dietary intake of carotenoids (Kardinaal et al. 1995). β-carotene concentration in adipose tissue increased 5 days after a large oral dose consumption (Johnson et al. 1995). Lutein and zeaxanthin levels in adipose tissue significantly increased after spinach and corn supplementation in healthy subjects, with a maximum at 8 weeks of intervention (Johnson et al. 2000). Finally, tomato-oleoresin supplementation significantly increased lycopene concentration in adipose tissue (Walfisch et al. 2003). Dietary carotenoid intakes were strongly correlated with abdomen adipose tissue concentration (a lower correlation was found for buttock or thigh adipose tissue) for α-, β-carotene, β-cryptoxanthin, *cis*-lycopene, and total carotenoids

(Chung et al. 2009). However, these correlations vary largely and are strongly influenced by the gender. Notably, El-Sohemy et al. reported correlation coefficients in women between intake and concentration in adipose tissues of α-, β-carotene, β-cryptoxanthin, and lutein/zeaxanthin (0.25, 0.29, 0.44 and 0.17 respectively), but not in men (0.04, 0.07, 0.23, and 0.06, respectively) (El-Sohemy et al. 2002). The origin of the discrepancy is presently unknown but suggests that carotenoids adipose tissue concentration may be affected by factors other than intake, or that carotenoid intake is not appropriately estimated.

Adipose tissue carotenoids content is not only correlated with dietary intake, but also with other tissues concentration. Thus, lutein adipose tissue content has also been reported to be positively correlated with macular pigment density in men, but not in women (Broekmans et al. 2002). In addition, total carotenoid content in adipose tissue is strongly associated to serum levels (Chung et al. 2009), except for lycopene and lutein + zeaxanthin. β-carotene content in adipose tissue is correlated to plasma level with a correlation coefficient of 0.20 (Kardinaal et al. 1995; Su et al. 1998). Similarly, breast adipose tissue carotenoids content correlates with plasma levels, except for β-cryptoxanthin (Yeum et al. 1998).

21.3.2 Carotenoids Are Metabolized in Adipocytes and Adipose Tissue

β-carotene 15, 15′-monooxygenase (BCO1; involved in centric cleavage of carotenoids) and β-carotene 9′, 10′-dioxygenase (BCDO2; involved in eccentric cleavage of carotenoids) are expressed in adipocytes (Hessel et al. 2007), raising the possibility that carotenoid cleavage products, including retinal, derivatives and apocarotenoids could be found in adipocytes (Coronel et al. 2019; Amengual et al. 2011; Tourniaire et al. 2009). In agreement retinal (Ziouzenkova et al. 2007), as well as free retinol has been identified in the adipocyte fraction of adipose tissue (Tsutsumi et al. 1992). Several isomers of retinol, including all-*trans*, 9-*cis* and 13-*cis* isomers, were also quantified in white adipose tissue (Tsutsumi et al. 1992; Kane 2012; Sima et al. 2011), together with several isomers of retinoic acid, except 9-*cis* retinoic acid (Kane 2012; O'Byrne et al. 2005; Landrier et al. 2017). Adipocytes express BCO1 and BCO2, as well as the enzymes necessary for vitamin A metabolism, suggesting that part of the effect of provitamin A carotenoids are mediated via vitamin A production. This topic will not be developed in the present review and the reader is referred to the excellent review of Dr. Blaner (2019).

Besides these retinoids, β-10′-apocarotenal has been identified in adipose tissue (Amengual et al. 2011). It is highly probable that other apocarotenoids are produced in adipose tissue, nevertheless, the function of those compounds in adipocyte biology still needs to be investigated.

21.3.3 Carotenoids Regulate Gene Expression in Adipocytes and Adipose Tissue

Several molecular mechanisms mediating the effects of carotenoids on gene expression have been described. In the case of provitamin A carotenoids, leading to retinoic acid synthesis, retinoic acid receptors (RARs), and retinoid X receptors (RXRs) constitute specific signaling targets. Two families of receptors mediate the effects of retinoids (Germain et al. 2006a, b). Three subtypes of each have been described (RARα RARβ, RARγ, RXRα, RXRβ, and RXRγ). These receptors work as ligand-dependent transcriptional regulators by binding specific DNA sequences [Retinoic Acid Response Element (RARE) or Retinoid X Response Element (RXRE)] found in the promoter region of retinoid target genes either as RAR-RXR or RXR-RXR dimers. RARs and RXRs subtypes are found in every cell type. Furthermore, RXRs are dimerization partners for other nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR), farnesoid X receptor (FXR), pregnane X receptor (PXR), RARs, thyroid hormone receptor (TR), and vitamin D receptor (VDR), leading to the regulation of a huge number of genes. In addition, several other transcription factors and signaling pathways are modulated by retinoic acid (Yasmeen et al. 2012), including PPARβ ((Berry and Noy 2009). Lycopene (Aydemir et al. 2012) and apo-10′-lycopenoic acid (Gouranton et al. 2011) are also able to activate RAR. Many carotenoids regulate gene expression via ubiquitous signaling pathways such as NF-κB and MAP kinases (Rao et al. 2006; Sharoni et al. 2012), or via transcription factors involved in detoxification such as AhR, NRF2, or PXR (Ben-Dor et al. 2005; Landrier 2012).

21.4 Carotenoids and/or Metabolites Impact Adipocyte Biology in in Vitro Studies

The impact of some carotenoids has been documented in adipogenesis, which could contribute to obesity management, via a limitation of lipid accumulation in adipocytes. Most of the reported effects inhibited adipocyte differentiation (Kawada et al. 2000) by interfering with nuclear receptors such as RAR, RXR, or PPAR. Indeed, β-carotene inhibited adipogenesis not only through the production of β-apo-14′-carotenal and repression of PPARα, PPARγ, and RXR activation (Ziouzenkova et al. 2007), but also through the production of all-trans retinoic acid (Lobo et al. 2010). Similarly, β-cryptoxanthin suppresses adipogenesis via activation of RAR (Shirakura et al. 2011), and astaxanthin inhibits rosiglitazone-induced adipocyte differentiation by antagonizing transcriptional activity of PPARγ (Inoue et al. 2012). Zeaxanthin (Liu et al. 2017) and fucoxanthin (Maeda et al. 2006; Seo et al. 2016) exhibited anti-adipogenic effect via a down-regulation of adipogenic transcription factors C/EBPα and PPARγ, which blunted lipid accumulation. Conversely, lycopene (unpublished personal data) and apo-10′-lycopenoic acid (Gouranton et al. 2011)

showed no effect on adipogenesis. Beside these effects, there is also evidence that some effects of provitamin A carotenoids are mediated through retinol and its metabolites production which are known to regulate adipogenesis (reviewed in Blaner 2019).

Molecules with anti-inflammatory effects are supposed to limit the risk of obesity-associated disorders, including insulin resistance. Such anti-inflammatory effects of β-carotene in 3T3-L1 adipocytes were suggested to arise through limitation of TNFα-mediated down-regulation of genes linked to adipocyte biology (Kameji et al. 2010). β-carotene also counteracted oxidative stress-mediated dysregulation of adiponectin secretion, chemokines expression, and NF-κB activation in 3T3-L1 adipocytes (Cho et al. 2018). Fucoxanthin blunted TNFα-mediated induction of pro-inflammatory cytokines in adipocytes (Hosokawa et al. 2010) and in adipocytes/macrophages coculture systems (Maeda et al. 2015). The most studied anti-inflammatory carotenoid is lycopene (all-trans), and we demonstrated its ability to inhibit pro-inflammatory cytokine and chemokine expression in vitro (in murine and human adipocytes) (Gouranton et al. 2011). These data were also reproduced ex vivo on adipose tissue explants from mice subjected to a high fat diet (characterized by low-grade inflammation). The molecular mechanism was investigated and the involvement of NF-κB was confirmed. Similar results (i.e., inhibition of cytokine and chemokine expression in various in vitro and ex vivo models) were obtained with apo-10′-lycopenoic acid, a metabolite of lycopene (Gouranton et al. 2011). Lycopene also attenuated LPS-mediated induction of TNFα in macrophages via NF-κB and JNK (Marcotorchino et al. 2012), as well as macrophage migration in vitro. Consequently, lycopene decreased macrophage-induced cytokine, acute phase protein and chemokine mRNA in adipocytes. Interestingly, all-trans and 5-cis lycopene, the two main isoforms of lycopene found in vivo, displayed similar effect in terms of inflammation control and glucose uptake in adipocytes (Fenni et al. 2019). A few studies have shown that retinoids, similar to carotenoids, have positive effects by decreasing the expression of adipocyte-derived inflammatory mediators such as adipsin (Antras et al. 1991) and resistin (Felipe et al. 2004). Our group has also shown that ATRA blunts TNF-α mediated cytokine expression in 3T3-L1 cells (Gouranton et al. 2011). More recently, we demonstrated that ATRA limits the expression of a large range of chemokines in vivo and in vitro. Such anti-inflammatory effect of ATRA was associated with a reduction in the phosphorylation levels of IκB and p65, probably mediated by PGC1α expression (Karkeni et al. 2017).

The browning process of white adipose tissue has been proposed as a putative mechanism controlling energy homeostasis and insulin sensitivity (Chondronikola et al. 2014). Recently an AMPK1-mediated effect on adipocyte browning and mitochondrial biogenesis has been demonstrated for zeaxanthin (Liu et al. 2019). We reported similar mitochondrial biogenesis, induction of OXPHOS, and adipocyte browning in adipocytes incubated with ATRA (Tourniaire et al. 2016), whereas fucoxanthin and its metabolite fucoxanthinol were inefficient to induce adipocyte browning (Rebello et al. 2017).

21.5 Carotenoids and/or Metabolites Are Involved in Body Weight Management and Limitation of Obesity Comorbidities

Significant research has been dedicated to the study of the impact of β-carotene on energy metabolism and consequences on obesity (Coronel et al. 2019). Its anti-obesity effect has subsequently been demonstrated to be linked to the provitaminic A effect (Amengual et al. 2011; van Helden et al. 2011) since BCMO1$^{-/-}$ mice did not display adipose tissue weight modification. This effect was found to be linked to decreased expression of PPARγ in adipose tissue and the involvement of RAR signaling in this regulation (Lobo et al. 2010).

Astaxanthin prevented obesity in mice fed with a high fat diet (Ikeuchi et al. 2007), via the limitation of adipose tissue expansion. Similar anti-obesity effects have been documented in mice subjected to a high fat and high fructose diet (Arunkumar et al. 2012) where insulin sensitivity and inflammation were also improved by astaxanthin. Preventive effect of astaxanthin regarding hepatic steatosis (Ni et al. 2015) and inflammation and fibrosis in the liver in a NASH and DIO mice model (Kim et al. 2017).

Anti-adiposity properties have also been reported for β-cryptoxanthin (Takayanagi et al. 2011), but the mechanism has not been unveiled. In addition, β-cryptoxanthin reversed liver steatosis and insulin resistance in DIO mice; such effect may be related to the anti-inflammatory effect of this carotenoid in the liver (Ni et al. 2015). The potentiality of fucoxanthin in weight management has been extensively studied (for review (Maeda 2015). This carotenoid limited weight gain, hyperglycemia, and inhibited the expression of several pro-inflammatory cytokines in adipose tissue of KK-a(y) mice (Hosokawa et al. 2010). Similar effects have been depicted in DIO mice, not only through modulation of lipogenesis, adiponectin production, and inflammation in adipose tissue (Grasa-Lopez et al. 2016), but also via browning of white adipose tissue (Maeda 2015).

Zeaxanthin inhibited obesity in high fat fed mice, presumably by inducing AMPK activation, and inhibition on lipogenesis in adipose tissue (Liu et al. 2017).

The anti-obesity effect of lycopene was demonstrated in mice submitted to high fat diet, where adiposity was reduced after supplementation. Several comorbidities were consequently reduced such as glucose tolerance insulin sensitivity steatosis (Singh et al. 2016). We and others confirmed this beneficial effect of lycopene and/or tomato powder rich in lycopene in DIO mice model, on adiposity, glucose homeostasis, adipose tissue, and liver inflammation and steatosis (Fenni et al. 2017; Li et al. 2018; Wang et al. 2019).

It is also obvious that part of the effects of carotenoids (provitamin A or not) are due to the vitamin A effect and are mediated by RAR. Such effects have been extensively reviewed elsewhere (Bonet et al. 2015; Coronel et al. 2019; Blaner 2019) and will not be detailed in the present review.

21.6 The Impact of the Carotenoids on the Control of Energy Homeostasis by Brain

As mentioned above, carotenoids can affect the biology of the adipose tissue and then modulate the production of leptin as well as the inflammatory cytokines (Landrier et al. 2012), and consequently may have an indirect effect on brain function. However, several food compounds including carotenoids could reach directly the hypothalamus (Aragones et al. 2016), where they regulate leptin signaling pathway. More precisely, they could either cross the blood brain barrier or pass through fenestrated capillaries of circumventricular organs and target the arcuate nucleus neurons. In the context of the central control of feeding behavior, it is important to notice that other structures as hippocampus plays an important role and that they could be targeted by the carotenoids as indicated below.

It is presently not clear if carotenoids act indirectly via adipose tissue or directly on brain, nevertheless, several studies suggested an involvement of brain in body weight management under carotenoids effect. Indeed, the continuous intake of lycopene-rich food as well as the intraperitoneal administration of lycopene increased neuronal activity within the paraventricular and ventromedial nuclei as shown by c-Fos, a marker of neuronal activity, immunoreactivity (Takayama et al. 2013). This study suggested that lycopene may have some influence on feeding behavior. In accordance with this hypothesis, a nice work of the group of Dr. Bishnoi showed that lycopene prevented weight and adiposity in mice in a HFD model (Singh et al. 2016). Interestingly, this effect was associated with a modulation of hypothalamic anorexigenic and orexigenic genes expression. To date, the direct effect of lycopene on neuronal activity is unclear and more studies are necessary to thoroughly understand this mechanism. As evoked above, lycopene can impact brain function by limiting peripheral inflammation. In accordance with this hypothesis, Kuhad et al. showed that chronic treatment with lycopene significantly and dose-dependently attenuated cognitive deficit associated with inflammation in diabetic rats (Kuhad et al. 2008).

Interestingly, recent work by Zhao et al. suggest that fucoxanthin may modulate neuro-inflammation (Zhao et al. 2017). In this work, fucoxanthin increased Nrf-2 activation in LPS-activated microglia. This interesting observation needs to be studied in in vivo model and especially in brain structures (*i.e.,* hypothalamus or hippocampus) involved in feeding behavior. In accordance with this idea, a recent paper indicated that fucoxanthin treatment reversed LPS-induced defect in body weight and food intake in mice (Jiang et al. 2019). The authors also showed that fucoxanthin inhibited LPS-induced overexpression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the hippocampus and hypothalamus, via the modulation of AMPK- NF-κB signaling pathway. Interestingly, current studies have shown that the activation of AMPK pathway is essential to maintain energy homeostasis, as they are involved in the anorexigenic effect of leptin (Hardie 2014).

21.7 Conclusion

In vitro and preclinical studies clearly indicate beneficial effects of carotenoid consumption on obesity and associated physiopathological disorders including metabolic inflammation, insulin resistance, hepatic steatosis. Molecular mechanisms begin to be unveiled, even if it is not always clear if carotenoids are active under their native form or after cleavage and metabolization, and adipose tissue appears as a major target of those molecules. Nevertheless, recent but limited data suggest that carotenoids or metabolites might also act at the central level, probably by preventing or decreasing obesity-associated neuro-inflammation and comorbidities. Randomized clinical trials using pure carotenoid(s) should be urgently conducted to confirm preclinical and observational studies.

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Chapter 22
Phycobiliproteins as Food Additives

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Abstract The phycobiliproteins (FBPs) are antenna pigments composed of apoprotein covalently bound to phycobilin (tetrapyrrole chain chromophores). The main FBPs are phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) that absorb at 550, 620, and 650 nm, respectively, and are associated with phycobilisomes in cyanobacteria and rhodophytes. At present, it is of industrial-biotechnological interest to use natural dyes mainly blue pigments with food grade quality. The main known source is phycocyanin (PC) although unstable for use in industrial food processes because it degrades at temperatures above 45 °C and it is sensible to light and pH. Recently, it was reported that some extremophile microorganisms, such as the thermophilic red algae *Cyanidioschyzon merolae*, are able to produce PC which is stable to pH 5 at 83 °C, but with a half-lifetime of 40 min. Another interesting example is a halophylic cyanobacteria *Euhalothece* sp. that can grow at 45 °C, pH 6–9, and 12% NaCl which allows low risk of microbial contamination. The PC from this cyanobacteria was purified up to analytical grade (purity >2) and was stable at 45 °C, pH 5–8. These strains open the possibility to explore more biotechnological conditions that would allow to generate a pigment with a longer half time for industrial use. The last studies about phycobiliproteins have been focused on the way to prevent the loss of color due to PC degradation. In this regard several preservatives substances were tested such as citric acid, fructose, beet pectin, crosslinking with methylglyoxal (MGO). Formulation in anionic micelles was also investigated. The PC from *Spirulina platensis* improved its stability in the presence of citric acid (4 mg/mL) at 35 °C during 15 days. It has been shown a correlation between sugars (mainly fructose) and PC stability, but the use is limited for pastry and confectionery due to the high sugar content. On the other hand Sodium Dodecyl Sulfate micelles

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stabilize the non-protonated (blue) forms of PC and prevent the formation of protonated forms (green) at low pH. The challenge in the field of phycobiliproteins is to improve the stability at high temperatures for its use in food in order to guarantee food safety and overall quality. This can be achieved by using genetic, molecular tools and biotechnological approaches including extremophile microorganisms.

Keywords Microalgae · Phycoerytrin · Phycocyanin · Allophycocyanin · Food grade

22.1 Introduction

Phycobiliproteins are classified into three main groups, phycoerythrin (red), phycocyanin (blue), and allophycocyanin (blue). Phycocyanin includes C-phycocyanin (C-PC) and R-phycocyanin (R-PC). The phycobiliproteins are located on a specialized cellular structure called phycobilisome, they are organized in two domains, and are attached to the surface of the thylakoid membrane. Phycobilisome consists of a core of allophycocyanin (APC) surrounded on the periphery by phycocyanin and phycoerythrin (PE). The organization of the phycobilisome is supported by polypeptides called linkers, which interact with phycocyanin and phycoerythrin to assemble the rods (Gantt and Conti 1966; Eisele et al. 2000; Samsonoff and MacColl 2001). The maximum absorption for phycoerythrin is estimated between 540 and 570 nm, phycocyanin between 610 and 620 nm and allophycocyanin between 650 and 655 nm (Kannaujiya and Sinha 2016; Kaur et al. 2019).

Phycobiliproteins (PBPs) are antenna pigments composed of apoprotein covalently linked by cysteine residues to tetrapyrrole chromophores, called bilins (phycocyanobilin for allophycocyanin and C-phycocyanin; phycoerythrobilin for C-phycoerythrin). Phycocyanin is composed of two polypeptide chains (apoprotein): a small subunit called α containing a chromophore and another subunit called β that contains two chromophores (Seibert and Connolly 1984; Samsonoff and MacColl 2001; Selig et al. 2018). It is estimated that subunit α has a molecular weight between 12 and 19 kDa and unit β between 14 and 21 kDa (Kaur et al. 2019).

Phycobiliproteins form aggregates. The phycocyanin subunits are found as dimers, trimer $(\alpha\beta)3$ of about 120 kDa, hexamers $(\alpha\beta)$ 6 of about 240 kDa, and even high molecular weight dodecamers (Samsonoff and MacColl 2001; Selig et al. 2018; Kaur et al. 2019).

The structure of phycobiliprotein is expressed as a component (pigment) of photosynthesis, bright in color, highly fluorescent (due to phycobilins), and soluble in water (Kuddus et al. 2013; Kannaujiya and Sinha 2016). In cyanobacteria and red algae, the energy of light is absorbed by phycoerythrin, then migrates to phycocyanin, to allophycocyanin and finally to chlorophyll, a process that is associated with photosystem II (Samsonoff and MacColl 2001; Edwards et al. 1997).

At present, it is of industrial-biotechnological interest to use natural dyes mainly blue pigments with food grade (Fig. 22.1). The main known source is phycocyanin

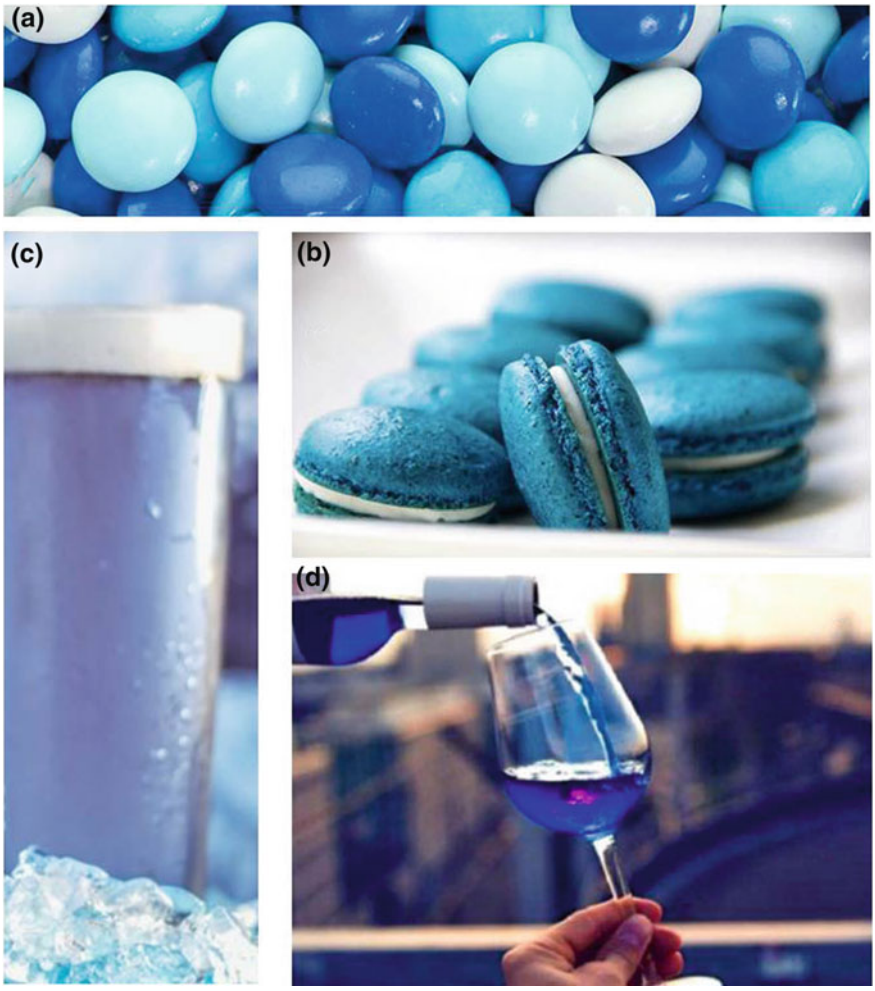

Fig. 22.1 Blue food is FANCY ! (**a** candies, **b** macaron cookies, **c** beer, **d** wine). Nature and researchers should be able to provide safe blue food colorants to the industry and consumers

(PC) although unstable for use in some industrial food processes because it degrades at temperatures above 45 °C and it is sensible to light and pH. Recently, was reported that some extremophile microorganisms, such as thermophilic red algae *Cyanidioschyzon merolae*, is able to produce PC which was stable to pH 5 at 83 °C, but with a half-lifetime of 40 min. Another example is a halophilic cyanobacteria *Euhalothece* sp. that can grow at 45 °C, pH 6–9, and 12% NaCl, conditions which allows low risk of microbial contamination. The PC from this cyanobacteria is purified with analytical grade (purity >2) and is stable at 45 °C and pH 5–8. This leaves open the possibility to explore biotechnological conditions that allows to generate a pigment with a longer half-lifetime for industrial use (Mogany et al. 2018).

The last studies about phycobiliproteins have been focused on the prevention of the loss of color due to PC degradation. In this regard several preservative substances were tested such as citric acid, fructose, beet pectin, crosslinking with methylglyoxal (MGO) and anionic micelles. The PC from *Spirulina platensis* improved the stability in presence of citric acid (4 mg/mL) at 35 °C during 15 days. It has showed a correlation between sugars (mainly fructose) and PC stability, but the use is limited for pastry and confectionery due to the high sugar content. On the other hand SDS micelles stabilize the non-protonated (blue) forms of PC and prevent the formation of protonated forms (green) at low pH (Martelli et al. 2014; Falkeborg et al. 2018).

In this chapter, the structural characteristics of phycobiliproteins, chromophores, and phycobilisomes are related to the stability of these structures, mainly at pH and temperature. In addition, the biochemical differences found between phycobiliproteins of mesophilic and thermophilic organisms and the potential use in biotechnological applications are analyzed. Likewise, is considered the use of food grade phycobiliproteins as additive and on stability studies at pH, temperature, light, as well as the use of preservatives and microencapsulation to increase the stability of these pigments.

22.2 Structural Characteristics of Phycobiliproteins

The cyanobacteria are located in diverse environments such as the desert, sea water, Antarctic region, lakes, soils, lithic substrates (halite and quartz), and their adaptation is due to the synthesis of compounds (polysaccharides, osmolytes, and pigments among others) that serve as a survival strategy, to tolerate a wide range of factors such as temperature, pH, ultraviolet radiation, and salinity (Rippka et al. 1979; Tandeau de Marsac and Houmard 1993). Several studies have been dedicated to test the stability of phycobiliproteins in high-temperature environments and compare it with those of microorganisms in moderate temperature environments. The following question arises: are there differences in the structure and stability of the phycobiliproteins of thermophilic and mesophilic cyanobacteria?

In this sense, the presence of cyanobacteria in environments with high temperatures such as hot springs and geysers (Pumas et al. 2012; Rahman et al. 2017) has been widely reported and studies have focused mainly on C-phycocyanin cyanobacteria isolated from thermal environments.

The cyanobacterium *Synechoccoccus lividus* isolated from thermal alkaline hot springs of Yellowstone National Park grows at 73 °C and produces C-phycocyanin, which denaturates at a higher temperature than mesophilic microorganisms. Unlike other phycocyanins, this pigment has a maximum absorption at 608 nm, which may be due to a biline-apoprotein interaction, which is different from that found in mesophilic phycocyanins. A second strain of this cyanobacterium grows at 55 °C and at temperatures of 49 °C, the aggregation increases, it needs a higher temperature to acquire the native structure. The aggregation of phycobiliprotein monomers has been associated

with an increase in temperature stability (Samsonoff and MacColl 2001; Pumas et al. 2011).

High molecular weight aggregates have been found in thermophilic cyanobacteria. The thermophilic cyanobacterium *Chroococcidiopsis* sp. strain TS-821 isolated from hot spring in Thailand grows at 50 °C, over a wide pH range. Its phycocyanin has a molecular weight of 600 kDa, suggesting that it is added in the form of three-stacked hexamer in a similar way phycoerythrin from *Leptolyngbya* sp. KC45 isolated from hot spring in its native form is a 235 kDa hexamer (Hayashi et al. 1997; Pumas et al. 2011).

Thermophilic monomers are more stable than those of mesophiles. In the thermophilic cyanobacterium *Synechococcus lividus* the monomers are denaturated at 60–70 °C. The instability of the monomers at physiological temperatures (66–73 °C) suggests that they should be added quickly to trimers to avoid denaturation (Edwards et al. 1997).

Additionally, to the formation of high molecular weight aggregates, the stability of phycobiliproteins of thermophiles has been linked to the presence of specific amino acids at key points in the structure of the protein. In the phycocyanins of a thermophilic cyanobacterium (*Synechococcus lividus*) and another mesophilic cyanobacterium (*Phormidium luridum*) that grow at 52 °C and 25 °C, respectively, it was observed that there are minimal differences in the amino acid composition. Phycocyanin has apolar amino acids which are important due to the hydrophobic interaction between their side chains and which could be responsible for their temperature stability, rather than the hydrogen bonding interactions. Similarly in the PC of *Synechococcus* PCC6715 has been detected the substitution of conserved amino acids that allows the stabilization of interactions between protein subunits when compared to mesophilic proteins (Berks et al. 1963; Chen et al. 1994; Liang et al. 2018).

Likewise, the stability to the temperature and pH of C-phycocyanin of *T. elongatus* TA-1, a thermophilic cyanobacterium, may be due to the substitution of certain amino acid residues at specific positions that allow ionic interactions in the phycobiliproteins. The difference in the evolutionarily conserved sequence of amino acids found in the C-phycocyanin of thermophilic and mesophilic cyanobacteria lies in the alpha chain, and a pattern of amino acid substitution in the beta chain has not been found (Leu et al. 2013; Pumas et al. 2011; Liang et al. 2018).

The C-phycocyanin of *Synechococcus* PCC6715 has a sequence in amino acids in the alpha chain with identity greater than 97% with respect to the alpha chain of *T. vulcanus* and *T. elongatus*. The comparison in the amino acid sequence of C-phycocyanins of highly thermostable cyanobacteria (*Synechococcus* PCC6715 and *Thermosynechococcus* sp.) and mild-thermostable (*Cyanidium caldarium* and *Galdiera sulphuraria*) and mesophilic (*Synechococcus elongatus* PCC 7942, Spirulina, *Leptolyngbya* sp. N62DM) showed that there are differences in the sequence of amino acid residues in the alpha chain, mainly in those amino acids that affect the formation of salt bridges and hydrogen bridges (Liang et al. 2018).

Liang et al. (2018) showed that the salt bridge that stabilizes the structure in the vicinity of phycobilin between Asp 28 and Arg33 alpha chain residues, is absent in mesophilic and mild-thermophilic proteins, where the Asp is replaced by amino

acids with different polarity characteristics as Phe an aromatic amino acid and Asn that is a polar noncharged amino acid to neutral pH, respectively.

In relation to hydrogen bonds, the Arg33-Gln145 pair is present in phycocyanin sequences of thermophiles but absent in other less stable proteins. Another amino acid residue related to the stability of phycocyanins is Gln 111 located in the alpha chain whose position is close to the phycocyanobilin. It is believed that the substitution of this amino acid can affect the thermostability since this type of amino acid forms hydrogen bonds that stabilize the protein structure. In addition, *Synechococcus* PCC6715 cyanobacteria, as in other thermostable phycocyanins, have Ala 43 and Asn 42 in the alpha and beta chains, respectively, which are important for the stability of the protein because they can form hydrogen bonds (Liang et al. 2018).

The phycobilisomes of cyanobacteria and mesophilic algae lose color (bleaching) above 60–65 °C, this could occur by the covalent modification of the chromophores and not by modifications in the apoprotein because the absorption band of the chromophore disappears completely (Zhao and Brand 1989). On the other hand, Murthy et al. (2004) showed that the temperature above 50 °C, affects the energy transfer in the phycobilisome modifying the interaction between the PC chromophore of *Spirulina* and its apoprotein due to disorganization of the PC structure.

Phycobilisomes are more stable when they are purified in solutions with high ionic strength. In the alga *Anacystis nidulans* phycobilisomes purified in solutions with high concentrations of phosphate tolerated temperatures exceeding 80 °C. This factor should be considered in the purification processes of phycobiliproteins (Zhao and Brand 1989).

22.3 Stability of Phycobiliproteins

Phycobiliproteins of cyanobacteria and mesophilic and thermophilic red algae have a very similar secondary and tertiary protein structure. However, as discussed in the previous paragraphs, the differences in stability may be due in part to the individual interaction of some amino acids in apoproteins of these pigments. These interactions can be affected by pH and temperature (Edwards et al. 1997).

22.3.1 Phycobiliproteins of Thermophilic Cyanobacteria

Studies show that the phycobiliproteins of thermophilic cyanobacteria are more stable than those of mesophiles, in a wide range of temperature and pH. However, phycocyanin from the thermophilic cyanobacterium *Phormidium laminosum* does not have a longer fluorescence lifetime than mesophilic phycocyanin, presumably because the specific environment of tetrapyrrole more than other factors such as aggregation, concentration, or thermophilic nature of the microorganism (Seibert and Connolly 1984).

Phycobiliprotein stability measurements are made over a wide range of temperature and pH, measuring changes in the structure and function of the protein, commonly using spectrophotometric techniques, fluorescence, and measurement of antioxidant capacity. The thermostable phycobiliproteins have been extracted from cyanobacteria that live in warmer waters, e.g., the C-phycocyanin of *Thermosynechococcus elongatus* TA-1, isolated from Taiwan hot springs, is stable between 4 and 60 °C and pH values between 4 and 9, after 4 h of treatment. However, as with mesophilic phycocyanins above 75 °C, the protein loses its fluorescence due to denaturation (Leu et al. 2013). Interestingly, the phycoerythrin of the cyanobacterium *Leptolyngbya* sp. KC 45 also isolated from hot springs, after being exposed to 60 °C for 30 min maintains 80% of its antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Pumas et al. 2012).

Phycocyanin from *Synechococcus lividus* PCC 6715 isolated from Yellowstone National Park hot springs is one of the most stable proteins reported. It showed 90% stability, after 5 h of incubation at 50 °C and about 70% after two weeks at the same temperature. Phycocyanin from *Synechococcus* PCC6715 is slightly more stable than *Thermosynechococcus elongatus* TA-1, with 5 h exposure at 50 °C. The phycocyanin stability of *Synechococcus* PCC6715 is high at pH 4–8 at 4–50 °C (Liang et al. 2018).

Another reported phycocyanin is that of the thermophilic red alga *Cyanidioschyzon merolae* isolated from hot springs and geysers has a midpoint denaturation point at pH 5 at 83 °C with an average lifetime of 40 min (Rahman et al. 2017).

The structural and genomic study of thermostable phycobiliproteins, will allow to modify the genetic engineering phycobiliproteins to improve the thermostability in sterilization processes in order to use them safely in different foods.

22.3.2 Phycobiliproteins of Mesophilic Cyanobacteria

Studies in phycobiliproteins in mesophilic cyanobacteria have focused on evaluating the effect of temperature, pH, light, storage form (dry or in solution) as factors to preserve the stability with food grade and antioxidant capacity of phycobiliproteins for use in food or other purposes.

The studies on phycocyanin stability of pH and temperature have been carried out mainly in the cyanobacterium *Spirulina platensis*. The phycocyanin of this cyanobacterium is stable in a range of pH 4–8 but at pH 5 the stability of the protein is increased. At temperatures above 45 °C and pH 7, phycocyanin is denaturated, but when it is at pH 5–6 its stability is higher, independent of the exposure temperature (Couteau et al. 2004; Patel et al. 2004; Antelo et al. 2008). In agreement with the above, in other cyanobacteria and red algae has been observed the disappearance of the color of the phycobiliproteins when exposed to 60–65 °C (Zhao and Brand 1989; Topchishvili et al. 2002).

In the cyanobacterium *Phormidium rubidium* A09DM, the phycobiliproteins were stable in a temperature range of 4–40 °C, but their concentrations decrease to 60–80 °C, as in *Spirulina fusiformis* (Prasad et al. 2015; Munawaroh et al. 2018).

The content of PBPs decreases to pH very acidic (2–4) and alkaline pH 8–12, this is because changes in pH alter the electrostatic properties and hydrogen bonds of the protein-producing changes in the structure of the chromophore and apoprotein (Rastogi et al. 2015).

In this sense, the stability of phycocyanin is greater than 4 °C and in darkness (Choi and Lee 2018). PC Anabaena fertilissima PUPPCC 410.5 and PE of Lyngbya arboricola to terrestrial desiccation cyanobacterium are more stable lyophilized than in solution (Tripathi et al. 2007 and Kaur et al. 2019). The half-life of dry lyophilized PC is 100 days stored at 4 °C in darkness. PC is highly unstable at 42 °C in light decreased 50% in 4 days. The phycocyanin of Anabaena fertilissima was added to dairy milk products such as milk and yogurt, stable for 6–9 days at 4 °C in the dark and maintained its antioxidant properties (Kaur et al. 2019).

Another way to preserve and store phycocyanin from the PC of *Spirulina* sp. strain LEB-18 is by drying system known as spouted bed drying (at 80 °C) or tray drying (at 55 °C) with both techniques the PC had high stability measured as PC content, color, and antioxidant activity (Larrosa et al. 2018).

Interestingly, temperature can induce changes in the content of each of the phycobiliproteins, in a similar way as in the case of complementary chromatic adaptation, in which the type of wavelength that is influenced by cyanobacterial cultures produces increases in PE or PC. The thermostable cyanobacterium *Oscillatoria* sp. N9DM when grown at 30–42 °C produces more PE, but if the cultures are grown at 55 °C they produce only PC (Singh et al. 2012). The latter is a simple way to enrich cultures with the phycobiliprotein of interest.

22.4 Food Grade Phycobiliproteins

The term food grade is used in the food industry to refer to the quality of the ingredient (phycobiliprotein) measured in terms of concentration and purity in order to ensure the innocuousness of the pigment. In phycobiliproteins the food grade is calculated from the absorbance ratios at the maximum absorption of each phycobiliprotein (PC at 620 nm, PE at 550 nm) in relation to the absorbance of total proteins at 280 nm. Figure 22.1 shows some examples of the application of phycobiliproteins in foods.

The absorbance ratios for PC A620/A280 between 0.7 and 4 are considered food, > 4 reactive and analytical grade. The A620/A280 ratio of 2 is accepted in the food industry (Kuddus et al. 2013; Munawaroh et al. 2018). Classical biochemical techniques such as precipitation with ammonium sulfate and ion exchange chromatography, commonly used in the purification of phycobiliproteins, allow these pigments to be obtained with reactive and analytical grade (Table 22.1).

Table 22.1 Food grade (purity) of phycocyanin from diverse species according to extraction methods

Authors/ratio	Species[a]	Step purification	Purity
Kaur et al. (2019) A615/A280	*A. fertilisima*	Crude extract	0.56 ± 0.04
		Ammonium sulfate saturation (20–75%)	1.96 ± 0.12
		Gel filtration chromatography	3.28 ± 0.2
Munawaroh et al. (2018) A620/A280	*Spirulina fusiformis*	Crude Extract Ammonium Sulfate precipitation	0.53
		Step 1	1.36
		Step 2	1.70
		Step 3	2.06
Khazi et al. (2018) A620/A280	*A. platensis* [$NaNo_3$]	Crude Extract $CaCl_2$	1.18
		70% NH_4SO_4 fractionation	1.77
		Dialysis	2.31
		Sephadex-25	3.28
		DEAE-Sephadex	4.33
	A. platensis [KNO_3]	Crude Extract $CaCl_2$	1.07
		70% NH_4SO_4 fractionation	2.03
		Dialysis	2.19
		Sephadex-25	3.0
		DEAE-Sephadex	4.11
	Phormidium sp. [NH_4Cl]	Crude Extract $CaCl_2$	1.15
		70% NH_4SO_4 fractionation	2.34
		Dialysis	2.44
		Sephadex-25	2.94
		DEAE-Sephadex	4.14
	Pseudoscillatoria sp. [NH_4Cl]	Crude Extract $CaCl_2$	1.12
		70% NH_4SO_4 fractionation	1.92
		Dialysis	2.18
		Sephadex-25	3.31
		DEAE-Sephadex	4.4
Sala et al. (2018) A620/A280	*S. platensis*	Initial	1.08 ± 0.17
		Retained—ultrafiltration	1.62 ± 0.04

[a]Between brackets, nitrogen sources are listed

22.5 Preservative Substances and Microencapsulation of Phycobiliproteins

Natural pigments such as phycobiliproteins have the disadvantage of being more sensitive to temperature, pH, humidity, and light compared to chemical dyes. Due to the above, the use of preservatives such as citric acid, sodium chloride, calcium chloride, benzoic acid, sugars among others, and microencapsulation have been used to increase stability against these abiotic factors and can be used in food.

In C-phycocyanin from *Arthrospira platensis*, citric acid was used as an edible preservative at 80 °C, for one hour and an increase in the stability of this pigment was observed (Pan-utai et al. 2018). The work of Mishra et al. (2008) in the cyanobacterium *Spirulina platensis*, also shows that citric acid (4 mg/mL) was the best preservative for PC at 35 °C, in aqueous solution for 15 days, without loss of stability when compared to C-PC at 0 °C. It is also recommended to use preservatives substances before lyophilizing the purified C-PC with food grade and using amber or dark-colored bottles, to avoid the degradation and loss of color due to effects of light, temperature, and oxygen.

In the phycoerythrin of *Pseudanabaena* sp. sodium chloride, sucrose, calcium chloride, and citric acid (4 mg/mL) were used. The citric acid was the best preservative between 0 and 35 °C at pH 7 for 45 days. The amount of phycoerythrin remaining was 46 and 38% at 35 °C (Mishra et al. 2010).

Additionally, the PE and PC of the cyanobacterium *Nostoc* sp. HKAR-2 strain improved stability in the presence of edibles preservatives, benzoic acid, citric acid, sucrose, ascorbic acid, and calcium chloride over 30 days of incubation at 4, 25, and 40 °C. The best preservative was benzoic acid, the average lifetime was 100 and 86 days at 25 and 40 °C, respectively, at pH 7 (Kannaujiya and Sinha 2016).

In the case of the PC *Spirulina platensis* is stable at pH 5–6, low temperature, dark, and with the addition of edible stabilizers. Sucrose, glucose, and sodium chloride were added to PC solutions at pH 5–8 at a concentration of 20% (w/v) of each of the compounds and were incubated at 65 °C for 30 min, the best condition was at pH 5 with NaCl (Wu et al. 2016).

Recently was reported, that the halophilic *Euhalothece* sp. which tolerates temperatures up to 45 °C and is highly stable at pH 5–8. After 2 h of incubation at 45 °C, 80% of C-PC remained in solution. The addition of sodium azide and citrate preservatives at 4 °C improves preservation for up to 42 weeks (Mogany et al. 2018).

The phycocyanin of *Spirulina platensis* was stabilized using different sugars, such as methylglyoxal (MGO), which modifies the microenvironment of the chromophore by interacting with apoprotein. The stability of phycocyanin improved by doing crosslinking with MGO, present in high concentrations in honey Manuka honey, but when testing with conventional honey (with lower MGO content), no differences in stability were observed, suggesting that sugars (mono and disaccharides) present in honey, are responsible for the stability of phycocyanin. The presence of 62% fructose has the same effect as honey in the stabilization of phycocyanin. The stability

of phycocyanin correlates with the concentration of glucose, sucrose, or fructose (Martelli et al. 2014).

Another polysaccharide used in *Spirulina* phycocyanin to prevent its thermal and proteolytic degradation is beet-pectin (anionic). This compound improved the stability of phycocyanin at 65 °C and protects the pigment from proteolysis by alcalase, papain, and bromelain. The complexation of phycocyanin with beet pectin produces an increase in the exposure of tryptophan residues, which interacts with aromatic ferulic acid groups of the beet pectin, suggesting that a negatively charged environment in which the chromophore is immersed, may be important in the stability of the color of phycocyanin (Selig et al. 2018).

In accordance with the above, in the work of Hadiyanto et al. (2018), the protein portion of phycocyanin from *Spirulina* sp., binds to sugars (fructose, sucrose, and glucose) via N-linked glycosidic, increasing its stability. Phycocyanin was more stable with glucose than with sucrose or fructose at 40, 60, and 80 °C for 60 min.

Interestingly, phycocyanin from *Spirulina platensis* was subjected to HTST (high-temperature short time) at 74 °C for 1 min at pH 5–7, maintaining 96% of the phycocyanin concentration. This is of interest if phycocyanin or phycoerythrin is added to dairy products that must be pasteurized before consumption. According to this study, the addition of glucose 20%, sucrose 20%, and NaCl 2.5% increase the stability of phycocyanin at pH 7 at 60 °C. However, this would imply adding a high concentration of sugars and salt in foods (Chaiklahan et al. 2012).

The use of SDS detergent has been used to increase the stability of PC from *Spirulina* 1% w/w (commercial *Spirulina* contains 5% tri-sodium citrate, 55% d-trehalose) was mixed with 0.7% w/w SDS to achieve PC-SDS solutions at pH 2 and 6 exposed at 65 °C for one hour. The anionic micelles SDS stabilize the non-protonated (blue) forms of phycocyanin and prevent the formation of protonated forms (green) at low pH. The phycocyanobilin chromophore is stabilized by hydrophobic interactions remaining inside the micelles (Falkeborg et al. 2018).

Recently, the PC of S*pirulina* sp. have been microencapsulated using spray drying method in a range of 90–130 °C using maltodextrin and k-carrageenan as the coating material. In this work it was observed that at 110 °C there was good encapsulation yield, efficiency, moisture content (3.2% and PC concentration (1.46%) (Purnamayati et al. 2018). Maltodextin and carrageenan maintain the PC color due to the formation of gels that protect the PC from degradation. In addition, maltodextrin and alginate contribute to bulk density and alginate and carrageenan prevent moisture loss from the microcapsule (Dewi et al. 2018).

The PC of *Spirulina* sp. was microencapsulated using the spray-dried technique and applied as blue colorant in jelly candy. The PC was encapsulated using maltodextrin and Na-alginate as coating materials at a temperature of 80 °C. PC 0–5% concentrations were used, the concentrations influenced the color, ashes, and moisture content. The jelly candy with 5% PC showed strong blue color, but with a high percentage of moisture in the microcapsule (Dewi et al. 2017).

22.6 Concluding Remarks

Microorganisms and algae are the main sources of wonderful natural pigments. The interest in the search for alternative sources to synthetic dyes has made pigments such as carotenes and phycobiliproteins the focus of scientific studies that show their structural characteristics, toxicity, antioxidant properties, and stability to support and encourage their use with food and analytical grade in cosmetic, food, and pharmaceutical industry.

The blue phycocyanin pigment of algae and cyanobacteria is commercially appreciated because blue pigments are difficult to find in nature. The disadvantage of natural pigments is their low stability due to exposure to high temperatures, light, pH, humidity, and therefore loss of color. Due to the above, the addition of edible preservatives has helped improve the stability of phycobiliproteins as well as the recent use of microencapsulation in confectionery, pastry, and dairy products.

The challenge in the field of phycobiliproteins is to improve the stability at high temperatures for its use in food in order to guarantee food safety. Another point of interest, in the study of phycobiliproteins, is the increasing knowledge about biochemistry and molecular biology of these pigments in microorganisms that are able to grow in extreme environments. This information undoubtedly opens a field of research to improve the stabilization of phycobiliproteins using molecular tools and their subsequent application in biotechnology.

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Chapter 23
Nutraceutical and Pharmaceutical Applications of Phycobiliproteins

Emmanuel Manirafasha, Li Guo, and Keju Jing

Abstract For eradication of current pressing human health challenges including the chronic disease, there is a need for microalgae exploitation. Microalgal resources have various advantageous and potentials associated with their products and properties. Microalgae including cyanobacteria are considered as a promising, prominent, and sustainable source of enormous novel bioactive compounds including nutraceutical and pharmaceutical products such as phycobiliproteins. Phycobiliproteins (PBPs) are water-soluble pigments with widespread biotechnological applications due to their mesmerizing physicochemical properties. Phycobiliproteins can be classified into three categories based on some properties: phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC). Microalgal phycobiliproteins, especially phycocyanin, have commercial and industrial applications due to their primary functions including nutraceutical and therapeutic values (due to their pharmacology and biological activities including anticarcinogenic, antioxidative, anti-inflammatory activities, as well as, protective effect against various conditions), over-the-top fluorescent properties (high quantum yield, high Stokes shift, and an essential insensitivity to quenching), and natural colorants. This chapter focuses on highlighting and describing the microalgal phycobiliproteins potentials and modern technologies regarding their nutraceutical and pharmaceutical applications.

Keywords Microalgae exploitation · Nutraceuticals · Pharmaceuticals · Pigments

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23.1 Introduction

Nutraceutical also referred to as functional food, is nutrient/food that has properties of health-promoting or disease-preventing including chronic diseases. The word "nutraceutical" is derived from **nutr**ition and pharm**aceutical** words (Nasri et al. 2014; Pandey et al. 2013). The microalgal technology is a promising and sustainable source of wide broad bioactive compounds with nutraceutical and pharmaceutical applications. Phycobiliproteins (PBPs) are one of those functional ingredients produced by microalgae resources and applied in different industrial fields including food (for example, they are applied as food colorants), nutraceutical (for example, they are applied as free-radical scavengers, antioxidants), therapeutic (for example, they are applied as anti-aging, anti-Alzheimeric, anti-cancer), cosmetic (for example, they are applied as lipstick, sun protecting cream, eyeshadow pallets), pharmaceutical (for example, they are applied as anti-inflammatory, neuroprotective, hepatoprotective), and biotechnology (for example, they are applied as protein markers for gel electrophoretic, PBPs derived conjugates) (Sonani et al. 2016) due to their potent biological and pharmaceutical properties (Manirafasha et al. 2016). In other words, the nutraceutical products are the substance with physiological health benefits that may generally improve health through body growth and health maintenance by delaying the aging process and preventing, controlling, and treating various illnesses (Nasri et al. 2014). This chapter focuses on nutraceutical and pharmaceutical applications of (PBPs) where it highlights and describes the potentials of (PBPs).

23.2 Why There Is an Urgent Need of Developing the Potent Nutraceutical and Natural Pharmaceutical Compounds

The world is pressed by chronic diseases that are increasing worldwide, especially in lower and middle-income countries (Wilcox 2004, Landrigan et al. 2016). Chronic diseases also referred to as non-communicable diseases (NCDs), are the leading global cause of morbidity, disability, and account for 70% of deaths worldwide (WHO 2014, 2017). Chronic diseases are mainly caused by unhealthy food (i.e., poor nutrition), lack of physical activity, tobacco use, excessive alcohol consumption, and environmental pollution (WHO 2017; Bezner 2015; Landrigan et al. 2016). Diet-induced diseases count for the largest chronic disease epidemic in the human population (Anthony 2009). The chronic conditions are not just affecting people with chronic diseases; they are also affecting the world economy and sustainable development; the burden of family, which has to take good care of the sick person, to the community, to the country, and finally worldwide (Chaparro-Díaz 2016; Schirnding and Yach 2002).

The WHO developed a global action plan for prevention and control of NCD and the WHO in collaboration with its member states established a time-bound set of nine

voluntary global targets to be attained by 2025. They are aimed "*to reduce harmful use of alcohol, insufficient physical activity, salt/sodium intake, tobacco use, and hypertension, halt the rise in diabetes and of obesity, and improve coverage of treatment for prevention of heart attacks and strokes*" (WHO 2014). Regrettably, NCDs progress monitor 2017 reported that despite many government political commitments to prevent and control NCDs, these commitments are still insufficient to meet the Sustainable Development Goal (WHO 2017). Apart from affecting human health, NCDs affect the world economy; for example, the cumulative economic losses are estimated at US$ 7 trillion during 2011–2025 (WHO 2014). Thus, it is evident that the prevention and control of chronic diseases will contribute to the achievement of sustainable development (WHO 2005). One strategy to prevent and control chronic diseases is to eat fruits, vegetables, and wholegrain foods due to their nutritional contents with abilities to boost the body's immune system and self-healing, thus leading to the reduction of the development of those diseases. In other words, nutrition is a key part of life, disease prevention, cancer treatment, and illness recovery (Byers et al. 2002).

The safe, nutritious, and healthy food production and supply should be one of many priority strategies and policies for development and implementation in each country for tackling NCDs. New studies publicized that a diet with essential nutrients and enriched with bioactive food compounds (such as polyphenol, omega-3 polyunsaturated fatty acids, and phytochemicals) can prevent, control, as well as treat NCDs (Warburton et al. 2018; Hennig et al. 2018). Unfortunately, climate change has threatened agriculture, which in turn leads to the lack of enough nutrient-rich foods including fruits and vegetables (Gillespie and Bold 2017). There is a need to invest significantly in an ordinary agriculture supportive strategy, which will contribute to the production of enough nutrient-rich foods. Therefore, this chapter highlights the linkage between algal-derived phycobiliproteins as potent nutraceutical and pharmaceutical agents, and chronic disease prevention and control. Based on algal macro and micro-nutritional contents, adequate integration of algal-derived functional foods and nutraceuticals into food security and nutrition will contribute to the improvement of human health, thus leading to prevention and control of NCDs. For example, algal resources contain antioxidant constituents, including phytonutrients such as phycobiliproteins, superoxide dismutase (SOD), with abilities to scavenge the free radicals and reactive oxygen species (ROS) in the body that have been reported to play a role in many diseases including heart diseases, cancer, hypertension, diabetes, and atherosclerosis (Lee et al. 2007).

Moreover, free radicals and ROS play an important role in aging, another reason why it is crucial to eat food that contains nutrients that can scavenge them for a healthy life (Simm and Brömme 2005; Naidoo and Birch-Machin 2017; Ahsanuddin 2016). There is also a need for well-established systems for monitoring and evaluating the linkage between nutrient-rich food production, nutrition, and human health. Those systems require a strong inter-collaboration between food producers, researchers, nutritionists, health practitioners, policymakers, food industry technicians, and others that will help establish adequate interconnected research, strategies, and policies based on optimal nutrition for optimal health and healthy living. The collaboration

will reduce the gap between research outcomes and their implementation for the betterment and well-being of global human health. Also, it will facilitate monitoring and share information related to NCDs.

Antimicrobial resistance is another growing menace to global human health (Daglia 2012) that needs urgent preventive action, where the effort and awareness of every person (CDC 2017a, b) are required to reduce the usage of antibiotics, otherwise it will keep causing premature deaths and continue to deteriorate the world economy, and be hindrance to sustainable development. For example, in 2013, the CDC estimated that, in the United States, more than two million people are annually afflicted with at least 23,000 dying as a result of antibiotic-resistant infections (CDC 2013).

The single most important origin factor of antibiotic resistance is the overuse of antibiotics in human treatment and livestock production as a disease preventer and growth promoter (CDC 2017b). Antibiotic resistance is spread into the environment and food chain through two main channels: human wastes (including domestic waste and pharmaceutical industry waste), misuse of antibiotics in livestock, aquaculture, and crops (Wongsuvan et al. 2018).

The misuse of antibiotics in food production is the main factor contributing to antibiotic resistance (Sprenger 2017). Most disease-causing microorganisms are living in the environment. Once the antibiotics are discharged in the environment, especially water resources, those microorganisms become familiar with the antibiotic, then through natural and genetic adaptation, they become resistant to antibiotics as discussed by Walsh (2000).

The introduction of antibiotics was considered as a new era in modern medicine. Antibiotics are necessary critical tools to protect people against infection (CDC 2017a), but it is necessary to properly use the necessary antibiotic at the right time, in the right dose, and patients' encouragement to take them as indicated by the physician. These precautions and awareness can help fight antibiotic resistance, reduce unnecessary side effects from antibiotics, and help make sure life-saving antibiotics will work when we need them in the future (CDC 2017a).

Adequate policies, action plans, and strategies have to be regularly established, implemented, and monitored to stop antibiotic resistance. First, doctors, nurses, veterinarians, and other health workers are urged to avoid unnecessary prescription of antibiotics and to prevent infections by promoting wash and sanitation and employing vaccines where appropriate. Furthermore, they should pay more attention to human patient and animal treatment to prescribe appropriate antibiotics when necessary. Second, farmers and agriculture practitioners should only give antibiotics to their animals for controlling and treating infections; there are alternative ways such as clean and uncrowded conditions and vaccination of animals, use healthy feed that contains prebiotics (as feed) can reduce the need of antibiotics. Third, governments and development organizations are urged to set policies and strategies to tackle antibiotic resistance such as the promotion of sanitation, rules, and regulations concerning supply and usage of quality medicines, availability and affordability of essential antibiotics to be used when necessary, training about the usage and side effects of overuse of medicines. Furthermore, governments should develop their national

antimicrobial resistance action plans and put more financial support into research and development of new antibiotics and antibiotic alternatives. Fourth, researchers, pharmacists, and engineers, among others are urged to research and develop new antibiotics with the ability to treat the new infections (Sprenger 2017).

The WHO suggests that a new and more global approach is needed. Therefore, a global collaboration among various organizations and institutions (including the WHO, pharmaceutical industry, universities, and governments, among others) is also needed for the creation of a global antibiotic research and development facility (Sprenger 2017). It is in the same line that; this chapter highlights that algal-derived resources can contribute in several ways at once to the reduction of antibiotic use. The first way algal-derived resources can contribute to the reduction of antibiotic resistance is through the production of algal-derived functional food and nutraceuticals. Safe and nutrient-rich food can boost body self-healing and auto-protection (i.e., immunity). If the nutrient-rich food helps to prevent and control various diseases, it will contribute to the reduction of illness cases (i.e., the implication of reduction of antibiotic prescription and usage), hence leading to the reduction of antibiotic resistance. The second way algal-derived resources can contribute to the reduction of antibiotic resistance is the removal of antibiotics from the environment through wastes and wastewater treatment (Abdel-Raouf et al. 2012). Gentili and Fick (2017) investigated whether pharmaceutical pollutants in urban wastewater can be reduced during algal cultivation; the results show that algal cultivation can remove partially or entirely pharmaceutical pollutants including antibiotic Clarithromycin. Yu et al. (2017) also investigated the algal removal of antibiotic ceftazidime and its basic parent structure 7-aminocephalosporanic acid, and the results showed that 92.7% and 96.07% of both compounds were removed after algal treatment, respectively. The third way algal-derived resources can contribute to the reduction of antibiotic resistance is through the development and production of new antibiotics and/or new potent antimicrobial agents as accessories to antibiotic therapy (Daglia 2012), with the ability to fight new infections, due to their bioactive compounds with various pharmacological activities (Borowitzka 1995; Mimouni et al. 2012; Thomas and Kim 2011) including antimicrobial effect (Eom et al. 2012; Thomas and Kim 2011); and physiological activities (Manirafasha et al. 2016). The fourth way algal-derived resources can be used is to feed fish and animal as prebiotic feeds; in that case, they will contribute to the reduction of using antibiotics in livestock. Algal-derived products used as animal and fish feed in agriculture and aquaculture, respectively, will promote the animals' self-healing and protection against various infections, and speed up animal growth as growth agents, thereby leading to the elimination of the overuse of antibiotic in livestock.

Governments' intervention and investment in wastes and wastewater treatment as an efficient way to reduce pharmaceutical pollutants in the environment, especially water bodies, will contribute to the reduction/eradication of antibiotic resistance. Besides human health benefits, algal resources play a role in animal and fish feeds, as well as environmental bioremediation. Unfortunately, still, there is a big gap between research findings and algal resource applicability to solving pressing real-world challenges due to some challenges.

23.3 A Glance at Phycobiliproteins (PBPs)

Microalgae resources are regarded as sustainable sources of bioactive compounds with various industrial applications. Phycobiliproteins are one of those compounds with several biological and pharmacological properties thus making them potentials assets with a wide range of industrial applications (see Fig. 23.1). Phycobiliproteins are a group of water-soluble fluorescent pigment proteins accumulated under different culture conditions by microalgae, especially *Arthrospira* (*Spirulina*) *platensis*, though heme metabolism. PBPs upstream process in the microalgae cells followed by downstream processes (including cell disruption, extraction, and purification) for high purity PBPs (Manirafasha et al. 2016). PBPs are mainly classified into three main classes based on their color and absorption properties: phycoerythrin (PE, red color and maximum absorbance at 565 nm), phycocyanin (PC, intense blue color and maximum absorbance between 615 and 620 nm), and allophycocyanin (APC, bluish-green color and maximum absorbance at 652 nm) (Pandey et al. 2013). Phycocyanin is major pigment among the phycobiliproteins protein group that consists of two subunits α (approximatively 18kDA) and β (approximatively 19 kDa) subunits chains bound to phycocyanobilin and form αβ monomers; αβ monomers form trimmers $(\alpha\beta)_3$, and hexamers $(\alpha\beta)_6$ aggregates (Stanic-Vucinic et al. 2018). Phycocyanin is also characterized by intense and very stable blue color (Jespersen et al. 2005). The intense blue color and fluorescence features of PC originated from linkage to phycocyanobilin molecules covalently attached to cysteine-84 of α-subunits and Cysteine-82 and-153 residues of β-subunits.

The various applications of PBPs rely on their nontoxic characteristic and imperative biological and pharmaceutical activities including antioxidative, anticarcinogenic, anti-inflammatory, anti-aging, protective effect against various conditions, as well as particular spectral features of over-the-top fluorescent properties (high

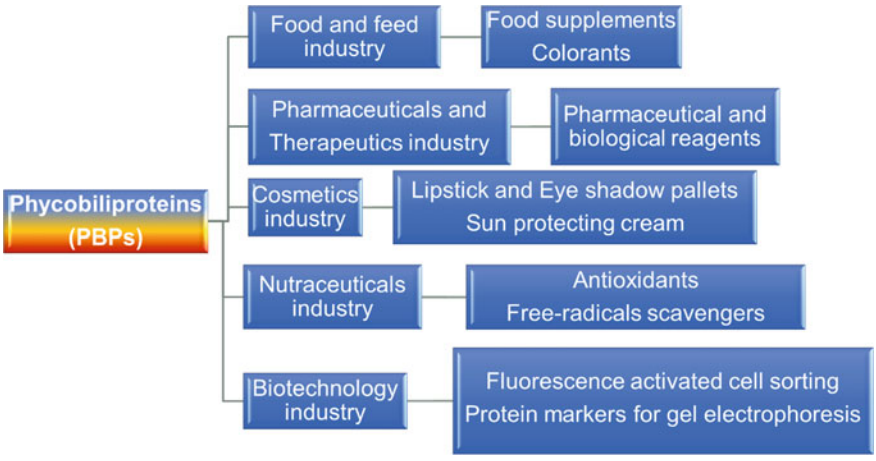

Fig. 23.1 Various industrial applications of microalgae-derived phycobiliproteins

quantum yield, high Stokes shift, and an essential insensitivity to quenching), and natural colorants (Sonani et al. 2016). The quality of PBPs also determines their applications. For example, the phycocyanin with purity grade less than 0.7 is known as food grade and used in the food industry, phycocyanin with purity grade of 3.9 is used as a reactive agent while the purity grade greater than 4.0 is applied as an analytical grade (Kuddus et al. 2013). The purity grade is determined by calculating the absorption ratio (absorption of phycocyanin at 620 nm to the absorption of total proteins at 280 nm.

23.4 Phycobiliproteins for Nutraceutical and Pharmaceutical Applications

The climate change conditions, unhealthy food, tobacco, and alcohol consumption may be the leading cause of the increase of harmful free-radicals production in the body. In turn, the overproduction of free radicals leads to oxidative stress, which is one of the leading causes of pressing human body health challenges including chronic diseases (Pandey et al. 2013). Those harsh conditions increase the new cases of chronic diseases and the costs of related health care. Nutraceutical compounds are considered as a natural and sustainable solution to achieve the prevention, control, and treatment of various diseases with minimal or no side effects. Furthermore, the approach of utilizing natural nutraceutical compounds as health-promoting products is also expected to reduce the health care costs, and improve health quality and lifestyle.

It is in the same line that PBPs are applied as potent nutraceutical and pharmaceutical compounds to support the body self-healing and to manufacture new pharmaceutical products with the potential to prevent and cure the new infections. Different scientific studies reported a variety of biological and pharmacological activities (such as antioxidant, anti-inflammatory, anti-cancer, hepatoprotective, free-radicals scavenger, among others) of PBPs that impeccably impel them, and sustainable health-promoting agents (Romay et al. 2003; Stanic-Vucinic et al. 2018). The PBPs exhibit the strong ability for scavenging the harmful free radicals (for example, peroxynitrile due to interaction with apoprotein residues such as tryptophan and tyrosine), thus leading to the promotion of body health and life maintenance including prevention of oxidative stress-induced diseases such as cancer. The scavenging ability of PBPs, especially phycocyanin is linked to its prominent antioxidant property. For example, Renugadevi et al. (2018) evaluated PC extracts for their antioxidant property through various methods: phosphomolybdenum essay, ferric ions reducing power essay, hydrogen peroxide free-radical scavenging activity, anti-lipid peroxidation activities; the results from those essays revealed that PC pigments possess a prominent antioxidant property where the efficiency of hydrogen peroxide scavenging activity reached 95.27%. Apart from scavenging the harmful free radicals, PBPs have other potent physiological and pharmacological activities. PBPs are used

in nutraceutical and pharmaceutical industries for two main purposes: the first target as a natural and nontoxic colorant, and second target as a therapeutic agent. Phycocyanin (PC) has the most application in the above-mentioned industries. PC to be used in the pharmaceutical industry should have high quality with purity ratio at least 4.0, this remark indicates that the PBPs efficiency also depends on the applied production processing (Dejsungkranont et al. 2017).

The PC is commercialized and consumed in a different form, particularly as powder, capsules, and tablets. In some cases, the PBPs are used to fortify other foodstuffs such as biscuits, bread, pasta, dairy products, bioactive drinks, among others. The recent scientific reports have proven that phycobiliproteins, especially PC, can be taken for therapeutic effects including antioxidant (Renugadevi et al. 2018), improvement of immune system functions, anticancer effect (Ravi et al. 2015; Jiang et al. 2018), anti-inflammatory effect (Romay et al. 2003), antimicrobial effect, different organs (liver, kidney) protection (Niu et al. 2017),

Therefore, PBPs are natural phytochemical compounds with potent pharmacological properties with chronic diseases-protective activities that can be used in pharmaceutical and nutraceutical industries. Even though the PBPs exhibit a prominent health promotion, there are still some challenges such as their availability and affordability (García et al. 2017). The main challenges are also associated with technology for the production of high-efficiency natural nutraceutical compounds. For example, the stability of PBPs in aqueous solution is still questionable due to their interaction with the light and denaturation by high temperature at various pH values.

23.5 Conclusion

It is inevitable to pay attention to nutraceutical and pharmaceutical agents from microalgal resources. The microalgal resources can produce a broad range of bioactive compounds with health benefits within a short time, without interfere with the conventional food crops. Furthermore, microalgal resources production does not destroy the environment and ecosystem but they participate bioremediation.

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Chapter 24
Diagnostic Applications of Phycobiliproteins

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Abstract Phycobiliproteins (PBPs) are widely used as fluorescent detection system in both medical and non-medical diagnostics. This chapter focuses research arena with extensive coverage of patents and commercial products. Being unique and brilliantly colored proteinaceous photosynthetic accessory pigments of algal kingdom, they were mainly employed in the diagnosis of human metabolic disorders, infectious diseases, cancers, and immune disorders. It was further extended to the prognosis of few human diseases too. They find novel applications in human theranostics either as fluorescent detection component or both as detection system and therapeutic molecule. Apart from medical diagnosis, they were largely used in the diagnosis of cell components, cell lineages and subsets, and other biological and environmental probing too. It was further extended to the diagnosis of pathogenic diseases in birds and mammals. There are available extensive patented research on the development of methods of using them as detection probes in the form of molecular tags, protein conjugates, fusion proteins, antibody conjugates, and with DNA probes. Their incorporation into micro beads, magnetic beads, and biochips were elaborate. Globally, several companies market diagnostic products of PBPs in the form of preparations like streptavidin conjugates, activated PBPs, fluorescein isothiocyanate (FITC) cross-linked forms, purified and activated forms, antibody conjugates, and as multi-color detection systems including custom designing. Among various phycobiliproteins, phycoerythrins find common application followed by allophycocyanin and phycocyanin. Generation of novel hybrids and conjugates coupled with improvement in signal generation and amplification that helps in early diagnosis are in the offing.

Keywords Phycoerythrin · Allophycocyanin · Phycocyanin · Fluorochrome · Fluorescent detection · Diagnostic patents

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24.1 Introduction

Diagnosis denotes the act of distinguishing or discerning among possibilities. Though it is commonly used in conjunction with medical, it rather includes non-medical problems too. It broadly refers to the examination of root causes for any problem and aimed to promote remedial measures. The major aspect of diagnosis is on the identification of or nature of an illness by examination of certain root causes or biomolecular signatures associated with the disease. The brilliantly colored proteinaceous pigments from algal kingdom, namely phycobiliproteins (PBPs) have fine application as a component of diagnostics in terms of aiding investigation as fluorescent detection moieties in medical, cellular, biological, and non-medical fields. In medical diagnosis, the application further extends to the development of "prognosis" and "theranostic" aids representing prediction of diseases and synchronizing diagnosis with treatment, respectively. Though the research literature on diagnostic applications of PBPs is limited, there are extensive activities of patenting and commercial ventures. Hence review of research will be merely indicative of certain diagnostic applications but may not be comprehensive in revealing the depth of understanding that exists in this field. To avoid this vacuum, this chapter presents the pile of information from strategic patent mining incorporating research articles and ongoing commercial activities in the field of diagnostic applications of PBPs. The patent analysis was carried out from data retrieved from public databases of global nature using a wide range of general, specific, and combination of keywords of this domain. The body of information from relevant patents coupled with literature was segregated into various categories.

24.2 Kinds of Diagnostics Developed Using PBPs as Fluorophores/Fluorochromes/Fluorescent Probes/Fluorescent Detection Labels/Fluorescent Markers/Detection System

Analysis of research literature and patents revealed the extensive use of PBPs as a fluorescent detection system in diverse fields of diagnosis. It is widely used in the diagnosis of various human ailments. For example, diagnosis of an array of human metabolic disorders/syndromes, infectious diseases, cancers, and immune disorders. Additionally, they are used in the prognosis of a few human diseases and also in human cancer theranostics. Similarly, diagnosis of human cell components, lineages, and cell subsets were also performed using PBPs. Their application is also extended to the diagnosis of many infectious diseases in birds and mammals which may pose a health risk to human beings. These pigments also find application in various biological diagnostic investigations involving proteins, genomics, plant transformants, microbial detection, food and environmental aspects. Finally, a pile of information and knowledge is available over the development of methods of using PBPs as a detection system exploiting their fluorescent behavior.

24.2.1 Diagnosis of Human Metabolic Disorders/Syndromes

Usage of PBPs as detection system in the diagnosis of many metabolic disorders/syndromes in human remains exhaustive. For example, functional ability of platelets in thrombosis and hemostasis could be diagnosed by the expression of Integrin αIIbβ3. Its expression was low on resting platelets. This diagnostic system used R-phycoerythrin conjugated with monoclonal antibody (mAb) against Integrin αIIbβ3 (Bergmeier et al. 2002). Alzheimer's disease was diagnosed by detecting the amyloid protein (Aβ) employing the fluorochrome phycoerythrin (PE) (Patel and Good 2007). Amyloid is the primary protein component of senile plaques in Alzheimer's disease. Similarly, down-regulation of CD5 expression on activated $CD8^+$ T cells in hemophagocytic lymphohistiocytosis was identified using immunofluorescence kit containing the corresponding antibody conjugated phycoerythrin and other dyes (Wada et al. 2013).

However, usage of PBPs particularly phycoerythrin in most of the cases was well documented among patents of various countries designed to diagnose a range of human illness (Table 24.1). Diagnosis of fitness of human body, common ailments, and major diseases of cardiac, brain and other central nervous system (CNS), multi-organs of the body, reproductive system, and other health issues were extensively worked out and patented exploiting PBPs (Table 24.1). It was illustrated that diagnostic kits to assess nutritional status, obesity, multiple pathology, and hereditary issues were profound using PBPs as an adduct system. Similarly, cardiac ailments like arrhythmia, myocardial infarction, cardiovascular diseases, etc., could be detected. Brain and other CNS ailments such as Alzheimer's, amyloid diseases, schizophrenia, and nerve imaging to detect neurodegenerative disorders were performed. In the case of multi-organ ailments, kits used for detecting problems of more than one organ were presented like a combination of pulmonary and cardiac ailments, cardiac and kidney, kidney and cardiovascular diseases, etc. Diseases pertaining to various organs like lung, kidney, liver, ear dysfunction, reproductive system, and certain hereditary diseases were addressed among diagnostic patents using PBPs as a detection component (Table 24.1).

24.2.2 Diagnosis of Human Infectious Diseases

Comparable to the utility of PBPs for the diagnosis of human metabolic disorders, an equivalent quantum of exploitation of these pigments for the diagnosis of human infectious diseases was made. Detection of food borne pathogen, *Escherichia. coli* O157:H7 in ready to eat food was done using polyclonal antibodies conjugated to R-phycoerythrin (Subires et al. 2014) On-chip nested multiplex amplification in microfluidic channels for analysis and detection of respiratory viruses from nasopharyngeal sample was investigated using streptavidin-conjugated R-phycoerythrin and real time-polymerase chain reaction (RT-PCR) probing (Ritzi-Lehnert et al. 2011).

Table 24.1 Patents on the diagnosis of human metabolic disorders/syndromes using PBPs as fluorophores

Disease/diagnostic parameters	Patents-source/country and number
(A) *Diagnosis of fitness or general ailments*	
Assessing nutritional and health status	IN 20187047753
Obesity and related disorders by adiponectin receptor	IN 4981/DELNP/2008
Detection of increase in the level of human tumor necrosis factor (TNF) -α indicating disease	US 8414894
Disease related to genetic alterations	US 5665540
Multiple pathologies by toll-like receptors	IN 201617043688
Detection of sensitivity to pain (algesia)	IN 6327/CHENP/2012, IN 6328/CHENP/2012, IN 7203/CHENP/2012
Detection of pain generators	US 9757476, US 10098970
Skeletal muscle injury	US 8748104
Hereditary angioedema type III (skin swelling on limbs, face, arms, etc.)	IN 4249/CHENP/2006
Mountain sickness	CN105385777 (E), CN105296631 (E), CN105296630 (E), CN105219872 (E)
(B) *Cardiac ailments*	
Cardiac arrhythmia	IN 4322/DELNP/2009
Diastolic heart failure	IN 421/DELNP/2009
Acute myocardial infarction	CN106950381 (E), CN101271108 (E)
Acute coronary syndrome	CN103163295 (E), CN103376313 (E), IN 1278/KOLNP/2009, CN102062735 (E)
Cardio vascular disease/cardiac failure	CN101825627 (E), IN 201741004864, IN 201838007033, IN 2807/CHENP/2008
Absolute blood flow	KR 1020177012463
(C) *Brain and other central nervous system-related ailments/assessments*	
Alzheimer's disease (β-peptide formation & cognitive impairment)	US 6770448, CN10246164 (E), CN107238711 (E), IN 1174/MUMNP/2012, IN 148/KOLNP/2010, IN 595/MUMNP/2012, IN 6811/CHENP/2013
Amyloid associated diseases (Due to Type II Diabetes mellitus, Alzheimer's disease, prions, etc.)	IN 380/CHENP/2006
Schizophrenia (mental disorder)	CN108387743 (E), IN 227/MUMNP/2011
Multiple neurodegenerative disorders	IN 201717043447, IN 201717043449
Diagnosis of fragile X mental retardation 1 protein causing Autism	US 2014127724
Nerve imaging	US 8361775, JP2014111614 (E)

(continued)

Table 24.1 (continued)

Disease/diagnostic parameters	Patents-source/country and number
Distinguishing nerve-tissue during surgery	JP2014111614 (E)
(D) *Multi-organ ailments*	
Pulmonary hypertension and cardiac dysfunction	IN 201617043331
Cardiac and kidney diseases using signal peptides	IN 201818036486
Damage to kidney and cardiovascular diseases	JP 2005-527813
Diabetes mellitus and associated cardiac diseases	IN 6924/DELNP/2010
Renal and periodontal diseases	IN 201817032598
Risk of atherosclerosis and stroke by lipoprotein phospholipase A2 marker	CN104820097 (E)
Lysosomal storage disorders	US 7615224
(E) *Ailments of other organs/body components*	
Chronic Obstructive Pulmonary Disorder (COPD)	IN 1489/KOLNP/2015
Kidney disease	CN 109154621
Liver fibrosis (necrosis of hepatocytes)	CN101178403 (E), IN 2894/DELNP/2004
Non-Alcoholic Fatty Liver Disease (NAFLD)	IN 201937011234
Inflammatory bowel disease	IN 3401/CHENP/2008
Ankylosing spondylitis (arthritis of spine & joints)	IN 695/MUMNP/2008
Bone metabolic marker	CN101221171 (E)
Assay of thyroid hormones	US 6423549, AU 2002255788
Congenital Adrenal hyperplasia	KR 1020070056018
Gall stone formation	US 8406859
Susceptibility to ocular disorders (exfoliation syndrome and glaucoma)	IN 146/DELNP/2010, IN 6616/DELNP/2010
Perilymph fistula (inner ear dysfunction)	US 9458210
Drug-induced deafness (e.g., antibiotics)	CN108486242 (E)
Hereditary deafness due to gene mutation	CN106480222 (E)
Gingival crevicular fluid (tissue inflammation around teeth)	CN103823054 (E)
Vaginal atrophy (post-menopause)	IN 201617042609, IN 201617043154
(F) *Reproductive ailments*	
Spontaneous preterm delivery	IN 201817045768
Detection of amniotic fluid in vagina to avoid prenatal risk	IN 5175/DELNP/2007, IN 965/DELNP/2005

(continued)

Table 24.1 (continued)

Disease/diagnostic parameters	Patents-source/country and number
Risk of preterm births	IN 679/MUMNP/2012
Premature rupture of fetal membrane	CN103267851 (E)
Fetal cells in maternal blood	US 5766843, US 5858649, US 5861253, IN 9840/DELNP/2015
Testicular spermatogenic function	CN107315092 (E)
(G) *Miscellaneous issues*	
Transplantation—MHC (Major Histocompatibility Complex) allo antibody testing for irreversible or terminal organ failure	IN 201811016151
Numerical chromosomal abnormalities—aneuploidy and polyploidy	IN 993/DELNP/2004
Detection of fish allergen—parvalbumin	CN102680674 (E)
Detection of insulin sensitivity enhancers	US 5466610
Exposure/immunity to dengue virus	IN 4605/KOLNP/2010

Abbreviations: AU—AusPat database, Australia; CN—CNIPA database, China; (E)—Espacenet database, European Patent Office; IN—InPASS database, India; JP—J-Plat Pat database, Japan; KR—KIPRIS database, Korea; US—USPTO database, United States of America
Patent numbers represent application numbers except numbers with prefix "CN" and suffix "(E)" that denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

Immunotyping of human papillomavirus using monoclonal antibody bound phycoerythrin in the form of luminex microsphere employing multiplexed assay system was developed (Opalka et al. 2003). Monitoring the efficacy of drug, anti-retroviral therapy (ART) in human immunodeficiency virus (HIV) patients was performed using phycoerythrin and fluorescein isothiocyanate (FITC) conjugated anti-CD38 monoclonal antibodies (Onlamoon et al. 2011).

However, the major exploitation of PBPs for such of these diagnostics resides in patents (Table 24.2). There were patents covering the diagnosis of any pathogenesis in human body by analyzing suitable marker proteins and inflammatory responses. Similarly, diagnosis of diseases caused by many bacterial pathogens and their toxins along with some fungal pathogens employing suitable antigens as markers for detection was performed. However, an array of viral diagnostics were prevalent among patents. Additionally, diagnosis of a group/combination of bacterial, fungal and viral pathogens, parasitic worms, and prions were also patented (Table 24.2).

Table 24.2 Patents on the diagnosis of human infectious diseases employing PBPs as fluorochromes

Disease/diagnostic parameters	Patents-source/country and number
(A) *General diagnostics*	
Microbial infections	US 6649356, US 7875426, JP3336406 (E), CN103131760 (E), IN 201617035849, IN 7254/CHENP/2009
Sepsis in infants	US 5830679
Septicemia	CN101246163 (E)
Quantification of pathogens in blood	US 8795633
Detection of localized infection by imaging the infiltration of inflammatory cells	IN 4497/KOLNP/2009
Bacterial and viral pathogens by acute inflammation	IN 201817043067
Detection of any pathological condition by human leukocyte antigen (HLA)—G expression	IN 201817047794
Hand-Food-and-Mouth disease	CN101629214 (E)
Tick borne pathogens	CN 103173567
(B) *Diagnostics of bacterial pathogens*	
Pseudomonas aeruginosa infection	US 5716829
Multidrug resistant *P. aeruginosa*	CN104313166 (E)
Mycobacterial infection	JP 2003-534810
Mycobacterium tuberculosis infection	CN101216491 (E), IN 201717037365, IN 3761/KOLNP/2008, IN 624/KOLNP/2011
Monitoring of tuberculosis upon therapy	US 9651552, US 9874561
Clostridium difficile toxins	IN 3239/CHENP/2006
Staphylococcus aureus	IN 201637031568
Drug resistant *Enterococcus* spp.	IN 835/CHENP/2010
Multidrug resistant *Acinetobacter baumannii*	CN107619871 (E)
Determination of protective antigen of anthrax pathogen	RU2478970 (E)
(C) *Diagnostics of fungal pathogens*	
Corneal pathogenic fungi	CN1014349933 (E)
(D) *Diagnostics of viral pathogens*	
Human Immunodeficiency Virus (HIV) infection	US 5108904, US 5156951, US 5597688, AU 1991081528
Monitoring HIV specific T cell responses	IN 8394/DELNP/2014
Human Papilloma Virus (HPV) infection/genotyping	US 7067258, US 7910883, US 8017757, US 8389217, KR 1020000013161, KR 1020120130910, KR100382703 (E), KR100541916 (E), KR20040083674 (E), RU2008139313 (E), IN 3104/DELNP/2007

(continued)

Table 24.2 (continued)

Disease/diagnostic parameters	Patents-source/country and number
Japanese encephalitis virus	US 9783596
Hepatitis B Virus (HBV) genotyping	US 9927439, US 10067135, KR 1020040002964, CN101144815 (E)
Hepatitis C Virus (HCV) genotyping	KR20040083940 (E), IN 9851/DELNP/2008
Influenza virus genotyping	US 8003314, JP2009-523451, CN103131794 (E), IN 3721/KOLNP/2008, IN 4765/DELNP/2010, IN 6266/DELNP/2008
Drug resistant Influenza virus	US 9874562
Dengue hemorrhagic fever	IN 161/DELNP/2013
Respiratory viruses	US 8003314, US 8137921, US 8975016, US 10088481
Respiratory syncytial viruses	US 7875426, US 8003314
West Nile and other viruses	US 7384785, US 7901883
Rabies and other Lyssaviruses	IN 201817042625
Foot-and-mouth disease viruses	KR 1020160168915, IN 201831029609
Viral meningoencephalitis	CN102643928 (E)
Rash and fever viruses	CN102876807 (E)
Insect/mosquito borne viruses	WO2014067390 (E), CN108660253, CN104120191 (E), CN102943128
Many intracellular viruses	CN101504414 (E)
(E) *Diagnosis of a group/combination of pathogens*	
Pathogens of Sexually Transmitted Diseases (STD)	KR 1020130012930
Respiratory pathogens	CN107488748 (E)
Bacterial pathogens of female genital organ	IN 2420/MUMNP/2012
Combination of bacterial and fungal pathogens	CN101504414 (E)
Many viral pathogens and *Mycobacterium tuberculosis*	IN 201818046493
(F) *Diagnosis of other pathogens*	
Parasitic worm infections	US 9588116
Prions—proteinaceous infections agents	US 8372593

Abbreviations: AU—AusPat database, Australia; CN—CNIPA database, China; (E)—Espacenet database, European Patent Office; IN—InPASS database, India; JP—J-Plat Pat database, Japan; KR—KIPRIS database, Korea; US—USPTO database, United States of America

Patent numbers represent application numbers except numbers with prefix "CN" and suffix "(E)" that denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

24.2.3 Diagnosis of Human Cancers

In succession to the diagnosis of human pathogenic and non-pathogenic diseases, a wide variety of human cancers could be detected employing PBPs as a component of detection. In one such embodiment, Chronic lymphocytic leukemia was diagnosed by CD5– FITC/CD19- phycoerythrin immunostaining (Gupta et al. 2004) where FITC was a cross-linker. The detection of liver cancer using the biomarkers, α-fetoprotein and carcinoembryonic antigen was made by the recombinant fusion protein, streptavidin-phycobiliproteins (SA-PBPs) in Sandwich ELISA. Streptavidin is a protein obtained from *Streptomyces avidinii* (Ge et al. 2017). Human breast cancer was diagnosed with fluorescein isothiocyanate-conjugated anti-human leukocytic antigen antibody and a phycoerythrin-conjugated anti-mouse pan-leukocyte CD45 antibody (Allan et al. 2005). Detection of colorectal cancer with microarray of surface protein profiles and multiplexing with fluorescently labeled (phycoerythrin) antibodies against particular cell types. Evaluation of heterogeneity of human leukocyte antigen (HLA) class I expression in solid tumors like cervical tumor was done. It involved simultaneous measurement of normal stromal cells (vimentin positive), inflammatory cells (CD45 positive), epithelial cells (keratin positive), and DNA content readily using R-phycoerythrin, allophycocyanin (APC) along with two other dyes (Corver et al. 2000).

In order to differentiate the leukemic B cell chronic lymphoproliferative disorders by quantitative flow cytometry, phycoerythrin-conjugated CD19, CD20, CD22, CD23, CD79b, and CD5 monoclonal antibodies and pre-calibrated beads were used to calculate the number of antigen molecules per cell (D'Arena et al. 2000). It resulted in the discrimination of chronic lymphocytic leukemias, prolymphocytic leukemias, hairy cell leukemias, splenic lymphomas with villous lymphocytes, and mantle cell lymphomas. Plasmonic chip (based on plasmon resonance) which is a metal coated substrate with grating structure was known to provide enhanced fluorescence supporting immunosensing and bioimaging and used in the sensitive diagnosis of breast cancer. In living breast cancer cell lines, multi-color stained with 4′,6-diamidino-2-phenylindole (DAPI) for nucleus and allophycocyanin-labeled anti-EpCAM antibody, to stain epithelial cell adhesion molecule (EpCAM) contained in the cell membrane was made (Tawa et al. 2016). Drug (imatinib mesylate) induced proteomic changes in leukemia cells was analyzed using streptavidin phycoerythrin conjugate as the fluorescent detection system (Stuchlý et al. 2012).

Commensurate with previous cases of applications, these pigments were vitally used in patents on the diagnosis of human cancers too (Table 24.3). It includes the detection of cancer or tumor in general along with organ-specific and blood-related cancers and certain cancer-related assessments. In organ specific, diagnosis of cancers of pancreas, liver, colorectum, oral and nasopharyngeal, lungs, bones, ovary, cervix, and prostate were frequent. Among blood-related cancers, diagnosis of leukemia, lymphoma, and myeloma are prominent. Additionally, many follow up cancer assessments were also performed and patented (Table 24.3).

Table 24.3 Patents on the diagnosis of human cancers applying PBPs as fluorescent probes

Disease/diagnostic parameters	Patents-source/country and number
(A) *Cancers in general*	
Tumor/cancer/multiple tumor markers	US 8071293, US 8354222, US 8361706, US 8535639, US 8940493, US 10018633, JP 2010-193876, JP 2016-029048, KR 1020047013664, KR 1020047020362, CN101526535 (E), TW200501989 (E), CN101144815 (E), IN 20167027880, IN 201727001584, IN 201817042344, IN 201838002349, IN 1019/CHENP/2014, IN 150/MUMNP/2014, IN 1966/DELNP/2010, IN 4262/DELNP/2005, IN 4566/KOLNP/2007, IN 6320/CHENP/2011, IN 6597/DELNP/2015, IN 9120/DELNP/2008, IN 928/CHENP/2006
Tumor-specific fusion proteins	US 7993850
Genomic abnormalities leading to pathology and cancer	IN 4049/DELNP/2010
Pro-vasopressin expressing cancers	US 7790162, US 10155810
(B) *Organ-specific cancers*	
Pancreatic cancer	US 7052859, US 7282567, US 8795662, US 8974784, US 9238084, US 9599619, IN 4773/CHENP/2012
Pancreatic adenocarcinoma	US 9238081, IN 4612/DELNP/2012
Hepatocellular carcinoma	US 9506925, CN109085362 (E), CN 107271661
Colorectal cancer	US 8563682, CN100342034 (E), CN 1766615
Oral and nasopharyngeal cancer	IN 1921/DELNP/2011
Lung cancer	CN103383395 (E), CN103439511 (E), CN103389375 (E), CN103305602 (E)
Mesothelioma (Asbestos-related lung cancer)	IN 635/KOLNP/2013
Cancer bone metastasis	CN101571545 (E)
Breast carcinoma	US 8329875, CN 1012013570, IN 201641017874, IN 201717038976
Ovarian cancer	US 7462458, US 8252904, IN 4156/CHENP/2008, IN 450/KOLNP/2007
Ovarian and breast cancer	IN 48/MUMNP/2010
Cervical cancer	US 7067268, US 7901883, US 8017757, US 8389217, IN 2414/CHENP/2007, IN 3382/DELNP/2015, IN 4185/KOLNP/2007, IN 554/CHENP/2005
Prostate carcinoma	US 8691183, US 9891224, CN 101942523
(C) *Blood-related cancers*	

(continued)

Table 24.3 (continued)

Disease/diagnostic parameters	Patents-source/country and number
Leukemia	KR100759289 (E)
Leukemia fusion gene	CN203021568 (E), WO2011120398 (E), WO2011157222 (E), CN101838682 (E), CN 101955995, CN 101955991
Stem cell cancer	IN 636/DELNP/2015, IN 7062/CHENP/2012
B cell leukemia	CN103018463 (E)
Polycythemia vera (slow growing blood cancer)	BG1777 (E)
Acute leukemia and lymphoblastic lymphoma	IN 8730/DELNP/2007
Pediatric acute lymphoblastic leukemia	IN 2963/DEL/2013
Monitoring of multiple myeloma	RU2639382 (E)
(D) *Other cancer assessments*	
Recurrence of blood cancer after surgery	RU2017105638 (E)
Prognosis of survival time of solid tumor patients and treatment responsiveness	IN 201817045553
IF-α producing T-lymphocytes in chronic lymphatic leukemia	RU2526797 (E)
Phosphorylation status of retinoblastoma (eye cancer)	US 6821740

Abbreviations: CN—CNIPA database, China; (E)—Espacenet database, European Patent Office; IN—InPASS database, India; JP—J-Plat Pat database, Japan; KR—KIPRIS database, Korea; US—USPTO database, United States of America

Patent numbers represent application numbers except numbers with prefix "CN" and suffix "(E)" that denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

24.2.4 Diagnosis of Human Immune Disorders

Parallel to the previously reported cases of diagnostics, detection of human immune disorders using PBPs tags was also patented extensively. In this case, diagnosis of general immune disorders, antibody-mediated immune disorders, and certain assessments of immunity were patented (Table 24.4).

Table 24.4 Patents on the diagnosis of human immune disorders employing PBPs as fluorescent detection labels

Disease/diagnostic parameters	Patents-source/country and number
(A) *General immune disorders*	
Autoimmune diseases	US 6596501, US 7674632, US 7771932, CN1866013 (E), IN 4492/DELNP/2015, IN 5860/DELNP/2009
Inflammation	US 8116984
Allergy	US 7491553, CN103454412 (E), CN 1866013, IN 4492/DELNP/2015
(B) *Antibody-mediated immune disorders*	
IgE related diseases	US 7491553, US 9823248
Rheumatoid arthritis (autoimmune attack of joints)	JP 2005-527813, KR 1020080036215, IN 2179/MUMNP/2013
Systemic lupus erythematosus (autoimmune attack on body tissues)	IN 2013/MUMNP/2012, IN 6790/DELNP/2015
Antiphospholipid antibodies	US 5840587, US 7867723
Antinuclear antibody	CN 108802375 (E)
Antihistone antibodies	US 8987421, US 10040848
Autoantibodies to blood group antigens	CN 101365946
Autoantibodies to platelet	CN107422111(E), CN101246173 (E)
Autoantibodies to drugs	IN 7324/DELNP/2013
(C) *Immunity oriented assessments*	
Screening of transplant recipients	US 9528988
Immune status after transplantation	US 9448229
Detection of therapeutic responsiveness to immune disorder	IN 8029/DELNP/2009
Follicular regulatory T cells of peripheral blood that reduce autoimmunity	CN108982339 (E)
CD4 lymphocytes/T-cell counting	US 8541227, US2004110122 (E)
TNF—α expression	US 8414894
Cell secretion of cytokine promoting antitumor and antiviral abilities	CN107796944 (E)

Abbreviations: CN—CNIPA database, China; (E)—Espacenet database, European Patent Office; IN—InPASS database, India; JP—J-Plat Pat database, Japan; KR—KIPRIS database, Korea; US—USPTO database, United States of America

Patent numbers represent application numbers except numbers with prefix "CN" and suffix "(E)" that denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

Table 24.5 Patents on the prognosis of certain human diseases applying PBPs as fluorochromes

Diseases prognosed	Patents-source/country and number
(A) *Cancer in general*	
Circulating rare cells denoting cancer	US 9000130
Cancer by *p53* status	IN 3233/DELNP/2007
Solid tumor	IN 3794/DELNP/2007
(B) *Organ-specific cancers*	
Colon pre-cancer (dyspepsia)	US 8901276
Colorectal cancer	IN 6372/DELNP/2008
Pancreatic cancer	US 8795662
Breast carcinoma	US 8329875
Urinary bladder cancer	IN 229/MUMNP/2011
(C) *Susceptibility to autoimmune diseases*	US 5356779

Abbreviations: IN—InPASS database, India; US—USPTO database, United States of America
Numbers with prefix 'IN' represent application number and prefix 'US' represent publication numbers patents. Numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database

24.2.5 Prognosis of Certain Human Diseases

Prognosis of diseases is a precautious and preventive approach which safeguards the health of individuals. It requires basic understanding of markers that can act as indicators of disease prediction. By using PBPs as fluorescent molecular tags, mostly certain types of cancers were prognosed (Table 24.5). Among those patents, some of them were prognosis of cancers in general using circulating rare cells and *p53* gene as markers. Similarly, organ-specific cancers could also be prognosed apart from susceptibility to autoimmune disorders.

24.2.6 Human Theranostics

Theranostics is advantageous in terms of disease detection coupled with therapy. They can be target specific and confer efficacy in treatment. In this type of application too, PBPs also find application as detection agents. In one such embodiment, usage of nanomedical platforms having multimodality in both diagnosis and treatment was envisaged. Single wall carbon nanohorns (SWNHs) were used as theranostic system

Table 24.6 Patents on human theranostics using PBPs as fluorophores

Diseases theranosed/theranostic tool	Patents-source/country and number
Bispecific antibodies and drug delivery	US 7560110, IN 201717043418
Ly75 membrane protein target as cancer theranostic	IN 3537/DELNP/2015
Tumor cell identification and drug delivery	US 7238786
Colorectal cancer diagnosis and drug delivery	US 8563682
Pancreatic cancer theranostic	US 8414894
Rheumatoid arthritis theranostic	KR 1020080036215
Acute myeloid leukemia theranostic	IN 6324/DELNP/2007

Abbreviations: IN—InPASS database, India; KR—KIPRIS database, Korea; US—USPTO database, United States of America Numbers with prefixes 'IN' and 'KR' represent application numbers and prefix 'US' represent publication numbers of patents. Numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database

in cancer satisfying the dual role of targeting and killing cancer cells with phycobiliproteins. Phycocyanin (PC) conjugated nanohorns were effective in imaging to visualize cancer progression and efficacy of treatment in vivo. In this case, it is interesting to note that phycocyanin was known to mediate the production of Reactive Oxygen Species (ROS) photodynamically to kill cancer cells (Lin et al. 2019). Similarly, many cancer theranostics were patented applying PBPs (Table 24.6). It is common to find the usage of bispecific antibodies which carry both the diagnostic system and drug for treatment.

24.2.7 Human Cellular/Cell Subset Diagnostic Applications

It is vital to note that certain cellular and subcellular features could be diagnosed using PBPs as markers. To cite, identification of antigen-specific T cells using enzymatically biotinylated tetramers of major histocompatibility complex (MHC) coupled with mutant streptavidin-phycoerythrin conjugate was made. Such T cells find application as an essential tool in vaccine development, tumor immunology, autoimmunity, etc. (Ramachandiran et al. 2007). Subset analysis of leukocyte of new world monkeys was performed using the fluorochrome phycoerythrin (Brok et al. 2001). Co-localization of subcellular molecular interaction of macromolecules (like protein, transcription factor, DNA, etc.) using three-dimensional multi-color

Table 24.7 Patents on human cellular/cell subset diagnostic applications of PBPs as fluorescent markers

Diagnostic parameters	Patents-source/country and number
Identification of nucleated cells	US 6329158
Method and device for the isolation and diagnostic analysis of single cell	IN 2555/MUMNP/2014
Subpopulation of blood cells	US 4727020
Identification of stem cells	US 7223549
Lineages of hematopoietic cells	US 5137809
WBC subset analysis	US5994089 (E)
T cell/Th cell subset analysis	US 5206143, US 4677061, US 5085985, US 5206143, US 10156562
Detection of 2 types of CD26 markers in T cells	US 6265551
NK cell subset analysis	US 4599304, US 4607007, US 5786160

Abbreviations: (E)—Espacenet database, European Patent Office; IN—InPASS database, India; US—USPTO database, United States of America
Numbers with suffix "(E)" and prefix "US" represent publication numbers and prefix "IN" represent application number of patents. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

fluorescence microscopy employing multiple fluorochromes of different excitation and emission wavelengths have been performed with phycoerythrin and other fluorescent dyes (Abraham et al. 2010). Alike to these, identification of cellular attributes like nucleated cells, stem cells, etc., could be patented exploiting the exhibition of fluorescence by PBPs (Table 24.7). Further, patents pertinent to subset analysis of cells like white blood corpuscle (WBC), T cells, and natural killer (NK) cells were patented with specific reference to pigments such as phycoerythrin.

24.2.8 Diagnosis of Animal Infectious Diseases

It is crucial to note that the application of phycobiliproteins was not only restricted to human diagnostics but also extended primarily to the infectious diseases of animals that are closer to humans and thus pose health risks. In mice fecal pellets, diagnosis coupled with quantification of *Cryptosporidium parvum* oocysts using monoclonal antibody OW50, specific to the cell wall of oocysts conjugated to superparamagnetic particles, to fluorescein isothiocyanate and to R-phycoerythrin was performed (Moss and Arrowood 2001). Among patents, PBPs were widely used to detect bacterial and viral pathogens of poultry and swine in addition to other animals such as monkey, mouse, pig, rat, rabbit, and equines (Table 24.8).

Table 24.8 Patents on the diagnosis of animal infectious diseases employing PBPs as fluorochromes

Disease/diagnostic parameters	Patents-source/country and number
Detection of poultry viruses	CN107991482 (E), CN106290867 (E), CN106544447 (E), CN106282417 (E), CN106191312 (E), CN106011313 (E), CN106286241 (E), CN101975856 (E), CN106282416 (E)
Bursal disease in poultry	IN 3709/CHENP/2007
Poultry respiratory pathogens	CN106191319 (E)
Chicken mycoplasmosis	CN106222256 (E), CN106191312 (E), CN106011313 (E)
Detection of porcine (pig) viruses	CN105154589 (E)
Porcine parvovirus	CN108918870 (E)
Monkey viruses	CN106636461(E)
Respiratory viruses of mouse (murine)	WO2019001187 (E), CN106566897 (E), CN106755589 (E), CN106636474 (E), CN103173566 (E)
Guinea pig viruses	CN106191311 (E)
Canine (dog) viruses	CN106319091 (E)
Rat viruses	CN106167836 (E), CN106755589 (E)
Rabbit viruses	WO2018233448 (E), CN 107326098
Detection of Burkholderia mallei in equines (horse) and other animals	IN 3610/DEL/2015

Abbreviations: (E)—Espacenet database, European Patent Office; IN—InPASS database, India
Numbers with suffix "(E)" represent publication numbers and prefix "IN" represent application number of patents. For numbers with prefix "IN," numbers alone (without prefix alphabets) have to be entered in the suitable search field of the database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

24.2.9 Varied Biological Diagnostic Applications

Most of the diagnostic applications of phycobiliproteins are in the realm of human diseases. However, their diagnostic applications are expanded further to many other biological aspects and cellular analysis apart from environmental issues. Evaluation of post-thaw viability and acrosome integrity of bovine spermatozoa was performed employing the lectin, peanut agglutinin which was conjugated with phycoerythrin using the cross-linker FITC. PNA binds specifically to the outer acrosomal membrane thus represents viability and integrity of sperms (Nagy et al. 2003). Detection of aspirin resistance among long-term users after carotid endarterectomy was performed using phycoerythrin-labeled anti-P-selectin (CD62p) antibody assessing the reduction in platelet activity by aspirin (Assadian et al. 2007). mRNA abundance of p21 and proliferating cell nuclear antigen indicating diseased state was performed. For which

RT-PCR labeled with both digoxigenin and biotin during amplification was done. Subsequently, amplicons were simultaneously bound to anti-digoxigenin microparticles and fluorescently labeled with streptavidin-R-phycoerythrin (Wedemeyer et al. 2000).

Aberrant expression of various antigens by leukemic blasts or lymphoma cells was assessed performing fluorescent assay using monoclonal antibodies conjugated with phycoerythrin (Gratama et al. 1998). Detection of apoptosis in cultured cells was performed by four-color flow cytometric assay. In which, the nucleated cells were selected with CD45-phycoerythrin conjugates. Further, detection of lymphocytes, apoptosis and distinguishing early and late apoptosis were performed using other dyes (Hasper et al. 2000). Quantifying melanosome transfer from melanocytes to keratinocytes and surface of skin was investigated using phycoerythrin as a tool. It employed double label with FITC-conjugated antibody against the melanosome-specific protein gp100, and with phycoerythrin-conjugated antibody against the keratinocyte-specific marker cytokeratin (Ma et al. 2010). Detection of intracellular signaling of cytokines by dimerization of interleukin, IL-7 receptor could be detected using specific antibodies conjugated with phycoerythrin (Andersen et al. 2013). Measurement of intracellular signaling proteins using calibrated standardized phycoerythrin-conjugated beads was performed which had potential applications in monitoring efficacy of targeted therapy (Chang et al. 2010).

It is interesting to note that applicability of phycobiliproteins is expanded to the environmental issues too. Detection of diarrhetic shellfish poisoning toxin, Okadaic acid (OA) produced by toxigenic dinoflagellates was performed using magnetic beads coated with streptavidin. It prevents hazard to public health (Pan et al. 2018). Using R-phycoerythrin-AgNPs (silver nanoparticle) construct, detection of Cu^{2+} ions based on the quenching effect between R-PE-AgNPs and Cu^{2+} ions, in combination with the fluorimetric method was made (Xu et al. 2019).

In the case of patents, the diagnostic potentials of PBPs were exploited in the investigation of proteins and proteomics, genomics, transgenomics, microbial detection, food safety, and environmental aspects (Table 24.9). For example, studies pertinent to the interaction of proteins, detection of enzymes and receptors, nucleic acid sequences, genetic polymorphism, analysis of transgenes in plants, authentication of microbial detection, and analysis of food contaminants were patented wherein phycobiliprotein fluorescence detection systems were coupled.

24.2.10 Development of Methods of Using PBPs as a Detection Component of Diagnostic/Prognostic/Theranostic Products

Though the aforesaid research literature and patents also include the methods of using phycobiliproteins in each one of the applications, there are exclusive work and patents on the development of methods in terms of usage of phycobiliproteins,

Table 24.9 Patents on varied biological diagnostic applications of PBPs as fluorescent probes

Diagnostic parameters	Patents-source/country and number
(A) *Proteins/Enzymes/Receptors*	
Detection of single biomolecules like protein	IN 7968/DELNP/2008
Detection of interacting proteins	US 4764462, US 7413862
Quantification of protein	US 5536382
Detection of β-lactamase in samples	US 4764462
Detection of telomerase protein	IN 7980/DELNP/2007
Protein detection to replace western blot	US 7049151
Enumeration of receptors in blood cells	US 5814468
Detection of Fc gamma R1 receptors in IgG	US 6146837
Screening of nucleic acid ligase modulators	US 6153384
(B) *Genomics*	
Quantification of nucleic acid	US 6060240
Amplification of nucleic acid sequences	US 6977148
Detectable label for nucleic acid sequences (molecular beacons)	US 5976802, US 6060240, US 7211414, US 7220847, US 7468261, US 7541165
Gene polymorphism of cytochrome P450	CN102399899 (E)
(C) *Plant biotechnology*	
Typing of Bt proteins in Genetically Modified (GM) crops	CN105092853 (E), CN 102288769
Detection of germline transformants in soybean	IN 7701/DELNP/2015
(D) *Microbial detection*	
Eubacterial detection	US 5935804, US 6090573
Pan-fungal and pan-bacterial identification	US 6159719
Detection of Tobacco Mosiac Virus (TMV)	CN 1715924
(E) *Food/Environmental aspects*	
Detection of nitrofuran (antimicrobial) in animal food	CN103884837 (E)
Detection of aflatoxin B1	CN108732346 (E), CN108508214 (E)
Detection of soybean trypsin inhibitor for adulteration of soybean in surimi food products	CN103087197 (E), CN103087197 (E)
Oxidation resistance of food	CN 107741414
Plural small molecular harmful residues (drugs, pesticides, and additives)	CN101871937(E)

Abbreviations: CN—CNIPA database, China, (E)—Espacenet database, European Patent Office; IN—InPASS database, India; US—USPTO database, United States of America
Patent numbers with prefix "IN" represent application numbers and prefix "CN" and suffix "(E)" denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

creation of protein conjugates, fusion proteins, adducts, linking of antibodies with PBPs, preparation of micro beads and magnetic beads coated with PBPs, and linking of these pigments with DNA probes. Patents pertaining to the development of such methods are listed in the global context (Table 24.10). It primarily includes patents from United States Patent and Trademark Office (USPTO) dealing with core methods of developments followed by many patents from China National Intellectual Property Administration (CNIPA), China.

The following types of preparations containing PBPs as detection component were commonly encountered among them.

Recombinant fusion proteins

The recombinant fusion protein, streptavidin-phycobiliprotein conjugates was produced in *E. coli* by combinational biosynthesis. There were two recombinant fusion proteins—SA-PCA-PEB (streptavidin-phycocyanin α subunit-phycoerythrobilin), and SA-PCA-PCB (streptavidin-phycocyanin α subunit-phycocyanobilin). Purification of these conjugates was efficient using biotinylated substances due to the higher affinity of streptavidin to biotin. This combination served the dual purpose of purification and fluorescence detection. These unnatural recombinant phycobiliproteins were cross-linked products of phycobiliproteins with phycobilins and exhibit improved fluorescence property (Ge et al. 2017). Another recombinant fusion protein containing streptavidin and allophycocyanin α-subunit (holo-ApcA) was biosynthesized in *E. coli* by a dual plasmid system. The rate of chromophorylation of the fusion protein with the prosthetic group phycocyanobilin (PCB) was increased with an in vitro chromophore attachment reaction system containing PCB and lyase gene *cpcS* yielding strong signal for detection (Wu et al. 2018). A Similar system of fusion protein with tandem repeats of allophycocyanin holo-α-subunits to improve brightness of fluorescence has been carried out (Chen and Jiang 2018). Flow cytometric evaluation of coupled microspheres of anti-IgG-PE and anti-HBsAg-PE conjugates was performed for quality control (de Almeida Santiago et al. 2016).

Magnetic beads

Preparations based on magnetic beads ease handling and purification using a magnetic system in which the beads were coated with streptavidin. The shellfish toxin, Okadaic acid was biotinylated which in turn binds to the magnetic beads by the attraction of biotin to streptavidin. OA bind with the anti-OA monoclonal antibodies which in turn bind with the secondary antibody IgG coupled with R-phycoerythrin. This preparation thus facilitates fluorescence detection (Pan et al. 2018). Such magnetic particle-based detection method and tool using phycoerythrin was developed (Leigh et al. 2005).

Micro beads

Performance evaluation of polystyrene beads (0.86 μm) of phycoerythrin in terms of quantity of antibodies bound per cell was made (Pannu et al. 2001).

Table 24.10 Patents on the development of methods of using PBPs as a detection component of diagnostic/prognostic/theranostic products

Country/union and database	Patent numbers
United States Patent and Trademark Office (USPTO)	US 4542104, US 4666862, US 4708931, US 4745285, US 4822733, US 4857451, US 4859582, US 4520110, US 5068178, US 5093234, US 5171846, US 5501952, US 5571680, US 5652093, US 5695928, US 5714386, US 5716784, US 5798276, US 5807879, US 5863401, US 5891738, US 6133429, US 6280618, US 6503702, US 6828109, US 6960478, US 7014839, US 7081336, US 7179658, US 7205160, US 7256050, US 7271265, US 7399591, US7465538, US 7511811, US 7556932, US 7674589, US 8354239, US 8632994, US 9383353, US 10209245
Korea Intellectual Property Rights Information Service (KIPRIS)	KR 1020140055649, KR 1020150126029, KR 1020170076403
European Patent Office (Espacenet- World Wide Database)	US5891741, CN106706898, CN 1657912, JPH079426, US2004142380, CN101408541, CN 101824426, CN 101824427, CN101824425, CN101824428, CN 101750482, CN 101412996, CN101469332, CN101469331, CN 101397554, CN 101397557, CN101397556, CN104313089, US 5171846, CA 2114837, US 5783673, US5272257, WO9405701, CN 101759788, CN 101759795, CN101759794, CN102308214, US 2004009559, EP 0509718, WO2017198334, US6020212, JP 2007112803, CN 101759793, CN101759792, CN101759791, US 6423549, CN 105018552, CN106916839, CN101838661, CN 108341904, CN 101481698, CN101475952, CN101463356, CN 101475950, CN 101481701, CN101481700, CN101481699, CN 101475951, US 2001055783, CN108841846, CN106596920
China National Intellectual Property Administration (CNIPA)	CN107064490, CN101759796, CN101759790, CN105962947, CN108414740, CN10677949, CN1721446, CN108037103, CN101560558, CN101139587, CN101121931, CN101412998, CN101412996, CN104313089, CN104372053, CN102731644, CN101759787, CN102127059, CN101759786, CN101759785, CN101759793, CN101759792, CN101759791, CN101759789, CN101759797, CN105018552, CN106916839, CN101838661, CN103992402, CN108101980, CN103667406, CN101144815, CN101980020, CN101481700, CN101481701, CN101481699, CN101475949, CN101475951, CN101481697, CN108841846, CN108148140, CN106596920

(continued)

Table 24.10 (continued)

Country/union and database	Patent numbers
Indian Patent Advanced Search System (InPASS)	IN 201717043828, IN 201817038711, IN 201847009727, IN 1282/KOL/2014, IN 5201/CHENP/2008, IN 6024/DELNP/2011, IN 6819/DELNP/2013

Abbreviations: CN—CNIPA database, China; (E)—Espacenet database, European Patent Office; IN—InPASS database, India; KR—KIPRIS database, Korea; US—USPTO database, United States of America
Patent numbers represent application numbers except numbers with prefix "CN" and suffix "(E)" that denote publication numbers. Except Espacenet, numbers alone (without prefix alphabets) have to be entered in the suitable search field of the corresponding country's database. Such prefix alphabets are meant for identifying the database. In Espacenet alone (tagged with "(E)" as suffix of numbers), given alphabetic prefix along with the publication numbers have to be fed to access patent details

Nucleic acid probes (molecular beacons)

Nucleic acid probes recognize and base pair with complementary nucleic acid targets. They undergo fluorogenic conformational changes upon pairing with targets. It specifically pairs with perfect complementary targets but not with mismatched nucleotides. These are called as "molecular beacons" and denote fluorescence-quenched nucleic acid probes. They emit fluorescent signal upon hybridization with target molecules. They have dual label—one with reporter fluorophore at one end and a dark quencher at the opposite end of the probe. In such kind of molecular beacons, phycoerythrin, allophycocyanin, and phycocyanin were employed as fluorophores (Bao et al. 2006).

A variety of diagnostic kits have been designed exploiting products of PBPs. They were based on the excitation and emission properties of PBPs. The presence of a variety of PBPs enabled multi-color and multiplex detection system in the form of microarrays. The pigments in the diagnostic kits and products were of various forms such as micro beads, magnetic beads, micro fluidic channel, RT-PCR system, DNA array, Aptamer based kit, biochips, liquid chips, suspension chip, multiplex liquid array system, streptavidin conjugates, conjugate with antibodies, fusion protein, activated PE, molecular beacons (detection of nucleic acid sequences), etc.

24.3 Commercial Trends

Globally, numerous companies are marketing products of phycobiliproteins that can be directly or indirectly used for diagnostic purposes (Table 24.11) but solely as fluorescent detection agents. A search would reveal the complexity of various products developed based on the forms of phycobiliproteins. The details presented in the table are only indicative in terms of the range of companies but not comprehensive. The quantum of PBP products also runs to few thousand in many companies which are mostly USA in origin. Similarly, a vast array of PBP products

Table 24.11 Major companies marketing diagnostic products of phycobiliproteins

Company and country	URL
Thermo Fisher Scientific (Thermo Scientific, Fisher Scientific Applied Biosystems, Invitrogen and Unity Lab services), USA	https://www.thermofisher.com
Rockland Immunochemicals Inc. USA	https://rockland-inc.com
BioLegend, Inc., USA	https://www.biolegend.com
AAT Bioquest, Inc., USA	https://www.aatbio.com
Novus Biologicals, USA	https://www.novusbio.com
Miltenyi Biotec GmbH, Germany	www.miltenyibiotec.com
Europa Bioproducts Ltd, UK	https://www.europa-bioproducts.com
R&D Systems, Inc. Canada	https://www.rndsystems.com
Biogems International, Inc., USA	https://www.bio-gems.com
Agilent Technologies, Inc., USA	https://www.agilent.com
Sigma Aldrich, USA	https://www.sigmaaldrich.com
Becton, Dickinson and Company, USA	http://www.bdbiosciences.com
Fitzgerald Industries International, USA	https://www.fitzgerald-fii.com
AnaSpec, USA	https://www.anaspec.com
Biocompare, USA	https://www.biocompare.com
RayBiotech Life, USA	https://www.raybiotech.com
Vector Laboratories, Inc., USA	https://vectorlabs.com
Dojindo Molecular Technologies, Inc, USA	https://www.dojindo.com
Phyco Biotech, France	https://www.phyco-biotech.com
Abnova, Taiwan	http://www.abnova.com
Expedeon Inc., USA	https://www.expedeon.com
Biotium, Inc., USA	https://biotium.com
Jackson ImmunoResearch Inc., USA	https://www.jacksonimmuno.com
Bio-Rad Laboratories, Inc, USA	https://www.bio-rad-antibodies.com
Abcam, UK	https://www.abcam.com

(continued)

Table 24.11 (continued)

Company and country	URL
Creative Diagnostics, USA	https://www.cd-bioparticles.com

are marketed, for example, streptavidin-conjugated PE & APC, activated PE & APC, custom conjugated PE & APC, cross-linked APC, purified PC & PE, monoclonal antibody conjugates of PE, polyclonal antibody FITC cross-linked PE, peridinin-chlorophyll-PBP complex, SMCC (succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate) activated cross-linked APC, micro bead conjugated PE, magnetic bead conjugated PE, nano bead conjugated PE, dyes conjugated PE & APC, multi-color detection system using PE & APC, etc. Further, a variety of kits are also marketed like labeling kits with PE & APC, conjugation kits with PE, detection kits using PE, etc. Among all these products as well as in patents, mostly PE is employed followed by APC and PC. It indicates the preferential suitability of PE in terms of emission characteristics and amenabilities for modification and conjugation. Most of the products and kits marketed are commonly revealed in patents which indicate the commercial dimension of PBPs for diagnostic purposes as fluorescent tags.

24.4 Conclusion

Diagnostic applications of phycobiliproteins are apparently seems to indicate their role as an effective detection moiety by exhibiting intense fluorescence. Initial trends indicate their usage in the diagnosis of various illnesses of human-like metabolic disorders, infectious diseases, cancers, and immune disorders. But of late, they find their application in prognosis and theranostics also. It is vital to note that the therapeutic properties of phycobiliproteins were coupled with their diagnostic role in some of the theranostics. The dual role in diagnosis and therapy deserves futuristic attention. Further, the ambit of diagnosis is extended to cellular, subcellular, and biological investigations and animal diseases reaching to environmental concerns. Newer and novel hybrids and conjugates of PBPs are in the offing. Similarly, their use is intensified in kits based on antibodies/antigens/receptors/ligands/markers/proteins and traverse as molecular beacons in genomics coupled with improvements in methodology. Their ability as signal amplifiers helps in early diagnosis of diseases which is characterized by low-intensity expression of disease-specific markers. Usage of PBPs as imaging agents rather than chemical dyes is preferential due to the lack of toxicity. Such special attributes of these pigments induce a wave of research coupled with patenting and commercial activities that collectively contribute to the marketing of newer products and diagnostic kits.

Patent databases

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Espacenet database—https://worldwide.espacenet.com
InPASS database—http://ipindia.gov.in
J-Plat Pat database—https://www.jpo.go.jp
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USPTO database—http://patft.uspto.gov

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Chapter 25
Cosmetics and Cosmeceutical Applications of Microalgae Pigments

Ratih Pangestuti, Indyaswan Tegar Suryaningtyas, Evi Amelia Siahaan, and Se-Kwon Kim

Abstract Microalgae are a class of unicellular photosynthetic microorganisms found in aquatic environments (both marine and freshwater). In the marine environment, marine microalgae are the largest primary producers. These organisms are considered among the fastest growing creature in the world and potential resources of new biomass in fuels as well as cosmetics industries. Among bioactive materials identified from marine microalgae, natural pigments have received particular attention and substantial progress has been made in past few decades. This chapter describes the potential applications of microalgal pigments in cosmetics and cosmeceuticals. First, growth characteristics of marine microalgae and the effects of culture conditions on microalgal growth rate are described. Second, development of marine microalgae pigments in cosmetics and cosmeceuticals as well as environmental friendly method to isolate pigments from microalgae are presented. Third, potential applications of microalgae pigments in cosmetics and cosmeceuticals are assessed based on their biological activities. Finally, the pros and cons of microalgal pigments in cosmetics are overviewed.

Keywords Marine microalgae · Pigments · Cosmetics · Cosmeceuticals

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25.1 Microalgae

Ocean is a congested place of abundance biological diversity which offers tremendous resource for novel compounds. Each of them is unique and widely various, yet mostly undiscovered (Pangestuti and Arifin 2017). Microalgae is one of the most overflowing organisms living in this ecosystem, containing potential products such as carotenoids, fatty acids, polysaccharides, vitamins, enzymes, and other potential compounds that need to be discovered. In fact, from more than 50.000 of known microalgae species, only about 3.000 of them have been studied (Mata et al. 2010).

Microalgae is a group of prokaryotic and eukaryotic microorganisms with various cell morphology, ranging from 11 μm to less than a millimeter in size. In a single cell, microalgae is mostly invisible to the naked eye, they only can be seen when in a colony. In nature, microalgae has a symbiotic relationship with bacteria, as seen in Fig. 25.1.

Most of microalgae are autotrophic photosynthetic microorganism, but some have heterotrophic metabolism. Autotrophic microalgae only require inorganic components and light energy to grow, while the other group needs organic components as external source nutrients such as glucose, glutamate, and acetate. Some microalgae perform both metabolisms or sometimes referred as mixotrophy, which means they can do photosynthesis as well as obtain external organic nutrients depend on the environmental condition, for example, light intensity and nutrient availability in their environment (Hamed 2016).

As an omnipresent organism, microalgae is present almost in every water body on Earth. Widespread from freshwater such as lakes, ponds, rivers, reservoirs, to high-saline marine environments (William and Laurens 2010). They are also considered as the main producer of oxygenic layer of Earth's atmosphere (Saad and Atia 2014). Microalgae convert CO_2 into biomass and produce oxygen, provide food and energy for another species in their environment. As their capacity to recycle carbon dioxide, they play an important role in global biogeochemical cycles.

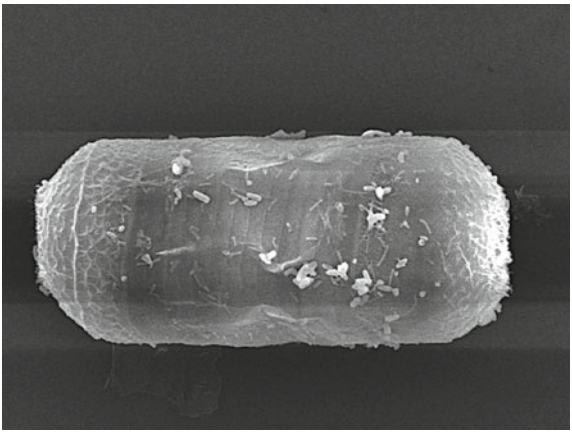

Fig. 25.1 Microalgae symbiotic with bacteria

Marine microalgae as well as another marine organism perform completely different in many biological aspects compared to them which living in terrestrial waters, as an adaptation to their environment (Lordan et al. 2011). Marine microalgae have an interesting potential as a natural producer of bioactive compounds. Cultured microalgae are known to produce intracellular and extracellular metabolites which are still difficult to synthesize chemically. In an optimal condition of temperature, pH, and nutrient in medium, incubation period, light intensity and salinity, diverse biological activities can be performed (Noaman et al. 2004). One of the interesting products of microalgae is pigment. Pigment contained in microalgae has a unique characteristic and function from each species. This has drawn the attention of researchers to study more about microalgae's pigments. This chapter focused only on marine microalgae and its pigment contents especially for cosmetics and cosmeceuticals.

25.1.1 Microalgal-Used History

In the past of thousands of years, microalgae were mostly used only as feed for fish larvae. The earliest documented microalgae for human consumption was reported in China, who consume *Nostoc* sp. during famine event (Spolaore et al. 2006). As a food diet, the most used genera of microalgae is *Spirulina* which started to be consumed traditionally (in small scale) in Maya civilization and by some African tribes in Chad and Niger (Belay 2008). Few decades later, this one celled organism has become a vital material for the needs of human life. People started microalgae biomass production, as they know their potential, especially for health supplements and biodiesel producers (Gouveia and Oliveira 2009). Japan started the first commercial huge scale microalgae biomass production in 1960s. As their company name, Nihon Chlorella, the main product is dried *Chlorella.* Through this company, Japan was the first one to introduce microalgae as health supplement in various forms (tablets, drinks, and food additives) (Blackburn and Volkman 2012). Microalgae based health supplements became more popular by the discovery of high polyunsaturated fatty acid (PUFA), in *Nannochloropsis* sp. Then followed by the establishment of mass scale culture and harvesting facility in Mexico in the early 1970s. The facility produced well-known high nutrient microalgae *Arthrospira* sp. which is commonly referred as Spirulina. The industry of Spirulina is a massive success. Spirulina was known as organic healthy food that have a big potential, so called "superfood" (Blackburn and Volkman 2012). While microalgae's pigments isolation and utilization as cosmetics is still new.

Cyprus is one of the pioneer countries which uses algae as cosmetic's raw material. Due to its extensive water resources, Cyprus has cultured alga in large scale as cosmetics material as well as herbal ingredients. Modern algal-based cosmetics manufacture was established in Japan with *Lina Blue* (*Spirulina* spp. and *Porphyra* spp.) as colorant for eye shadow. Followed by Monaco with Protulines which use *Spirulina platensis* as linoleic acid source; Poland with its product Bielenda which use *Spirulina platensis* as its raw material as antiaging cream and Dermochlorella

which use *Chlorella* spp. as carotenoid source which has a function to stimulate collagen production naturally (Ryu et al. 2015). Nowadays microalgae extracts are frequently found in cosmetics and skin care products as thickening agents, antiaging cream, antioxidants, and colorant of make-up products (Priyadarshani and Rath 2012) Microalgae are also found in UV-protection and hair treatment products.

25.1.2 Microalgae Growth Condition

Microalgae have an efficient and simple metabolism as advantages of becoming unicellular species. Because of that reason, microalgae can grow rapidly and become the fastest growing organism in this Earth. Most of the microalgae reproduce vegetatively by binary division several times depending on the species and environmental conditions. Some species of dinoflagellates takes two or more days to doubling the cell number, and shorter for cyanobacteria (blue-green algae) in which doubling takes place more than once a day (Blackburn and Parker 2005). Living with simple physiological metabolism, microalgae is adapted in extreme environmental conditions such as extreme temperature (heat or cold), anaerobes, high or low salinity, UV exposure, and pollution (Singh et al. 2019).

Even though microalgae has simple metabolism it needs several requirements to keep living. Basic growth requirements are different among autotrophic and heterotrophic microalgae. As a photosynthetic organism, autotrophic microalgae absolutely need light and CO_2 to perform photosynthesis to gain energy. Heterotrophic microalgae can use another organic carbon source. Apart from them, environmental parameters are important to microalgae growth such as, light intensity and wavelength, temperature, salinity, nutrient, and chemical contains. In cultured microalgae, these parameters can be manipulated to gain specific condition. For example, in some microalgae, salinity stress can improve lipid production. Different light wavelengths also effect lipid and pigment production.

Microalgae can be cultured in large scale using two main systems, open air system and photobioreactors (Borowitzka 1999). The uses of these systems are depending on cultured species characteristics, sunlight intensity, climate condition, nutrient and CO_2 supply, and the end product type. In open air system, contamination is one of the main problem in single species culture. Some algal species such as *Spirulina* and *Chlorella*, require specific culture condition, so that these species are a perfect example of species that are commonly cultured in open air system because other microalgae species are hardly contaminating this specific conditions (Wang et al. 2008). Open air system costs lower, and less complex in its construction. This system is perfectly used in tropical area with long beach line, where the climate is stable and humidity is consistent. Open air system also has some disadvantages such as water loss from evaporation that cause increase in salinity. The construction also needs more attention, it has to be shallow enough so that sunlight can not only reach the deeper cell, but also has to be deep enough to complete mixing. The biomass produced by this system is relatively lower than closed system, roughly 0.1–0.5 g

dry weight for 1 L culture (Borowitzka 1999). In closed system, usually known as photobioreactor, microalgae cells are well mixed with nutrients and CO_2, in closed vessel with controlled intensity of light. This system is highly sterile, with controlled parameter conditions, which allowed this system to culture various species of algae without worries about contamination. The productivity of this system is relatively higher than open air system, but it consumes huge amount of energy to operating this system, and of course spend more cost (Hosikian et al. 2010).

In a batch culture, microalgae growth shows a typical growth curve, which is divided into four phases in order to their cell number: lag phases where the growth is still low due to adaptation process, followed by logarithmic phase which means the cell growth is exponential. At this phase, growth rate is relatively high until some parameters become limited, for example, nutrient depletion, or accumulation of metabolic waste. Reaching this condition, growth rate decline leads to stationary phase, followed by death phase when environmental conditions do not meet the growth requirements. Recirculating water culture system can prolong the culture condition, where the metabolic waste can be minimized by treatment and nutrients can be added several times. But most of microalgae culture are intend to harvest at one time, so batch culture is preferable to use than recirculating water system. There are various techniques to perform batch culture, raceway ponds are the most popular and are the preferred production system for the large scale among them, some biggest microalgae companies use this system such as Hawaiian microalgae company, Cyanotech Corporation, Seambiotic in Israel, and Earthrise in California, in which their main business is spirulina in massive raceway ponds (Hosikian et al. 2010).

25.2 Pigments in Microalgae

Microalgae are excellent sources of natural pigments with chlorophylls, carotenoids (carotenes and xanthophylls), and phycobilins as the main types. Chlorophylls and carotenoids are generally fat soluble whereas, phycobilins are water soluble (Pangestuti and Kim 2011). Chlorophylls are greenish natural pigments which contain a porphyrin ring and found in all algae, higher plants, and cyanobacteria. Structurally, chlorophylls are substituted tetrapyrrole with a centrally bound magnesium atom; the porphyrin tetrapyrrole is further esterified to a diterpene alcohol, phytol, to form chlorophyll. There are several types of chlorophylls with differences in some macrocycle peripheral groups, which affect their absorption spectra. Differing with other types of chlorophylls, Chl c lacks the hydrocarbon tail which leads to differences in the absorption spectrum and therefore tonality, as Chl a appears blue green, Chl b brilliant green, Chl c yellow green, Chl d brilliant/forest green, and Chl f emerald green (Mulders et al. 2014). Due to the chemical structures, each chlorophyll showed different stability and Chl a showed better stability than other types of chlorophylls. Chl a is the most abundant pigment in all microalgae. The European current legislation (Regulation (EC) No 1333/2008 and its amendments) has allowed the use of

two natural green colorants from plants and microalgae, Chlorophyll (E140) and Cu-chlorophyll (E141) (Viera et al. 2019). In cosmetics industries Cu-chlorophyll (Cl 75810) is generally classified as non-toxic or harmful by a diverse source of expertise. CI 75810 is typically used in hair color products, color cosmetics, and bleaching products.

Carotenoids are the most ubiquitous and widespread natural pigments. These natural pigments are responsible for the red and brown color of plants, algae (both macro and micro), cyanobacteria, and some aquatic animals. Carotenoids consist of terpenoid which is derived from a 40 carbon polyene chain and may be complemented by cyclic groups and oxygen-containing functional groups. Based on these complementations, carotenoids may be classified into two types: carotenes and xanthophylls (oxygenated derivatives of carotenes). Compared to other types of pigments, carotenoids are more diverse and widely used in many products. Presently, astaxanthin and β-carotene from *Haematococcus pluvialis* and *Dunaliella salina*, respectively have been used in cosmetic products.

Phycobiliproteins are used as accessory or antenna pigments for photosynthetic light collection which absorb energy in portions of the visible spectrum (450–650 nm). In microalgae, phycobiliproteins are arranged in subcellular structures called phycobilisomes, which allow the pigments to be arranged geometrically in a manner that helps to optimize the capture of light and transfer of energy. The colors of the phycobiliproteins arise from the presence of covalently attached prosthetic groups, bilins which are linear tetrapyrroles derived biosynthetically from heme via biliverdin. There are four main classes of phycobiliproteins namely allophycocyanin (bluish green; λ_{max} 650–655 nm), phycocyanin (blue; λ_{max} 615–640 nm), phycoerythrin (red; λ_{max} 565–575 nm), and phycoerythrocyanin (orange; λ_{max} 577 nm) (Begum et al. 2016). Presently, *Spirulina* sp. and *Poprhyridium* sp. are the major producers of phycobiliproteins from microalgae (Rizzo et al. 2015).

25.3 Developments of Microalgae Pigments in Cosmetics and Cosmeceuticals

Increasing awareness on the various environmental problems has led a shift in the way consumers go about their life. In the past few years, there has been a change in consumer attitudes toward a green lifestyle. Consumers are actively trying and participate in reducing their impact on the environment (Cherian and Jacob 2012). In addition, increasing awareness of the environmental hazard of synthetic colors has increased demand for natural and environmentally sustainable products. Natural pigments derived from microalgal biomass have a significant market value in this regard. The demand for natural pigments from microalgae is estimated to rise each year due to the increasing consumer knowledge. Microalgae are viewed as "natural and healthy" by consumers, and this promotes a positive response in consumers, who often regard natural entities (Pangestuti and Kim 2017). Microalgae are the fastest

growing biomass in this planet. In addition, many studies have reported biological activities of marine algae pigments and cosmeceuticals properties of microalgae natural pigments. For example, phycobiliproteins (phycocyanin and phycoerythrin) are used as a natural dye in cosmetic industry in Japan. These natural pigments are considered as safe and non-toxic (Kuddus et al. 2015).

The concentration of natural pigments in microalgae is often too low; hence, microalgal-based natural pigment production is sometimes non-feasible economically. Production of natural pigments from microalgae requires a comprehensive study and knowledge to select a suitable production strain, various pigments present in the microalgae with respect to their in vivo roles and the metabolic pathways involved in their synthesis, suitable cultivation conditions, and a strategy for microalgae strain improvement (Mulders et al. 2014).

25.3.1 Environmental Friendly Technology for Recovery of Pigments from Microalgae

In addition to natural pigments production, industry and scientist should work out in sustainable ways to refine natural pigments derived from microalgae (Siahaan et al. 2017). Organic solvent extraction is the most common technique used to isolate natural pigments from microalgae. Ethanol, methanol, acetone, and ethyl acetate are the most common solvents used for the extraction of natural pigment. However, the volume of solvents used in the chemical process is extremely concerning. Organic solvents are a major contributor to the overall toxicity potential associated with many industrial processes and to the waste generation of chemical industries. The disposal of excessive solvent to the environment significantly contributes to the release of greenhouse gases and other emissions. Therefore, environmental friendly technologies in natural pigments extractions need to be developed. Environmental friendly technologies such as supercritical carbon dioxide (SC-CO_2), microwave assisted extraction (MAE), and ultrasound assisted extraction (UAE) have been demonstrated as potential technologies to obtain natural pigments from microalgae (Pangestuti et al. 2018).

The MAE and UAE, both are energy input-assisted extraction methods and have been used to isolate functional materials for many years. MAE involves the heating process of a solution in contact with a sample using microwave energy. Different from classical heating, microwaves heat the sample simultaneously without heating the vessel. Therefore, the solution reaches its boiling point very rapidly, leading to very short extraction times. The efficient recovery of natural pigments isolated from microalgae by environmental friendly technology has been reported (Table 25.1). Pasquet et al. (2011) compared conventional extraction, MAE, and UAE in pigment extraction from microalgae. UAE gives higher carotenoids and chlorophyll yield compared to conventional and MAE. However, MAE offers the advantage of homogeneous thermoregulation of the medium, as no heat transfers are required to heat

Table 25.1 Environmental friendly technology for the extraction of pigments from microalgae

Microalgae	Environmental friendly technology	Targeted pigment	References
Dunaliella tertiolecta	Cold and hot soaking, MAE, Vacuum MAE, UAE	Chl a, Fucoxanthin	Pasquet et al. (2011)
Cylindrotheca closterium	Cold and hot soaking, MAE, Vacuum MAE, UAE	Chl a, Fucoxanthin	Pasquet et al. (2011)
Porphyridium purpureum	MAE	Phycobiliprotein	Juin et al. (2015)
Scenedesmus obliquus	SC-CO_2	Carotenoids, Chl a, Chl b, Chl c	Guedes et al. (2013)
Scenedesmus almeriensis	SC-CO_2	Carotenoids	Macías-Sánchez et al. (2010)
Dunaliella salina	SC-CO_2, UAE	Carotenoids, Chl a,	Macías-Sánchez et al. (2009)
Nannochloropsis gaditana,	SC-CO_2	Carotenoids, Chl	Macías-Sánchez et al. (2008)
Synechococcus sp.	SC-CO_2	Carotenoids, Chl	Macías-Sánchez et al. (2008)
Dunaliella salina	SC-CO_2	Carotenoids, Chl	Macías-Sánchez et al. (2008)
Chlorella vulgaris	SC-CO_2	Lutein, β-carotene, Chl a, Chl b	Kitada et al. (2009)
Synechococcus sp.	SC-CO_2	Carotenoids, Chl a	Macías-Sánchez et al. (2007)
Chlorella vulgaris	SC-CO_2	Crude pigments	Gouveia et al. (2007)
Chlorella pyrenoidosa	SC-CO_2	Lutein	Wu et al. (2007)
Haematococcus pluvialis	SC-CO_2	Astaxanthin, β-carotene, Canthaxanthin, Lutein	Nobre et al. (2006)
Haematococcus pluvialis	SC-CO_2 with co-solvent	Astaxanthin	Krichnavaruk et al. (2008)
Haematococcus pluvialis	SC-CO_2	Astaxantine and Phycocyanine	Valderrama et al. (2003)
Nannochloropsis gaditana	SC-CO_2	Carotenoids, Chl a	Macıas-Sánchez et al. (2005)
Nannochloropsis sp	UAE	Chl	Parniakov et al. (2015)

cells located in the center of the flask, and no hot spots exist as in UAE. In addition, no degradation of carotenoids was observed during microwave irradiation since degradation of carotenoids during microwave heating usually starts at temperatures above 60 ◦C for the most thermosensitive carotenoids; while provitamin A carotenoids and lutein are more stable to heat (Pasquet et al. 2011).

Recently, SC-CO_2 has been widely employed as an alternative to organic solvent extractions including natural pigments. The SC-CO_2 allows extracting natural pigments from microalgae in an oxygen free situation with moderated temperatures and short extraction times, which reduces oxidation. Carbon dioxide (CO_2) is the most widely used supercritical fluid because of an inert gas, which is nontoxic, nonflammable, and available at low cost. The SC-CO_2 process can selectively extract low polar lipid compounds, avoiding the co-extraction of polar impurities such as some inorganic derivatives with heavy metals. In addition, after SC-CO_2 extraction of pigments from microalgae, the de-oiled portion of the microalgae could be used as a raw material for the extraction of bioactive materials, which may facilitate another value addition in microalgae (Fig. 25.2).

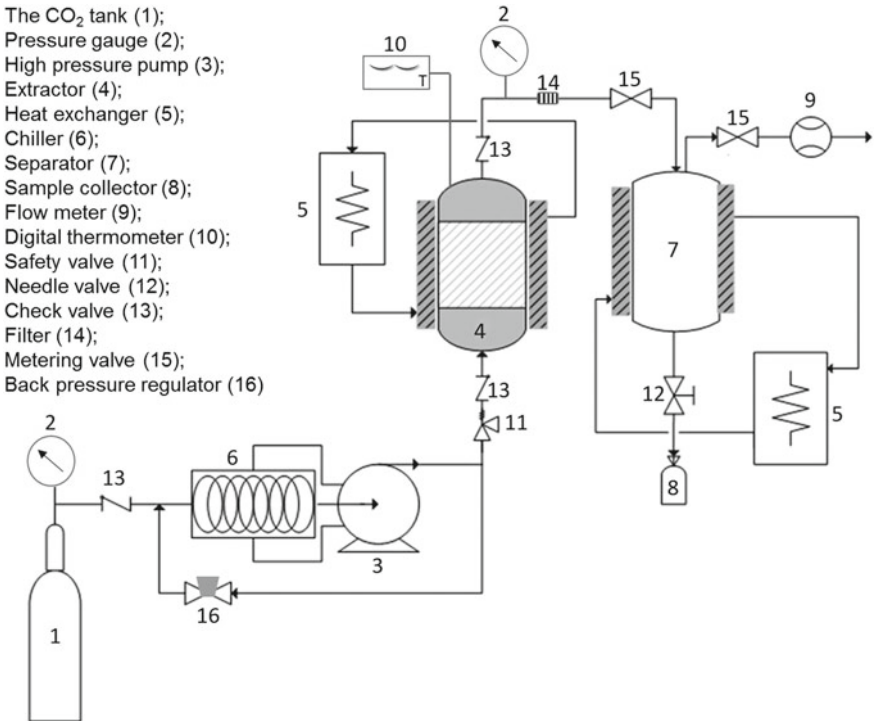

Fig. 25.2 Schematic diagram of supercritical CO_2 for the extraction of microalgal pigments

25.3.2 Future Prospects of Natural Pigment from Microalgae

Microalgae are the subject of increasing interest for their potential as a source of natural pigments in cosmetics industries for several reasons. These microorganisms are considered as the fastest growing organisms on Earth. Extensively available waters are potential areas for the culture of microalgae. In addition, culture techniques of microalgae commercial species such as *Spirulina* sp. and *Chlorella* sp. have developed rapidly. Microalgae can be found in many areas and has great biodiversity which can be seen as a potential field for the isolation of novel pigments. In addition, microalgae have exhibited unique chemical structures of natural pigments (i.e., Chl c, Chl d, Fucoxanthin) unlike those found in terrestrial counterparts. Additionally microalgae are viewed as "natural and healthy" by many people, and this promotes a positive response for consumers, who often regard natural and nontoxic entities. Many species of microalgae have been used as extracts and colorants in cosmetics, and there is no restriction for cosmetic use; microalgae can be considered a consumer-friendly source of skin care and cosmetic products. However, the health claims of natural pigments from microalgae required comprehensive studies on the mode of biological actions, and possible side effects have to be conducted in order to use natural pigments in skin care and cosmetic products.

The use of environmental friendly technologies to recover natural pigments is becoming very important since the volume of solvents used in the chemical process is extremely concerning. Further, advances in culture technologies of microalgae are important to bridge the gap between the challenges pertaining to the exploitation of microalgae. However, most natural pigments are sensitive to oxidation, isomerization, and polymerization. Many pigments derivatives may be formed through microalgae processing and preparation. As an example, sensitivity of chlorophylls to extreme pH and temperature allows the formation of several distinct derivatives (i.e., pheophytin and pheophorbide) through processing of microalgae. Therefore, technologies to improve natural pigments stability are required.

25.4 Potential Applications of Microalgae Pigments in Cosmetics and Cosmeceuticals

Investigative studies of skin health benefits of microalgae pigments have been reported extensively. It has been proved that microalgae pigments exhibit several cosmeceutical properties, including antiaging, antioxidant, skin protection, skin pigmentation, cosmetic colorants, and skin whitening. Moreover, a number of microalgae pigments have been used as active ingredients in commercialized cosmetics.

25.4.1 Sunscreen

Short-term ultraviolet (UV) exposure has several beneficial effects, including vitamin D synthesis in the skin, immediate pigmentation, skin thickening, and suntan (Webb and Engelsen 2006). However, long-term exposure to UV radiation results in skin damage including wrinkles, premature skin aging, and leads to skin cancer. Furthermore, excessive exposure of UV leads to reactive oxygen species (ROS) production, cytokine synthesis, and pyrimidine dimer formation in the genome of skin cells. These formations cause the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and trigger intracellular senescence (Kim et al. 2018). Based on these harmful effects, it is important to protect the skin during UV exposure by using skin care products, such as sunscreen, sunblock lotion, and antiaging serum.

Skin care products such as sunscreen creams, antiaging creams, emollient, and anti-irritants cream have been developed from microalgae-derived pigments(Venkatesan et al. 2015). The major pigment of microalgae, carotenoid (astaxanthin, lutein, zeaxanthin, and canthaxanthin) which is abundant in *Haematococcus* and *Dunaliella,* possess antioxidant activity and protective properties against sun damage (Ryu et al. 2015). Tominaga and colleagues stated astaxanthin treatment might normalize the corneocyte conditions protecting the keratinocyte differentiation and cornification from oxidative damage such as inflammation and improve the condition of skin layer in all layers (Tominaga et al. 2012). Another study has reported that astaxanthin derived from *Haematococcus pluvialis* showed the preventive effects toward UVA irradiation on filaggrin metabolism and desquamation in the epidermis and the extracellular matrix in the dermis (Komatsu et al. 2017).

Another carotenoid pigment, zeaxanthin, has been tested for its photoprotective effects on human skin by oral intake. Zeaxanthin dosage of 30–80 mg per day sufficiently generates a substantial increase in the person's ability to withstand elevated levels of UV exposure without subsequent pain (Gierhart and Fox 2013). Moreover, combined treatment of lutein and zeaxanthin has been reported to give multiple benefits to the skin, protection from detrimental effects of UV light as well as increase the surface lipids, skin hydration, and skin elasticity (Palombo et al. 2007).

Violaxanthin, an orange pigment from microalgae, has been proved to have photoprotective effects against UVB-irradiated human dermal fibroblasts. This pigment has been extracted from microalgae *Nannochloropsis oceania* and it significantly blocked UVB-induced damage including decreased cell viability and increased ROS production (Kim et al. 2018).

Another pigment from microalgae, fucoxanthin, has been investigated, fucoxanthin has the ability to suppress tyrosinase activity, melanogenesis in melanoma, and pigmentation in UV-irradiated through topical treatment (Shimoda et al. 2010). Fucoxanthin also plays an important role to significantly reduce intracellular ROS generated by exposure to UVB radiation in human fibroblast (Heo et al. 2008). Another work showed fucoxanthin can stimulate restoration of filaggrin by enhancing the metabolism in the dermis, leading to it exerting protective and therapeutic effect against sunburn (Matsui et al. 2016). Knowing all these photoprotective effects of

microalgae pigments, it might suggest that microalgae pigments can be used as an effective ingredient in cosmetic and sunscreen to protect skin from UV radiation.

25.4.2 Antiaging

The skin is the largest organ of human body which covers the entire external surface of the body. The skin is composed of three main layers: the outermost layer (a) epidermis, the inner layers, (b) dermis, and (c) hypodermis (Katiyar et al. 2007) The skin acts as a physical barrier to protect the body against harmful external agents such as pathogens, UV light, chemicals, and mechanical injuries (Pérez-Sánchez et al. 2018). In aged skin, the structural ability and physiological function are decreased, which increases the susceptibility to dermatologic disorder including laxity, wrinkles, dermatitis, xerosis, and cherry angioma (Kim et al. 2018). Skin aging can be affected by certain factors, those factors are genetic (dominant factor) and environmental factors that play an important role such as ultraviolet exposure, smoking, and weather (Guillerme et al. 2017).

Microalgae pigment is found to exhibit significant antiaging effects on the skin. It has been mentioned that β-carotene one of microalgae pigment has an excellent potential as aging preventive (de Jesus Raposo et al. 2013). β-carotene is commonly produced by microalgae *Dunaliella salina* that may produce up to 10% of its dry weight biomass in β-carotene. Sathasivam and colleagues stated β-carotene helps in preventing the formation of free radicals that can cause premature aging in skin cells (Sathasivam and Ki 2018).

Astaxanthin is known as one of the most powerful antioxidants. Based on its remarkable antioxidant property it is highly potential to apply astaxanthin in anti-aging care which is better than α-tocopherol (Guillerme et al. 2017). Cumulative cellular damage by ROS is related to the development of aging. Oxidative stress level is determined by the balance of ROS and antioxidant defense. A research work has investigated *Haematococcus pluvialis* contains high astaxanthin levels that was capable to protect mutant fruit flies against oxidative stress and increase its lifespan. Therefore those results strongly support the antiaging properties of microalgae *H. pluvialis* and its therapeutic potency (Huangfu et al. 2013).

Another work reported that carotenoid lutein showed protective reactions to UV radiation, especially in combination with other antioxidant systems (Lee et al. 2004). Lutein is a promising carotenoid found in several microalgae such as *Scenedesmus salina, Chlorella, Chlorella vulgaris, Scenedesmus obliquss, Dunaliella salina,* and *Mougeotia* sp. (Mäki-Arvela et al. 2014). Due to its similar physicochemical properties to astaxanthin, lutein has been used for applications related to human health such as age-related macular degeneration (Zuluaga et al. 2017). Together with zeaxanthin, lutein supports a nutrient relationship in preventing age-related cataracts and maculopathy (Bule et al. 2018).

Other microalgae carotenoids have been used in beauty care formulation as an anti-aging agent, such as lycopene. Lycopene is an efficient antioxidant that can neutralize

oxygen-derived free radicals which can lead to oxidative stress (Bhalamurugan et al. 2018; Mourelle et al. 2017).

25.4.3 Stress Protection

Microalgae metabolites products such as carotenoids are well-known to possess strong antioxidant properties and widely used in healthcare applications to protect from oxidative stress. Rinnerthaler et al. (2015) mentioned oxidative stress plays a major role in cornification and aging process of human skin (Rinnerthaler et al. 2015). Various stresses including daily exposure to ultraviolet radiation from the sun leading to the generation of ROS and disturb healthy skin conditions. Oxidative stress leads to premature signs of aging, uneven skin tone and texture, and even breaks down the essential proteins that support the skin.

Degradation of collagen and elastin in the dermal skin layer can be caused by oxidant events and molecular mechanisms of skin aging involve damage to DNA, the inflammatory response, decreased antioxidant production, and the generation matrix metalloprotein (MMP). Eventually those events lead to skin damage and reflect the aging process such as the loss of tensile strength and elasticity, the disruption of cellular dermal matrix, the appearance of wrinkles, age spots, and skin tone loss (Lephart 2016). Thus, dynamic equilibrium between antioxidant and ROS is needed as oxidative stress preventive.

Fucoxanthin possesses prominent antioxidant activity against H_2O_2-mediated cell damage and might be a potential therapeutic agent for treating or preventing oxidative stress (Heo et al. 2008). This pigment is present abundantly in several microalgae such as *Phaeodactylum tricornotum*, *Odontella aurita*, and *Isochrysis* aff. *galbana.* Astaxanthin is another excellent antioxidant that exhibits higher antioxidant activity than vitamins A and E. Davinelli et al. (2018) reported astaxanthin activities in the skin inhibit collagenases, MMP activity, inflammatory mediators and ROS induction, resulting in potent antiwrinkle and antioxidant effects (Davinelli et al. 2018).

25.4.4 Tanning Products/Pills

Tanning is an acclimatizational reaction of skin to increasing levels of UV exposure (Kalla 2007). It creates a natural shield against ultraviolet radiation. The melanin protects the skin by filtering the wavelength of sunlight that induce the formation of dangerous free radicals (Biba 2014). Tanning has become immensely popular over the years. Many people consider tan skin more attractive than pale skin. Tan makes the skin stand out, highlighting contours and giving it a glowing look. Moreover, being tan is often associated with leisure time, glamour, and good health.

It is well-known that UV exposure is the main cause of skin cancer and premature aging. According to public awareness of the high risk of UV tanning, tanning products

have become a particularly popular alternative including sunless tanner, bronzer, tanning preparations, tanning promoter, and tanning pills. To date, these products have gained a great interest of cosmetic industry since they provide a safer way to tan and aesthetically pleasing.

Research on cosmeceutical properties of microalgae pigments has led to the development of various beauty care products. One of those products comes as a tanning pill. The most common ingredient in tanning pills is canthaxanthin. Canthaxanthin is an intense red-orange carotenoid that can be found abundantly in green microalgae species such as *Chlorella zofingiensis*, *Chlorella vulgaris*, *Coelastrella striolata* var. *multistriata*, *Haematococcus pluvialis, Nannochloropsis gaditana, and Nannochloropsis salina* (Abe et al. 2007; Li et al. 2006; Lubián et al. 2000; Nobre et al. 2006; Raposo et al. 2015). This secondary carotenoid has been investigated to provide beneficial effects for human body, such as antioxidant, antitumor, and skin protector by creating skin tan color (Gong and Bassi 2016; Lober 1985).

Canthaxanthin darkens the skin color by depositing its red-orange color in the epidermis and subcutaneous fat. An orange-brown color appears through the skin at its sufficient concentration (Garone et al. 2015; Gupta et al. 1985; O'Leary et al. 2014). Oral tanning pill containing canthaxanthin has also been reported to be effectively used in the treatment of a variety of dermatology disorders (Wang et al. 2015). The effect of oral canthaxanthin in the treatment of vitiligo has been evaluated. Fifty six patients with vitiligo were treated with canthaxanthin serum as a pigmenting agent. Dosages were weight adjusted and were administered for at least 20 days. The evaluation showed that 73% of light skinned patients were satisfied with results and 27% of them were unsatisfied. Treatment appeared to be less effective in dark skinned patients. This study showed that oral canthaxanthin can give excellent result in selected cases of vitiligo. The excellent results of the canthaxanthin pill effects in the treatment of erythropoietic protoporphyria have been obtained as well (Eales 1978). Patients with polymorphous light eruptions have also been successfully treated with a combination of β-carotene and canthaxanthin (Hulisz and Boles 1993; Suhonen and Plosila 1981). However, the daily intake of canthaxanthin pill for tanning may cause urticarial, hepatitis, and fatal aplastic anemia. Food and Drug Administration (FDA) has not approved canthaxanthin use as a tanning agent. It is currently approved as a food colorant. Therefore, further study of canthaxanthin use as tanning pills is needed.

25.4.5 Colorants in Make-up Products

The market for natural dyes in cosmetics continues to grow on a global scale due to increasing popularity of green lifestyle based on naturally sustainable sources (Grifoni et al. 2009). Many cosmetic manufacturers are seeking natural colorants that offer additional health beneficial effects such as UV protection, antiaging, antioxidant and antibacterial in foundations, make-up products, beauty care products, hair

colorants, and other cosmetics (Patil and Datar 2016). Microalgae pigments meet these market demands and have been widely used as cosmetic ingredients.

It is well-known that microalgae contain a wide range of photosynthetic pigments. There are three major classes of pigments that can be found in microalgae, namely carotenoids, chlorophylls, and phycobiliproteins (phycocyanin and phycoerythrin). They exhibit brilliant colors ranging from green, yellow, and brown to red and are being utilized as an alternative to the synthetic colorants (Begum et al. 2016). In addition, these pigments have been reported to possess functional cosmeceutical effects or drug-like effects on skin.

Chlorophylls is an essential pigment not only used as an additive in pharmaceutical but also in cosmetic product. Chlorophyll α presents green color and has been used as a colorant due to its stability. This pigment is abundantly found in *Chlorella* and *Spirulina* species. *Chlorella vulgaris* has been extensively used to produce chlorophyll which is used for cosmetic colorants as a substitute for artificial color (Begum et al. 2016). Chlorophylls have also been applied as additive in deodorant, due to their ability to mask odors, as well as in toothpastes, due to their antioxidative properties (Mourelle et al. 2017).

Another microalgae pigment that widely applied as an additive for cosmetics is astaxanthin. The carotenoid pigment astaxanthin plays a role as a strong colorant and a potent antioxidant. Astaxanthin imparts red color to makeup and beauty care products (Chakdar and Pabbi 2017). Guerin et al. reported that this main carotenoid pigment can be efficiently produced from green microalgae, *Haematococcus pluvialis* (Guerin et al. 2003).

Phycobiliproteins (phycocyanin and phycoerythrin) are a group of colored proteins commonly present in cyanobacteria (formerly known as blue-green algae) and red algae. Phycocyanin and phycoerythrin can safely be used as food and cosmetic colorants as they are nontoxic and noncarcinogenic (Henrikson 1989). Phycocyanin is a florescent blue pigment and phycoerythrin imparts pink fluorescence. The main resource of phycocyanin is cyanobacterium *Spirulina platensis*, and red microalgae *Porphyridium cruentum* is exploited to produce phycoerythrin (de Jesus Raposo et al. 2013; Pangestuti and Kim 2011). Phycoerythrin is characterized to have intense pink color, heat stability, and pH tolerant and has already been formulated as lipsticks and eyeliners (Balboa et al. 2015). Moreover, Arad and Yaron have prepared pink and purple cosmetics, such as eye shadow, face make-up, lipstick, from various red microalgae. Phycocyanin isolated from cyanobacteria (*Arthrospira*) has already been marketed as cosmetic colorants in Japan and formulated as an eye shadow (Bermejo et al. 2000).

25.4.6 Thalassotherapy

Thalassotherapy is defined as the therapeutic use of seawater and all related marine elements (marine environment, seaweed, microalgae, sea mud, and sand) in the form of water baths, massages, and showers to promote health, wellness, and beauty. This

treatment is one of the traditional therapy in France that helps people to cure arthritis and rheumatism. This therapy has also been recognized by the Egyptians, the Greeks, and the Romans, as a healing method since ancient times.

The beneficial effects of thalassotherapy treatments have been widely documented. Different treatments have different effects, including pain relief, relaxing, detoxifying, slimming and toning, skin and tissue nourishment, and relief of skin problems (Mourelle et al. 2017; Pereira 2018). A rather wide range of marine macroalgae enter in the packs used in thalassotherapy, and algal powders or algal salts are sold for use in home BAHTtherapy. Most commonly used in this area are *Porphyra*, *Eucheuma*, *Laminaria,* and *Undaria* (Charlier and Chaineux 2009). Based on this fact, algae have been extensively used as thalassotherapy components, however, there has been limited use of microalgae. Microalgae pigments could be incorporated into thalassotherapy treatments due to their numerous bioactive substances that are of interest in skin care. Recently, a research group of the Applied Physics department at the University of Vigo, Spain, namely FA2, has described a pilot experiment to cultivate marine microalgae, *Nannochloropsis gaditana*, for application in thalassotherapy that entailed cultivating microalgae in seawater in the Talaso Atlántico Thalassotherapy Centre (in Oia, Pontevedra, Spain). *N. gaditana* is belonging to the green alga group that contained a high number of chlorophyll α and carotenoid (Yasmine et al. 2017). Both pigments have a great potency to be used in thalassotherapy treatments due to their beneficial effects for human skin, such as antiaging, skin protection, and relaxing. This research subsequently led to the development of thalassotherapy products from microalgae such as a microalgae bath and microalgae mud (Legido et al. 2013).

25.4.7 Skin Whitening

Currently, the applications of microalgae pigments in personal care products have gained more attention due to their beauty-enhancing effects. Besides all the functions as mentioned above, microalgae pigment is also known as an active ingredient for skin lightening products. Skin whitening is a very popular personal care product for woman all around the world, particularly in Asia. This is because most of Asian females preferred more fair skin tone, and thus skin whitening products have become the bestselling skin care product in Asia (Kim and Pangestuti 2011; Wang et al. 1997).

Tyrosinase is a key enzyme in melanin biosynthesis that determines the coloring of hair, skin, and eyes. The inhibition of tyrosinase has been distinguished as the common approach to treat hyperpigmentation from melanogenesis and to achieve skin lightening (Wang et al. 2015). UV radiation helps the formation of melanosomes which occurs in the melanocytes of the epidermis via a series of oxidation reactions, catalyzed by tyrosinase. Tyrosinase catalyzes the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-Dopa) and the conversion of L-Dopa to dopaquinone. Melanin is then converted from the dopaquinone formed (Hearing and Jiménez 1987).

The large amount of melanin formed promotes to skin pigmentation. Thus, tyrosinase inhibitors are used to catalyze rate-limiting step in the process of pigmentation (Joshi et al. 2018). It was found that several microalgae extract exhibits excellent antityrosinase activity (Matsukawa et al. 1997). An extract of *Nannochloropsis oculata* was found to be effective in inhibiting tyrosinase. Presence of zeaxanthin would be responsible for this efficiency (Shen et al. 2011). Recently, Rao and colleagues reported that astaxanthin isolated from *Haematococcus pluvalis* is successfully implemented as tyrosinase inhibitors. The potent inhibitory effects on L-Dopa oxidase activity of tyrosinase are increased with astaxanthin concentrations (Rao et al. 2013). These preliminary studies suggest that microalgae pigments such as zeaxanthin and astaxanthin are applicable for cosmetics as skin whitening ingredients and also as drugs for use in the treatment of abnormal pigmentation.

25.5 Pros and Cons of Microalgal Pigments in Cosmetics and Cosmeceuticals

Nowadays, people are more aware of health, causing a huge change in market demands from synthetic colorant to natural colorant. Research and development highly rise to found alternative natural colorant rather than artificial colorant, because of concerns that chemically synthetic colorant causes cancer, and cause liver and kidney damage. Colorant is important to manufacturers as they need to get uniformity of their products (Rymbai et al. 2011; Spears 1988). Pigments are one of the bioactive compounds contained in microalgae biomass. It is a valuable product that not only can be used as colorant but performs several biological activities that is important for health benefit. Chlorophyll is found to have the capacity to speed up wound healing process by 25%, that stimulates tissue growth and shows antibacterial activity after wound (Hosikian et al. 2010).

To collect pigment from microalgae, several processes should be performed. Each process has various difficulties and challenges. Microalgae biomass needs to go through desalting process before completely dried. From dried biomass, pigments then can be extracted using organic solvent extraction or supercritical fluid extraction methods. In plants, chlorophyll is located mostly or nearly only in chloroplast, and it is protected by hydrophobic membrane, and complexly binding with phospholipids, polypeptides, and tocopherols (Humphrey 2004). Chlorophyll becomes unstable when it is removed from its protective environment and can be easily broke only by different pH conditions. To solve this problem, copper ion often is used to substitute the magnesium ion in chlorophyll center, in order to form a more stable compound (Delgado-Vargas and Paredes-Lopez 2002). Copper complex is not absorbed by body, and removed together with excretion. This chemical modification is considered safe for the body, but it clearly costs more than artificial colorant (Spears 1988).

Compared to higher multicellular plants, microalgae growth are very fast and have much higher productivity, no seasonal variety, and it is easier to extract the compounds. Its simple structure and function, makes microalgae a suitable model for research purpose than any other terrestrial plants (Pulz and Gross 2004). As a photosynthetic organism, microalgae have a much more efficient utilization of sunlight energy to produce valuable compounds, compared to higher terrestrial plants. Unlike plants, microalgae can be easily cultivated in wastewater. Nutrients in wastewater can be absorbed by microalgae as their nutrient, with only sunlight and CO_2, microalgae can convert phosphate and nitrogen to biomass. This means, microalgae have dual role when cultivated in polluted water, as a wastewater treatment and to produce biomass (Rawat et al. 2011; Wang et al. 2015).

With the developments of advanced technology in biomass culture, and pigment extraction techniques, the opportunity in utilization of microalgae pigments is widely open. Several pigments products from microalgae are valuably used, such as chlorophyll, carotenoids, phycobiliproteins, and phycocyanin. Microalgae culture not only produces pigments itself but also a wide range of metabolites and compounds such as lipids, carbohydrates, proteins, vitamins, and minerals. The residual substances also can be used as fibers source or silica raw material. More efficient cultured technologies with addition of molecular engineering, lead microalgae to be a valuable future source of bioproducts. This combined with their capacity as cultured product, can be considered that microalgae are a renewable resource that has a high economic potential. Microalgal pigment-based cosmetics have high potential demand, especially when combined with antioxidant or other bioactive compounds and by product development to protect skin from UV radiation. A study of how these active compounds work has high potential commercially.

25.6 Conclusions

Natural cosmetics that incorporate microalgal extracts and compounds are being increasingly sought after in the industry, with more and more consumers demanding cosmetic products that are of natural origin. Microalgal pigment also showed biological activities and potential to be applied in cosmetics as well as cosmeceuticals. However, stability of microalgal pigments is still a significant challenge up to now. In addition, adequate clinical trials are needed in the development of cosmetics derived from microalgal pigments. More importantly, once their biological activities are demonstrated, new aspects need to be addressed before commercialization; these include integration and co-optimization of microalgal culture and downstream processes.

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Chapter 26
Encapsulation of Microalgal Pigments

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Abstract This review takes a critical approach on ways to increase the stability of pigments (phycobilins, chlorophylls, and carotenoids) using microencapsulation. Natural pigments are important nutraceuticals known for their potent antioxidant activities and they have been widely studied and used as high-quality health supplements. In this context, microalgae are the most promising sources and are devoid of the toxic effects associated with synthetic derivatives. Due to being subjected to extraction and purification treatments, such compounds may be more exposed to extrinsic actions of the environment, such as degradation by light, oxygen, adverse pH conditions, among others, in possible applications in food matrices or nutraceuticals. For this, microencapsulation appears as an alternative to protect these compounds and, in this way, increase the stability for a longer period than the conventional one. In this chapter, the main encapsulation techniques that may be useful for improving the stability of microalgal pigments, such as internal and external ionic gelling, complex coacervation, drying, and freeze drying, among others, as well as the main polymers can be used to coat these pigments, thus creating new possibilities and applications trends for human health.

Keywords Microalgae · Microencapsulation · Encapsulation techniques · Nutraceuticals · Health supplements

26.1 Microalgae

The microalgae are algal microorganisms considered one of the oldest living beings on the planet (Andrade and Filho 2014) that live predominantly in the aquatic environment and have heterogeneous characteristics. Because they are not considered a taxonomic classification, microalgae constitute a group in which different species or classes of living beings are accepted.

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Therefore, this group may assume prokaryotic or eukaryotic species or may be divided in relation to their life cycles. They are the main members of phytoplankton and can be found individually or in colonies capable of reaching large dimensions (Giordano et al. 2005).

This group of organisms is considered fundamental to the maintenance of life on earth since they participate, together with aquatic macrophytes, to produce most of the O_2 in the atmosphere. That is, about 60% of the primary oxygen production on the planet is carried out by microalgae (Chisti 2007). Likewise, these microorganisms are thought to together produce more O_2 than all other existing plants (Schmitz et al. 2012).

For the most part, microalgae biomass is composed of pigments, carbohydrates, proteins, and lipids. In this context, the presence of photosynthetic pigments in microalgae can be highlighted. These elements enable microalgae to perform oxygenic photosynthesis, and their systematic characterization involves the consideration of a series of criteria (Hoek et al. 1995; Eichhorn et al. 2001), as is the case with lighting conditions. Consequently, microalgae are often distinguished according to their pigmentation (Schmitz et al. 2012).

The use of microalgae in the context of feeding is important, since these beings produce several substances, such as vitamins, minerals, pigments, lipids, and fatty acids. In addition, most microalgae species are of interest for the production of pigments, proteins, lipids, and bioenergy. The concentration of these microalgae is distributed in the Eubacteria and Eukarya kingdoms, especially within the cyanophyte and chlorophyte groups (Lee 2011).

26.2 Microalgal Pigments

Because they present photosynthetic characteristics, microalgae have the capacity to produce elements with coloring properties. Pigments are chemical compounds that absorb radiation in the visible zone (Sampaio and Cruz 2014). These elements present different combinations according to the lighting conditions and the class of microalgae.

Several studies have shown that pigments have a great potential for application in several segments of the food industry—essentially in food production—and in the pharmaceutical field (Lourenço 2006). In addition, algal pigments are known for their potent antioxidant activities and are considered important nutraceuticals.

A wide variety of pigments can be found in microalgae biomass. Among the possible combinations, three fundamental types can be distinguished: carotenoids, chlorophylls, and phycobilins. The characteristics of this group of pigments are summarized in Table 26.1.

It is understood from Table 26.1 that different pigments may assume different categories according to a specific illumination condition. These characteristics are conditioned by the natural color of the microalgae, and the spectrum can be varied from greenish to orange or red in drastic conditions (Rangel 2000). Thus, the pigments

Table 26.1 Photosynthetic pigments, their respective colors, and distribution in various organisms

Photosynthetic pigments			
Pigment	Class	Color	Distribution
Chlorophylls	a	Green	Plants, algae, and cyanobacteria
	b		Plants and green algae
	c		Brown seaweed and diatoms
	d		Red algae
Carotenoids	Carotenes	Orange	Photosynthetic microorganisms
	Xanthophylls	Yellow	Brown seaweed and diatoms
Phycobilins	Phycoerythrin	Red	Red algae and cyanobacteria
	Phycocyanin	Blue	

can absorb light in the ultraviolet (UV) region and visible from the spectrum, with the rest being transmitted or reflected, thus presenting color.

All photosynthetic species have the chlorophyll a pigment, considered the most important pigment for photosynthesis (Lourenço 2006). They are liposoluble pigments found in the chloroplasts of all algae, upper plants and cyanobacteria, and can be colored from yellow-green to blue-green (Andrade and Filho 2014).

Along with chlorophyll, molecules of another type of pigment can be found: the carotenoids. This type of pigment is also characterized as a fat-soluble pigment and assumes stains that vary in shades of yellow, orange, or red. In addition, carotenoids are divided into two groups: carotenes (which rely on carbon and hydrogen) and xanthophylls (which count, in addition to carbon and hydrogen, with oxygen) (Lourenço 2006).

Phycobilins are soluble in water. Its presence is marked in red algae and cyanobacteria, so its pigment spectrum may be in shades of blue (phycocyanin and allophycocyanin) or red (phycoerythrins).

The use of pigments in the industry also varies according to their type. In the case of carotenoids, the main application is as natural dye, nutraceuticals, and animal feed additives (Cordero et al. 2011). Chlorophylls are used as a coloring agent in foods, cosmetics, and also in medicines, and in the treatment of wounds and ulcers (Ramirez 2013). However, microalgae with a high abundance of phycobilins are used as natural dyes (Chagas 2014; Lourenço 2006; Mulders et al. 2014).

26.2.1 Phycobilins

Phycobilins correspond to the pigments present in the Rhodophyta, Cyanophyta, and Cryptophyta divisions (Sampaio and Cruz 2014). They are water-soluble and are characterized by compounds with two units: alpha and beta (Andrade and Filho 2014). The main role of phycobilins is to assist in capturing solar energy in regions of the spectrum that chlorophyll has low absorption content. Thus, phycobilins can capture light even in low light conditions, as in deep ocean regions.

Therefore, the presence of chlorophyll is masked, that is, the energy captured by phycobilins is transferred to chlorophylls and provides bluish or reddish colorations to algae. Thus, phycobilins increase the spectrum of light uptake by photosynthesis. Also, as one of its main functions, phycobilins act as repositories for hydrogen reserves.

The main contexts of use of phycobilins are as fluorescent reactive agents in medical and scientific research, since the pigment absorbs part of the light (when exposed to a bright light) and reflects another part. This reflection makes it possible to trace elements and cells marked with the pigment.

Phycobilins also act as dyes in foods and cosmetic substances (Vargas-Rodriguez et al. 2006). In addition, they have applications in antioxidant, anti-inflammatory, antiviral activities, (Sampaio and Cruz 2014) and even in antitumor contexts, although research in this latter context is still scarce (Schmitz et al. 2012).

The phycobiliproteins are constituted by a protein part covalently attached to chromophores called bilins (prosthetic group) (Glazer 1981). They form a highly ordered complex called phycobilisomes, as shown in Fig. 26.1, located on the thylakoid membrane. The phycobilisomes are composed of two domains: nucleus and stems. The former is bound to the thylakoid membrane and composed of phycobiliprotein allophycocyanin. The second is associated with the nucleus, and is composed of hexameric arrangements of phycoerythrin and phycocyanin.

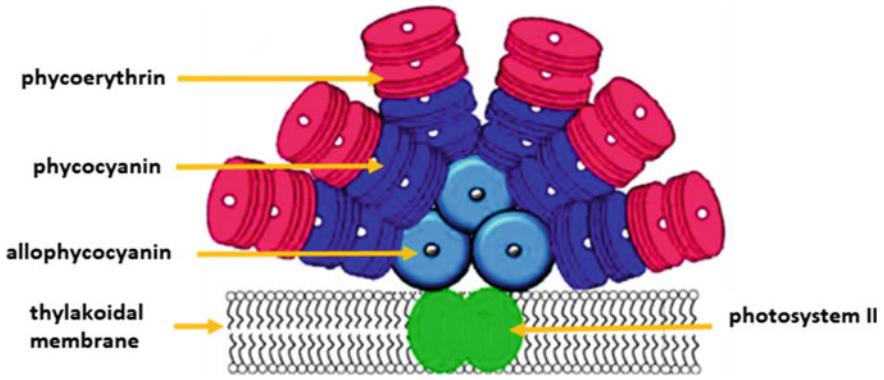

Fig. 26.1 Structure of a phycobilisome (Esteves 2016)

This pigment can absorb light from different wavelengths. In this context, there are three types of phycobiliproteins: phycoerythrin, phycocyanin, and allophycocyanin. Phycoerythrin has red pigment (it is present in red algae) and absorbs green energy with wavelengths around 480–570 nm. In turn, phycocyanin assumes blue tones and absorbs red light radiation at a maximum of 625 nm. Allophycocyanin also assumes blue color and absorbs energy in red light (maximum absorption at 650 nm) (Glazer 1981).

26.2.2 Carotenoids

Carotenoids are natural liposoluble pigments that exhibit coloration ranging from yellow to orange, also assuming red color in some cases (Lourenço 2006). This type of pigment is usually found in fruits, vegetables, and some marine animals, as is the case of salmon and some crustaceans (Valduga 2009).

They are characterized as the second of the most important pigments for photosynthesis. That is, although it is an accessory pigment that helps in the absorption and perception of light with chlorophyll. Its role for photosynthesis is extremely important: carotenoids act by ridding the chlorophyll of excess light when a large amount of energy is received. It is then the function of the carotenoids to dissipate excess energy and maintain the photosynthetic process stable.

In the context of the food industry, carotenoids are widely used as food dyes and as an agent for food quality improvement. On the other hand, carotenoids are also considered important for health, since their biological activities contribute to the inhibition of diseases from the strengthening of the immune system. Thus, this pigment can be used in the treatment of degenerative and cardiovascular diseases.

The commercial production of carotenoids from microorganisms competes mainly with the synthetic production by chemical procedures. Currently, industrially used carotenoids are obtained chemically or by extracting plants and/or algae. However, due to the concern with the use of chemical additives in foods, there was a growing interest in the carotenoids obtained naturally by biotechnological processes.

The chemical structure of the carotenoids is composed of eight isoprenoid (C5-isoprene) 40 carbon tetraterpenoid compounds with a linear central skeleton consisting of 22 carbon atoms, nine conjugated double bonds, and annular or near-annular final ends with nine carbon atoms in each ring (Andrade and Filho 2014). Carotenoid molecules have a double bond system that makes up the chromophore group responsible for the color they provide to food.

Further, carotenoids can be divided into two main groups: carotenes and xanthophylls. Figure 26.2 shows chemical structures of carotenoids of the xanthophyll groups (astaxanthin, zeaxanthin, and violaxanthin) and xanthophyll (beta-carotene, lycopene, and lutein).

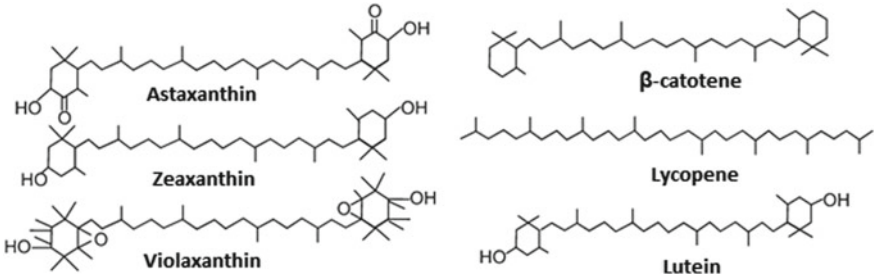

Fig. 26.2 Structure of some carotenes and xanthophylls (Andrade and Filho 2014)

26.2.3 Chlorophylls

Chlorophylls are green pigments often used as additives in food, as well as found in vegetables and fruits. These pigments are chemically unstable and can be altered or destroyed easily by modifying the quality and characteristics of products. In addition to being unstable, chlorophylls are sensitive to light, heating, oxygen, and chemical degradation (Schoefs 2002). They are substances that absorb the wavelengths corresponding to their absorption spectrum and reflect the green color.

Among the main uses of chlorophyll, its function as a natural colorant is widely explored in the food context. In this case, chlorophylls also act as antioxidants in order to restore the natural content of food products and protect healthy cells without the need for chemical additives.

There are four types of chlorophylls (chlorophyll a to chlorophyll d). The Chlorophyll a is present in all organisms that do oxygenic photosynthesis (Streit et al. 2005). This type is also responsible for performing the first stage of photosynthesis, so that the other pigments act as auxiliaries for the absorption of light and for the passage of energy to the reaction centers. For this reason, the remainder of the pigments (i.e., all but chlorophyll a) are called accessory pigments.

Chlorophyll molecules are present in the thylakoid membranes, which are lamellar structures located inside the chloroplasts. Figure 26.3 shows the chemical structure of chlorophylls a and b. Chlorophyll is a molecule of the group of porphyrins composed of rings that have carbon, hydrogen, and nitrogen in their composition and magnesium in the center. Magnesium plays an important role in photosynthesis from the formation of sugars, proteins, lipids, and vitamins.

The chemical compound shown in Fig. 26.3 describes a fully unsaturated asymmetric macrocyclic structure consisting of four pyrrole rings. These rings are numbered from 1 to 4 or from "a" to "d," according to Fisher's numbering system (Schoefs 2002). Chlorophylls a and b are in the nature at a ratio of 3:1, respectively, and differ in the C-3 carbon substituents. That is, the difference between the two chlorophylls is in the chemical composition, and the chlorophyll a has the CH_3 radical in place of the CHO present in chlorophyll b. The stability of chlorophyll b is due to the attractive effect of electrons of its aldehyde group in C-3 (Von Elbe 2000).

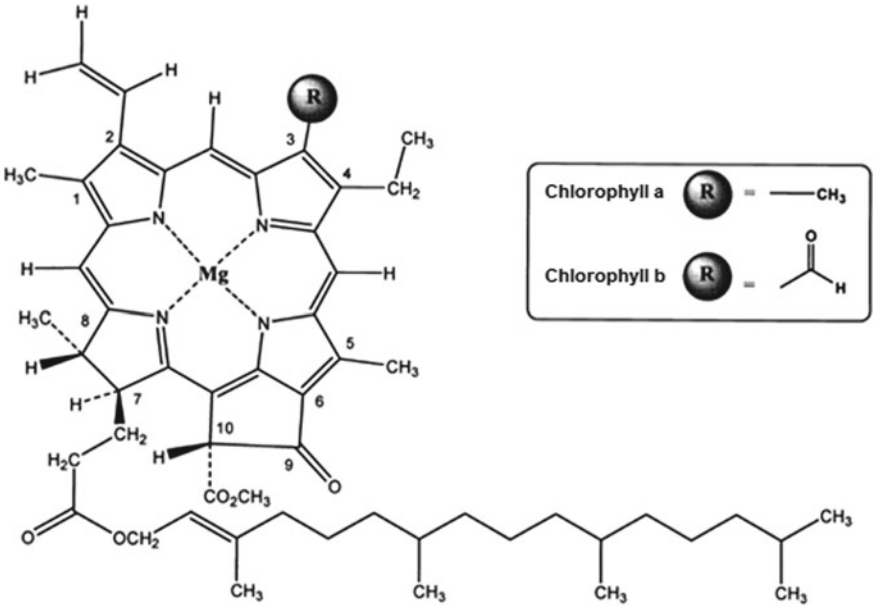

Fig. 26.3 Chemical structure of chlorophyll *a* and chlorophyll *b* (Streit et al. 2005)

Thus, it is proven that there is a vulnerability of these pigments under certain conditions. Once extracted from the microalgal matrix, they can be degraded by thermal or oxidative processes in the course of their processing and storage, which can cause the loss or diminution of their biological activity. In this sense, it is necessary to use techniques to increase and stability of these compounds. Thus, encapsulation is an extremely useful technique because it avoids the loss of the biological properties of sensitive substances under adverse environmental conditions by providing the protection of these substances (Niizawa et al. 2019).

26.3 Microencapsulation of Microalgal Compounds

The microencapsulation process is defined by the formation of a polymeric matrix or a protective layer around the biological substance (active material), thus protecting it (Martínez-Delgado et al. 2017). In addition to protecting the biological activity of the encapsulated compound, microencapsulation facilitates the manipulation of encapsulated material, enhances its stability, provides adequate particle size, mask flavors, and provides controlled release (Mortari 2018).

Knowing the sensitivity of microalgal compounds to adverse environmental conditions, microencapsulation is an alternative to these highly sensitive compounds. Through the encapsulation an outer membrane or coating of one material is created

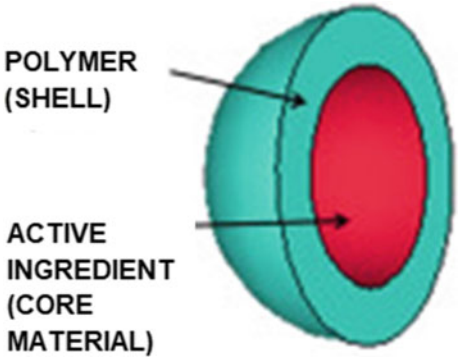

Fig. 26.4 Schematic representation of a microcapsule (Paulo and Santos 2017)

on another (Fig. 26.4), applied for the protection and/or preservation of its biological activity of factors such as oxygen, light, and temperature, preventing its oxidation and biochemical or thermal deterioration, and prolonging it. In addition to the microencapsulation of the microalgae biomass, the microencapsulation of substances synthesized by these microorganisms, mainly pigments and antioxidants, has been studied (Bustamante et al. 2016; Machado et al. 2016; Saifullah et al. 2019).

Table 26.2 shows the main microencapsulated substances synthesized by microalgae and the benefits that the microencapsulation process can offer to these substances.

The first reports related to the microencapsulation of microalgae appeared in 1999 and 2001, with very similar studies. Han et al. (1999) used microencapsulation to immobilize three species of microalgae (*Chlorella vulgaris, Dunaliella salina* and *Porphyridium purpureum*) by means of the extrusion technique, with the objective of developing an economic study for the high-density culture process and Joo et al. (2001) used the extrusion process to microencapsulate four species of microalgae (*Dunaliella bardawil, Chlorella minutissima, Pavlova lutheri,* and *Haematococcus*

Table 26.2 Microencapsulation of microalgae-based products (Derner et al. 2006; Martínez-Delgado et al. 2017)

Products synthesized by microalgae		Benefits of microencapsulation
Biomass	Biomass	Increased stability by limiting isomerization Protection against temperature and presence of light Improvement of oxidative stability Improvement of rheological characteristics Improvement of bioavailability Increased stability during processing and storage
Pigments and antioxidants	Xanthophylls (astaxanthin and canthaxanthin)	
	Lutein	
	Beta-carotene	
	Vitamin C and E	

pluvialis), also with the objective of developing an economical system for the cultivation of high-density microalgae. Thus, studies related to microencapsulation of microalgae biomass have been reported in the literature to date. Regarding microalgal compounds, such as pigments and antioxidants, microencapsulation studies are more recent and increasingly detailed in order to preserve these compounds and provide their application in a wide range of products. Thus, studies related to the microencapsulation of microalgal compounds are described below.

Because of their health benefits, microalgal pigments have great commercial value as natural dyes. However, they have low storage stability, high sensitivity to light, heat, and oxygen. In addition, there are few reports on encapsulation of algae extract and algal biomass, and only a few types of wall materials that may show improved stability, shelf-life, and controlled release are being investigated. In this sense, Bonilla-Ahumada et al. (2018) analyzed the microencapsulation of *Tetraselmis chuii* biomass and investigated the effect of wall material and spray dryer processing conditions on the preservation of β-carotene compounds and antioxidants. It was observed that the microencapsulation process preserved 80–92% of β-carotene and 46–81% of the phenolic compounds present in the microalgae for up to three months of storage in the absence of light at 25 °C and maltodextrin at 130 °C was the best wall material. In addition, the microencapsulation process does not cause loss of nutritional value, facilitates transport and handling, and preserves antioxidant capacity. Similarly, Bonilla-Ahumada and Khandual (2016) analyzed the microencapsulation of compounds extracted from the microalgae *Tetraselmis chuii*, where these compounds were microencapsulated in maltodextrin–gum arabic, pectin, and gelatin by spray drying. These authors verified that the microencapsulation process was able to preserve the nutritional characteristics as well as β-carotene, antioxidant properties, total amount of carotenoids, and to provide the use of these compounds as ingredients to increase the nutritional value of food products. In addition, maltodextrin and gum arabic provided greater preservation of β-catonene and that gelatin was more efficient in preserving antioxidant activity.

Among the microalgal pigments, astaxanthin is the pigment most widely studied in relation to microencapsulation, due to its significant antioxidant properties, which in human nutrition has been gaining popularity as a dietary supplement, precisely because of its powerful properties antioxidants (Higuera-Ciapara et al. 2004). However, its rapid degradation in the presence of light and oxygen limits its application in different food formulations (Bustos-Garza et al. 2013). Thus, Higuera-Ciapara et al. (2004) microencapsulated astaxanthin in glutaraldehyde crosslinked chitosan by the multiple emulsion/solvent evaporation method with the objective of studying its stability under different storage temperatures. In this study, it was observed that there was no isomerization or chemical degradation of astaxanthin under storage at 25, 35, and 45 °C for 8 weeks, the microencapsulation process being efficient for the protection and, consequently, the stability of this compound. Similarly, Bustos-Garza et al. (2013) microencapsulated astaxanthin in different encapsulating materials by spray drying in order to evaluate their stability against different pH conditions. It was observed that whey protein was the most suitable wall material because astaxanthin presented greater stability in relation to color and antioxidant activity. In

addition, pHs of 4–7 provide greater stability for application of astaxanthin and the microencapsulation process was adequate for its protection.

Also with respect to astaxanthin, Bustamante et al. (2016) encapsulated *Haematococcus pluvialis* oleoresin. However, astaxanthin loses its natural protection when extracted. Thus, *Haematococcus pluvialis* oleoresin containing astaxanthin was encapsulated in Capsul® by spray drying and its stability evaluated at 40, 50, and 70 °C. It was found that the microencapsulation process improved the stability of astaxanthin and tocopherol present in the oleoresin under exposure to different temperatures (40, 50, and 70 °C), prolonging its activity. These authors also emphasize that the encapsulation of *H. pluvialis* is an alternative for the development of a functional ingredient for use in food. Lastly, Vakarelova et al. (2017) encapsulated *Haematococcus pluvialis* astaxanthin in low methoxylation pectin and alginate by extrusion. The objective of this study was to evaluate the stability of astaxanthin in these encapsulating materials, as well as the technique used for microencapsulation and during storage for 52 weeks at refrigeration temperature. It was observed that the microencapsulation process was able to provide high encapsulation efficiency due to the minimal stress that the extrusion process offers and that mainly the low methoxylation pectin was suitable for microencapsulation and provided high stability of astaxanthin under light exposure, temperature, and oxygen, and preservation under storage at refrigeration temperature, making the process feasible for future applications.

Another pigment that has been well studied in relation to microencapsulation is phycocyanin. Phycocyanin is a light blue pigment that has antioxidant properties. Thus, the microencapsulation of phycocyanin is interesting for its protection, mainly due to its instability against light, temperature, and pH, during processing and storage (Dewi et al. 2018). Thus, Hadiyanto et al. (2017) encapsulated phycocyanin into alginate by extrusion in order to obtain an optimal condition for the encapsulation of phycocyanin into alginate and exposed the microcapsules under simulated gastrointestinal conditions. It was observed that the microencapsulation process was adequate to the phycocyanin, with high encapsulation efficiency and that the microcapsules were able to protect the phycocyanin in the gastric juice, releasing it only in the intestinal juice. Similarly, Yan et al. (2014) encapsulated phycocyanin into alginate and chitosan by extrusion in order to evaluate storage stability and under simulated gastrointestinal conditions. These authors observed that the stability of phycocyanin was preserved in storage for 40 days at up to 50 °C and that microcapsules resisted passage through gastric juice and released phycocyanin into the intestinal juice, preserving it. Finally, Dewi et al. (2018) encapsulated phycocyanin in maltodextrin and k-carrageenan by spray drying. The aim of this study was to evaluate the potential of these wall materials to protect phycocyanin. It was verified that the microencapsulation process was adequate for phycocyanin, where the encapsulating materials offered the same protection and high encapsulation efficiency.

Currently, different techniques and wall materials have been used, so it is extremely important to choose the most suitable ones for the type of compound that will be microencapsulated. According to Tyagi et al. (2011) the technique and wall materials (type, hydrophilicity, ratio of active material to wall material, among

others) directly influence the encapsulation characteristics, including the encapsulation efficiency, stability, solubility, and activity of the compound a be encapsulated. Table 26.3 shows the main wall materials used in microencapsulation techniques.

In relation to the microencapsulation process, the techniques are classified into three ways: (I) physical methods, (II) physical-chemical methods, and (III) chemical methods (Tyagi et al. 2011). Table 26.4 demonstrates the techniques and their classifications.

In this sense, there is a great need for the development of microencapsulation strategies based on the knowledge of the active agents and their desired beneficial effects, formulating coating materials with the required functional properties, as well as the selection of suitable encapsulation techniques and adjusting the specific conditions of processing for the core materials (Ye et al. 2018).

Thus, the importance of microencapsulation for the protection and supply of microalgae and its by-products is shown in Table 26.5, which shows a literature review of coating techniques and materials being used in microalgae research. In addition, the following will be covered a bit about each technique.

The solvent evaporation technique involves four major steps: (I) forming a suspension, emulsion, or solution, by dispersing the coating polymer and the active compound in an organic solvent; (II) by agitation, static mixing, extrusion, or dripping, emulsification of the organic phase (dispersed phase) is carried out in an aqueous phase (continuous phase); (III) removal of the solvent by evaporation or liquid extraction, and (IV) recovery of the particles by filtration or centrifugation, as well as drying thereof. It is considered an interesting technique, since it offers soft processing conditions, easy use, suitable for large scale, less amount of residual solvent, and does not interfere in the characteristics of the active compounds (Hwisa et al. 2013).

Complex coacervation can be basically defined as a phenomenon of liquid-liquid phase separation between biopolymers with opposing charges through electrostatic interaction. Factors such as pH, polymer concentration, polymer mixing ratio, ionic strength, and temperature may interfere with the formation of complex coacervates (Eghbal and Choudhary 2018). According to Ozkan et al. (2019) is superior to other microencapsulation techniques due to low processing temperature, high encapsulation efficiency, no need for specific equipment and simple conditions such as nontoxic solvents and low stirring. However, the complexity of the technique must be taken into account. Thus, its use for microalgae is still scarce.

The techniques based on supercritical fluids use these fluids as solvents that have properties (density, solvation power, and gases) between the liquids, as well as temperature, and pressure below the critical point. They are compounds such as carbon dioxide, water, propane, nitrogen, etc. Processes based on supercritical fluids are generally classified into three categories: (I) solvent; (II) anti-solvent, and (III) solute, depending on the function of the supercritical fluid. Furthermore, techniques based on supercritical fluids may be an alternative to the disadvantages presented by some conventional techniques, such as low size control and particle morphology, degradation and loss of biological activity in temperature sensitive compounds, and low encapsulation efficiency (Ozkan et al. 2019).

Table 26.3 Coating materials used in the encapsulation of microalgae-based products

Materials	Microencapsulated microalgal compound	References
Carbohydrates		
Sodium alginate	Phycocyanin	Yan et al. (2014)
	Astaxanthin	Vakarelova et al. (2017)
Capsul®	Oleoresin of *Haematococcus pluvialis*	Bustamante et al. (2016)
Carrageenan gum	Phycocyanin	Dewi et al. (2016)
Gum arabic	*Chlorella vulgaris*	Raposo and Morais (2011)
	Astaxanthin	Bustos-Garza et al. (2013)
	Extract of *Phormidium valderianum*	Chatterjee et al. (2014)
	Biomass of *Tetraselmis chuii*	Bonilla-Ahumada et al. (2018)
Inulin	Astaxanthin	Bustos-Garza et al. (2013)
Lactose	Astaxanthin and *Haematococcus pluvialis*	Chen et al. (2016)
Maltodextrin	*Chlorella vulgaris*	Raposo and Morais (2011)
	Astaxanthin	Bustos-Garza et al. (2013)
	Extract of *Phormidium valderianum*	Chatterjee et al. (2014)
	Phycocyanin	Dewi et al. (2016)
	Biomass of *Tetraselmis chuii*	Bonilla-Ahumada et al. (2018)
Low methoxylation pectin	Astaxanthin	Vakarelova et al. (2017)
Chitosan	Phycocyanin	Yan et al. (2014)
	Biomass of *Tetraselmis chuii*	Bonilla-Ahumada et al. (2018)
Proteins		
Casein	Astaxanthin and *Haematococcus pluvialis*	Chen et al. (2016)

(continued)

Table 26.3 (continued)

Materials	Microencapsulated microalgal compound	References
Gelatin	*Chlorella vulgaris*	Raposo and Morais (2011)
	Biomass of *Tetraselmis chuii*	Bonilla-Ahumada et al. (2018)
Whey protein	Astaxanthin	Bustos-Garza et al. (2013)

Table 26.4 Classification of microencapsulation techniques (Ozkan et al. 2019)

Classification
Physical methods
Spray drying
Freeze drying
Supercritical fluids-based techniques
Solvent evaporation
Physical-chemical methods
Coacervation
Liposomes
Ionic gelation
Chemical methods
Interfacial polymerization
Molecular inclusion complexation

Table 26.5 Application of microencapsulation to microalgae-based products

Technique	Coating materials	Core material	Authors
Multiple emulsion/solvent evaporation	Chitosan	Astaxanthin	Higuera-Ciapara et al. (2004)
Complex coacervation	Gelatin Gum arabic	Oil of *Schizochytrium* sp.	Zhang et al. (2012)
Supercritical fluids-based techniques	PHBV	Astaxanthin	Machado et al. (2014)
Freeze drying	Maltodextrin Tween-80	Pigment of *Sargassum* sp.	Indrawatia et al. (2015)
Spray drying	Gum arabic Chitosan Gelatin Maltodextrin	*Tetraselmis chuii*	Bonilla-Ahumada et al. (2018)
Ionic gelation	Alginate Calcium chloride	Astaxanthin	Niizawa et al. (2019)

The lyophilization encapsulation technique is composed of different stages: (I) freezing; (II) sublimation; (III) desorption; and (IV) storage (Ray et al. 2016). The advantages that lyophilization presents in relation to other techniques are the low temperature and the absence of air during the process, being a simple process, besides avoiding deterioration by oxidation or chemical reactions, being able to be used for natural oils, pigments, aromas, and water-soluble compounds, thus being the most suitable technique for drying thermosensitive substances.

Ionic gelation is based on the crosslinking of polyelectrolytes by multivalent ions such as Ca^{2+}, Ba^{2+}, and Al^{3+} and can be applied by extrusion or emulsification/gelation. Extrusion is more common and occurs by dripping an aqueous polymer solution through a syringe or an extruder nozzle into a solution containing $CaCl_2$. The emulsification/gelation basically occurs by the production of an emulsion containing the hydrophobic active component in a polymer solution and is dripped in a calcium solution, which can be carried out externally, where the Ca^{2+} ions are added from an external source to the polymer solution, or internally, where the calcium salts are added by dripping to the polymer solution, resulting in the production of particles.

This technique does not require specific equipment and application of heat treatments, making it advantageous and presenting as an alternative for the encapsulation of highly reactive and sensitive compounds, increasing their stability during storage. In addition, it is a technique that has been widely used for many years (Niizawa et al. 2019).

The spray drying technique consists in the production of highly dispersed powders from the atomization of a fluid within a drying chamber containing a heated air, causing the solvent to evaporate uniformly and rapidly by means of direct contact, obtaining the powdered product (Bora et al. 2018). It is an advantageous technique because it is considered fast, continuous, simple, economical, and easy reproducibility, besides high encapsulation efficiency and high physical, chemical, and microbial stability. In addition, it is the most commonly used technique for the encapsulation of compounds for the food industry (Wang et al. 2017).

Finally, from this review, it can be observed that the techniques of ionic gelation and spray drying are widely used for this purpose. Thus, Table 26.6 reports the diversity of researches conducted with microalgae and their by-products, using these techniques.

Future perspectives

The microencapsulation of microalgal compounds, nowadays, appears as an alternative to the vulnerability of these compounds. However, because its use in this sense is recent, it is necessary to develop further research involving pigments that have not yet been encapsulated, as well as, the more detailed study related to the different techniques and their materials, the applicability of microalgal compounds in different products, besides the possibility of the reduction to the nanoscale, which can be very promising for the protection and application of these compounds.

Table 26.6 Microencapsulation of microalgae-based products by ionic gelation and spray drying

Ionic gelation/extrusion	Microencapsulated compounds	Results
Pannier et al. (2014)	*Chlorella vulgaris*	Protection of *Chlorella vulgaris* against herbicide atrazine
Yan et al. (2014)	Phycocyanin	Preservation of phycocyanin under storage at a temperature of 50 °C
Hadiyanto et al. (2017)	Phycocyanin	Protection of phycocyanin in the simulated gastrointestinal tract
Nilamsari et al. (2018)	Phycocyanin	Preservation of phycocyanin
Niizawa et al. (2019)	Astaxanthin	Protection of astaxanthin under simulated gastrointestinal conditions
Spray drying		
Bustos-Garza et al. (2013)	Astaxanthin	Increased astaxanthin stability
Chatterjee et al. (2014)	Extract of *Phormidium valderianum*	Preservation of natural antioxidant activity
Lee and Hong (2015)	Extract of *Chlorella*	Preservation of chlorophyll stability in 40 days of storage
Bonilla-Ahumada and Khandual (2016)	Biomass of *Tetraselmis chuii*	Preservation of nutritional value Preservation of β-carotene Preservation of antioxidant activity
Bustamante et al. (2016)	Oleoresin of *Haematococcus pluvialis*	Improvement in the stability of astaxanthin and tocopherol
Chen et al. (2016)	Astaxanthin	Preservation of astaxanthin stability
Dewi et al. (2016)	Phycocyanin	Preservation of antioxidant activity
Bonilla-Ahumada et al. (2018)	Biomass of *Tetraselmis chuii*	Preservation of β-carotene and phenolic compounds
Dewi et al. (2018)	Phycocyanin	Preservation of phycocyanin

Final considerations

Microalgal pigments have several important qualities in the nutritional and food context. Known for their antioxidant and nutraceutical activities, this type of compound has been extensively studied. Thus, this work presented the characteristics of three known microalgal pigments: chlorophylls, carotenoids, and phycobilins.

Although they have several benefits, these pigments may be subject to adverse conditions, such as thermal or oxidative processes, after being extracted from the microalgae biomass. These processes can cause degradation of the pigment and

impair its biological activities. By virtue of this problem, techniques for increasing the stability of microalgal compounds are of paramount importance.

In view of the above, the microencapsulation of microalgae and its by-products have been extensively researched and used as an efficient way to aid in increasing the stability of pigments. Microencapsulation protects the substances involved in the process without loss of biological properties. That is, when applied to microalgae, encapsulation techniques allow the biological activities of the pigments to remain protected from adverse conditions. Thus, the microencapsulation process is promising in this sense, offering protection to the microalgae, facilitating the handling and transportation, besides increasing stability and efficiency in the delivery of highly sensitive by-products, proving to be also an effective and suitable process for such compounds.

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