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# Sustainable Production of Pigments from Cyanobacteria


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## Contents



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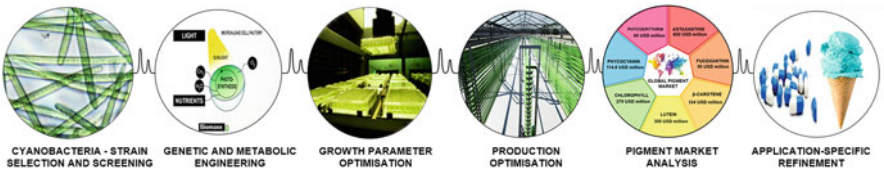
**Abstract** Pigments are intensely coloured compounds used in many industries to colour other materials. The demand for naturally synthesised pigments is increasing and their production can be incorporated into circular bioeconomy approaches. Natural pigments are produced by bacteria, cyanobacteria, microalgae, macroalgae, plants and animals. There is a huge unexplored biodiversity of prokaryotic cyanobacteria which are microscopic phototrophic microorganisms that have the ability to capture solar energy and $CO_2$ and use it to synthesise a diverse range of sugars, lipids, amino acids and biochemicals including pigments. This makes them attractive for the sustainable production of a wide range of high-value products including industrial chemicals, pharmaceuticals, nutraceuticals and animal-feed supplements. The advantages of cyanobacteria production platforms include comparatively high growth rates, their ability to use freshwater, seawater or brackish water and the ability to cultivate them on non-arable land. The pigments derived from cyanobacteria and microalgae include chlorophylls, carotenoids and phycobiliproteins that have useful properties for advanced technical and commercial products. Development and optimisation of strain-specific pigment-based cultivation strategies support the development of economically feasible pigment biorefinery scenarios with enhanced pigment yields, quality and price. Thus, this chapter discusses the origin, properties, strain selection, production techniques and market opportunities of cyanobacterial pigments.

**Graphical Abstract**




**Keywords** Astaxanthin, Chlorophyll, Fucoxanthin, Lutein, Phycocyanin, *Spirulina*

## Abbreviations

| | |
|---|---|
| ASE | Accelerated solvent extraction |
| ATP | Adenosine triphosphate |
| BDW | Biomass dry weight |
| CAGR | Compound annual growth rate |
| Chl | Chlorophyll |
| Cytb6 | Cytochrome b6 |
| EFSA | European Food Safety Authority |
| ETC | Electron transport chain |
| Fd | Ferredoxin |
| FDA | Food and Drug Administration |

| | |
|---|---|
| FNR | Ferredoxin $NADP^+$ reductase |
| FRP | Fluorescence recovery protein |
| HPH | High-pressure homogenisation |
| HRP | High-rate pond |
| LCA | Life-cycle assessment |
| $L_{CM}$ | Linker (protein) core membrane |
| MEP | Methylerythritol phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NPQ | Non-photochemical quenching |
| OCP | Orange carotenoid protein |
| PAR | Photosynthetically active radiation |
| PBP | Phycobiliproteins |
| PBR | Photobioreactor |
| PC | Phycocyanin |
| PCB | Phycocyanobilin |
| PE | Phycoerythrin |
| PEB | Phycoerythrobilin |
| PEF | Pulsed-electric field |
| PLE | Pressurised liquid extraction |
| PQ | Plastoquinone |
| PS | Photosystem |
| PUB | Phycourobilin |
| PVB | Phycoviolobilin |
| RC | Reaction centre |
| $SCCO_2$ | Super critical carbon dioxide |
| TEA | Techno-economic assessment |

## 1 Introduction

Earth formed around 4.6 billion years ago [1] and the Sun remains its largest energy source, delivering 3,020 ZJ $year^{-1}$ to the Earth's surface. The massive scale of this energy supply is highlighted by the fact that every 2 h Earth receives more energy than we need to power our total global economy for an entire year (~0.56 ZJ $year^{-1}$) [2]. Geological records indicate that around 3.4 billion years ago, early anoxygenic photosynthetic organisms evolved [3] using light absorbing pigments, today typified by chlorophylls and carotenoids bound as cofactors to proteins. These organisms were not yet able to catalyse the highly oxidising photosynthetic water splitting reaction of oxygenic photosynthesis. As a result, instead of water, purple bacteria, green sulphur bacteria, acidobacteria and heliobacteria used a range of alternative, available and more energetically accessible substrates as electron donors. These included hydrogen sulphide, dihydrogen, thiosulphate, elemental sulphur and ferrous iron [4]. Of these, early cyanobacteria evolved to use sulphides [5]. About 2.4 billion years ago, a genetic fusion event is thought to have taken place between two bacteria, one with a pheophytin-quinone reaction centre (Type II – an archetypal

form of Photosystem II; Q-type) and the other with an iron-sulphur reaction centre (Type I – an archetypal form of Photosystem I; FeS-type) to produce a chimeric photosynthetic organism with two unlinked photosystems [3]. Subsequently, these two archetypal photosystems evolved further and were linked into one operational photosynthetic electron transport chain. Development of the oxygen evolving complex of PSII [6, 7] enabled it to catalyse the most oxidising reaction in biology (water photolysis). This photosynthetic electron transport chain enabled cyanobacteria to use the huge energy resource of the Sun to split water into protons, electrons and oxygen to provide ATP and reducing equivalents such as NADPH [7]. Cyanobacteria remained the principal oxygenic photosynthetic organisms throughout the Proterozoic Eon (2,500 to 541 mya) and are thought to be responsible for the Great Oxidation Event (i.e. the rise of the oxygen concentrations in the atmosphere and oceans [8]). Later, capture of cyanobacteria by eukaryotes expanded oxygenic photosynthesis into a range of other organisms, including red algae, glaucophyta, green algae and higher plants, capable of producing and coordinating a range of pigments involved in photosynthesis to provide the food, fuel, biomaterials and atmospheric oxygen that support aerobic life on Earth [8]. This chapter elaborates on the many pigments coordinated within these intricate cyanobacterial cells and particularly their role in photosynthesis and the economic opportunities that these provide for commercial scale sustainable production platforms across the food, pharmaceutical, biomaterials and primary production (aquaculture and livestock feed) sectors.

*Cyanobacteria* are commonly referred to as blue-green algae but are strictly speaking microscopic prokaryotic photosynthetic bacteria. They exist as single cells, filaments, sheets or spherical clusters of cells and are found in diverse habitats including fresh, brackish and salt water. Under favourable environmental conditions, cyanobacteria can exhibit high growth rates but can also resist harsh environments through dormancy [9]. Cyanobacteria contain a range of pigments including chlorophylls (green), carotenoids (red, orange and yellow), phycobiliproteins (red and blue) and scytonemin (yellow-brown). These pigments function largely in photosynthesis and photoprotection and have useful properties that can be translated into advanced technical and commercial products [10, 11] and in certain cases (e.g. phycocyanin which has been explored to treat autoimmune encephalomyelitis [12]) are potentially beneficial to human health [13–15] and the environment (through biodegradability) [16].

*Pigments* are intensely coloured compounds that are used in a broad range of industries to colour other materials. They are extensively used to enhance the attractiveness of industrial products and are usually termed '*pigments'* in the pharmaceutical, ink and cosmetic industries and *'dyes'* in the food and textile industries [17]. They are broadly classified into organic vs. inorganic as well as natural vs. synthetic categories [17]. *Organic pigments* are carbon-based compounds with conjugated chains and rings, either synthetic or natural. *Inorganic pigments* are usually metals and metallic salts that are typically insoluble, heat stable opaque oxides such as Prussian blue (Iron (III) ferrocyanide, produced by the oxidation of ferrous ferrocyanide salts), cobalt blue, cadmium yellow, lead oxide and titanium yellow. *Natural pigments* are mainly organic and include chlorophyll, lutein,

β-carotene, astaxanthin, indole based dyes and anthocyanins and are widely used as food colourants (e.g. chlorophyll derivatives) and nutraceuticals (e.g. lutein from marigold flowers used in functional foods) for human consumption [18]. *Synthetic pigments* are usually carbon-based molecules chemically derived from petrochemical products, acids and other chemicals. Even when synthetic pigments are copies of natural products, their activity may not be the same. This is because natural products are often chiral in nature while their synthetic counterparts may be racemic. For example, synthetic astaxanthin produced from petrochemical products (e.g. the Wittig reaction) is reported to provide less antioxidative activity than natural astaxanthin (55x less singlet oxygen quenching capacity and 20x less free radical elimination [19]). Some synthetic pigments (e.g. citrus red II, metanil yellow and rhodamine B) are reported to have various toxicological effects, including carcinogenesis, oestrogenic activity and neurotoxicity [20] which has increased the desirability of natural pigments. Pigments in the food sector are strictly regulated due to health and safety concerns [21, 22]. Synthetic pigments are inexpensive and typically stable, but increasing health and environmental awareness has led to market-driven expansion of the naturally derived pigment sector as part of an expanding circular bioeconomy [23, 24]. In terms of industrial-scale pigment production it is important to note that pigments can be produced as isolated *coloured chromophores* such as chlorophylls, carotenoids and pheophytin (Fig. 1b), phycoerythrobilin (PEB) and phycocyanobilin (PCB; Fig. 1c), or as the *coloured proteins* that coordinate them (e.g. phycoerythrin, phycocyanin and allophycocyanin). To avoid confusion, isolated chromophores are here referred to as *chromophores* and chromophore binding proteins as *coloured proteins.* Collectively, along with other coloured molecules, they are referred to as *pigments*.

The *global pigment market* including both natural and synthetic pigments was estimated to be USD $36.4 billion in 2020 and based on a 5.1% Compound Annual Growth Rate (CAGR) between 2021–2028 is forecast to expand to USD $51.7 billion in 2028 [25]. Different market sectors comprising textiles (62%), leather (10%), printing inks (10%) and others (food, nutraceuticals, pharmaceuticals and cosmetics, 18%) provide significant opportunities for high quality natural pigments. Compared to plant and animal sources, microbial pigment production is more sustainable [26], providing opportunities for the production of biodegradable colourants (e.g. phycocyanin from *Arthrospira platensis* (*Spirulina*)). For large-scale production, cyanobacteria offer specific advantages for pigments unique to cyanobacteria (e.g. phycocyanin and scytonemin) or that they can deliver higher yields (e.g. lutein yields are reported to be three- to sixfold higher than in marigold). Other potential benefits of cyanobacterial systems include lower cultivation time (compared to plants; days/weeks vs season), lower cultivation cost [27], less arable land (ability to use non-arable land and floating systems), low freshwater demand (ability to grow in closed systems using recycled freshwater/seawater/brackish water) and labour requirements [28–30]. Furthermore, cyanobacteria are amenable to genetic engineering to support further improvement.

This chapter focusses specifically on natural pigment production from cyanobacteria – their properties, applications, current extraction technologies and market trends.

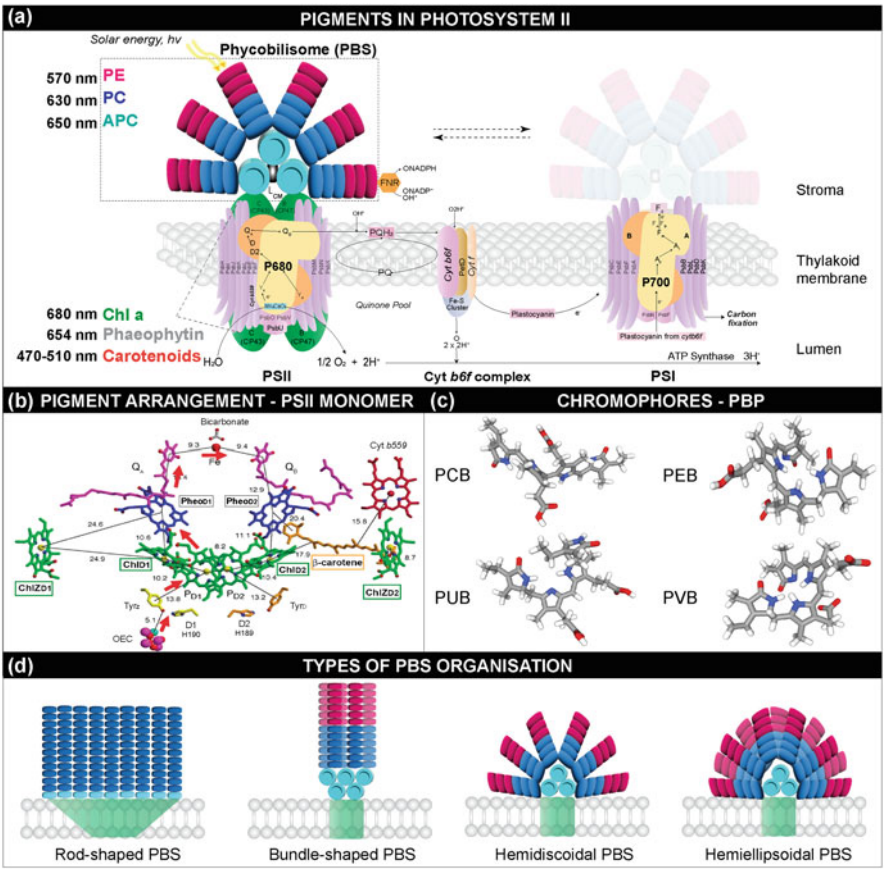



**Fig. 1** Cyanobacterial light harvesting antenna and pigment organisation. (**a**) Cyanobacterial photosynthetic electron transport chain including the dynamic extrinsic antenna system consisting of phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC) is connected to the stromal surface of the PSI and PSII core complexes via the Core-Membrane Linker ($L_{CM}$). (**b**) Example of pigment coordination within the PSII monomer. (**c**) Four major chromophores in cyanobacteria. The chromophores Phycocyanobilin (PCB; $C_{33}H_{40}N_4O_6$), Phycoerythrobilin (PEB; $C_{33}H_{38}N_4O_6$), Phycourobilin (PUB; $C_{33}H_{42}N_4O_6$) and Phycoviolobilin (PVB; $C_{33}H_{34}N_4O_6$). (**d**) Typical phycobilisome (PBS) organisation: rod-shaped, bundle-shaped, hemi-discoidal and hemi-ellipsoidal. In most cyanobacteria the hemi-discoidal organisation occurs but the pigment composition within these rods is species-specific

## 2 Cyanobacterial Pigments

The first step of photosynthesis is light capture, which is mediated by the light harvesting antenna proteins of photosystems I (PSI) and II (PSII). These light harvesting antenna systems are designed to capture Photosynthetically Active Radiation (PAR) in the visible spectrum (400–700 nm). In cyanobacteria, these antenna systems consist of pigment-protein complexes located on and in the thylakoid

membranes, which lie under the cell membrane (see Fig. 1), typically in a dense multilayered wrapping (Fig. 6, Sect. 5.2). The extrinsic and intrinsic antenna proteins have evolved to provide a dynamic scaffold that coordinates an intricate and excitonically coupled network of chromophores including phycoerythrobilin (PEB; Fig. 1c), phycocyanobilin (PCB; Fig. 1c), phycourobilin (PUB; Fig. 1c), phycoviolobilin (PVB; Fig. 1c), chlorophylls, pheophytins and carotenoids that collectively support the dual function of PSI and PSII light-driven charge separation and photoprotection. The extrinsic antenna systems include the light harvesting protein complexes (phycoerythrin, phycocyanin and allophycocyanin) which usually coordinate the chromophores phycoerythrobilin and phycocyanobilin within them and connect them into the excitonically coupled chromophore network coordinated by the PSI and PSII core complexes [31].

The cyanobacterial PSII core complex is composed of around 20 subunits (Fig. 1a). In 2001 a 3.8 Å resolution PSII core complex structure from *Synechococcus elongatus* was described [32]. Each 350 kDa PSII monomer (Fig. 1b) is reported to contain 17 membrane spanning protein subunits as well, three extrinsic proteins, 99 cofactors, 35 chlorophyll a, 12 β-carotene, 2 pheophytin, 2 plastoquinone and 2 heme molecules, the water splitting $Mn_4CaO_5$ cluster and one non-heme $Fe^{2+}$ [33]. The electrons extracted from water by PSII are passed, via the cytochrome $b_6f$ complex (a dimer which includes one chlorophyll and one carotenoid per monomer) to PSI, contributing to the generation of an electrochemical gradient across the membrane that drives ATP production [34]. At PSI, photons harvested by its phycoerythrin, phycocyanin and allophycocyanin antenna system are passed on to the PSI core complex to drive charge separation and raise the redox potential of the donated electrons [35]. Specifically, PSI catalyses the light-induced electron transfer from plastocyanin or cytochrome *c6* to ferredoxin or flavodoxin via its chain of electron carriers [36, 37]. The first crystal structure (2.5 Å resolution) of the cyanobacterial *Synechococcus elongatus* PSI complex was also reported in 2001 [38]. Cyanobacterial PSI core complexes are typically trimeric with each monomer core consisting of 12 subunits and 127 cofactors which include 96 chlorophylls, 22 carotenoids, two phylloquinones and three iron-sulphur (4Fe4S) clusters [36, 37]. The subunits collectively stabilise the core-antenna system and help them interconnect with peripheral antenna systems. Within the PSI core is the redox active PSI reaction complex which consists of PsaA and PsaB which coordinate the key intrinsic redox active cofactors in the membrane [37]. Plastocyanin/cytochrome *c6* are soluble electron carrier proteins that donate electrons at the luminal surface of PSI. Cytochrome c6 is likely the evolutionary older electron donor as it can be found in most cyanobacteria [39, 40]. Excitation energy transfer from the antenna chlorophylls leads to excitation of $P_{700}$ to the excited state $P_{700}$*, which catalyses the primary charge separation [41]. Upon illumination, electrons are transferred from plastocyanin/cytochrome c6 at the luminal surface of the PSI reaction centre to ferredoxin/flavodoxin at the PSI stromal surface.

## 2.1 Phycobiliproteins

*Definition:* Cyanobacterial phycobilisomes (PBS) (Fig. 1a) are large organised complexes of water-soluble phycobiliproteins (PBPs), phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC) and their chromophores [42, 43]. Their chromophores (phycocyanobilin and phycoerythrobilin) are synthesised from glutamic acid, which is converted to aminolevulinic acid (ALA), two molecules of which form porphobilinogen and ultimately protoporphyrin IX by the action of three enzymes (Fig. 2a). The enzyme Fe-chelatase catalyses the formation of protoheme from protoporphyrin IX. Subsequently, this protoheme is converted to biliverdin IX, from which phycocyanobilin and phycoerythrobilin are produced.

*Classes:* The 3 major PBPs (PE, PC and APC) [35] have been further classified into six groups based on their light absorption and fluorescence properties: phycoerythrocyanin, C-phycoerythrin (C-PE) and R-phycoerythrin (R-PE), C-phycocyanin (C-PC), allophycocyanin (APC) and allophycocyanin-B (AP-B) [35] (Table 1).

*Sources:* Phycobilisomes (PBS) are unique to cyanobacteria and some red macroalgae [45]. In green microalgae and higher plants they were replaced by transmembrane chlorophyll a/b binding proteins [46]. In cyanobacteria, phycobiliproteins make up a large proportion of soluble proteins; e.g. *Nostoc commune* (54%), *Scytonema* sp. (37%), *Lyngbya* sp. (32%) and *Anabaena* sp. (8%) [47].

*Structures & Properties:* The PBS consist of water-soluble phycobiliproteins (PBPs) and hydrophobic linker peptides and are classified into 4 structural types which are both species and light-dependent: rod-shaped, hemi-ellipsoidal, hemi-discoidal and bundle-shaped (Fig. 1b). The most common and stable type of PBS organisation is reported to be the hemi-discoidal form (4.5–15 MDa) [48]. It is thought to accommodate a maximum of 800 chromophores per PSII dimer [49]. The bundle-shaped PBS was found in *Gloeobacter violaceus* and reported to support among the fastest energy transfer rates [49]. The rod-shaped PBS was found in *Acaryochloris marina* and the excitation energy transfer is reported to be unidirectional and faster in PS II (compared to hemi-discoidal form) because of its differential organisation of APC and PC [50].

*PC* is ubiquitous in cyanobacteria and present at high intracellular levels. It consists of two subunits: α-PC (15 kDa) and β-PC (19 kDa). These subunits coordinate three PCBs via thioether bonds within each αβ PC monomer [51]. These αβ PC monomers can in turn form PC trimers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$. The fluorescence of PC has been attributed to the covalent linkage of phycocyanobilin to cysteine-84 of α-subunits as well as cysteine-82 and cysteine-153 residues of β-subunits [51]. These coordinated phycocyanobilins collectively contribute to the high Stokes shift of PC (i.e. the difference between the band maxima of the absorption and emission spectra [51]) and its high quantum yield, with maximum fluorescence emission at ~640 nm, and the molar extinction coefficient at $\varepsilon_{620}$ is $1.54 \times 10^6\ M^{-1}\ cm^{-1}$ for a 242 kDa C-PC hexamer [52].

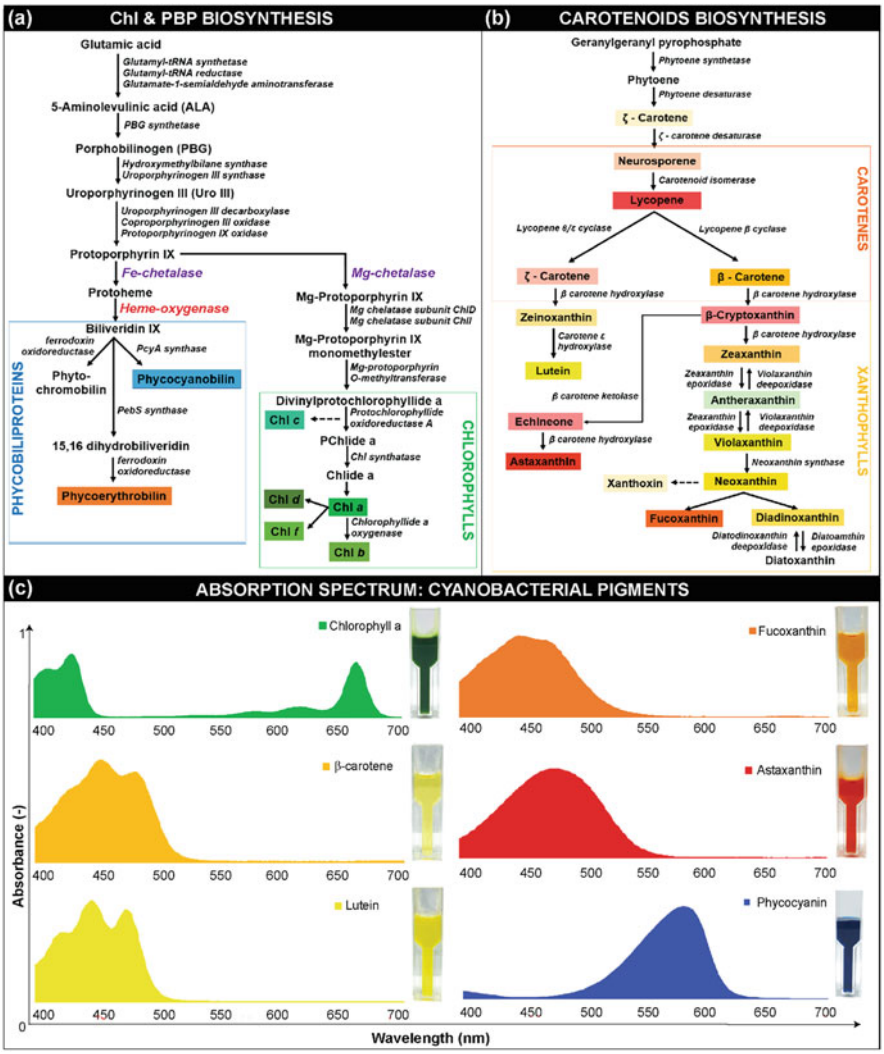
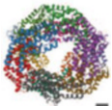
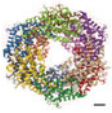
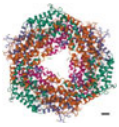



**Fig. 2** Cyanobacterial pigments – biosynthesis and absorption spectra. (**a**) Phycobiliprotein and Chlorophyll biosynthesis. The enzymes Fe-chelatase, Mg-chelatase and Heme oxygenase play important regulatory roles in chlorophyll and bilin synthesis. The enzymes PebS synthase and PcyA synthase catalyse key steps in phycoerythrobilin and phycocyanobilin synthesis, respectively, and are either NAD(P)H- or ferredoxin-dependent bilin reductases. During chlorophyll biosynthesis, Mg-chelatase catalyses the insertion of $Mg^{2+}$ into protoporphyrin IX at the branch point between bilin synthesis and chlorophyll biosynthesis [35]. (**b**) Carotenoid biosynthetic pathway via the Methyl-Erythritol 4-Phosphate (MEP) pathway [44]. Phytoene synthase and phytoene desaturase (red dotted boxes) are both important enzymes in carotenoid biosynthesis. The carotenes and xanthophyll pathways are highlighted by the orange and yellow boxes, respectively. (**c**) Absorption spectra of major cyanobacterial pigments of commercial interest – Chlorophyll (Chlorophyll a), Carotenoids (β-carotene, lutein, fucoxanthin, astaxanthin) and Phycobiliproteins (phycocyanin)

**Table 1** Phycobiliproteins structure (PDB; scale bar 10 nm) and spectral properties ($\lambda_{exc}$ – excitation wavelength)

| PBP pigments | Structure | Colour | Absorption maxima (nm) | Fluorescence emission maxima (nm) |
|---|---|---|---|---|
| Allophycocyanin (4RMP) |  | Bright blue | 652 | 657 ($\lambda_{exc} = 633$) |
| C-phycocyanin (1HA7) |  | Dark blue | 621 | 642 ($\lambda_{exc} = 620$) |
| R-phycocyanin (1F99) |  | Blue | 533,544 | 636 ($\lambda_{exc} = 580$) |
| C-phycoerythrin (5FVB) |  | Reddish pink | 565 | 573 ($\lambda_{exc} = 560$) |
| R-phycoerythrin (1B8D) |  | Red | 566 | 578 ($\lambda_{exc} = 561$) |
| B-phycoerythrin (3 V58) |  | Orange | 545 | 572 ($\lambda_{exc} = 545$) |

*APC* consists of the two subunits α-APC (15 kDa) and β-APC (17 kDa). They coordinate 2 PCB per αβ-APC monomer via thioether bonds [42, 53]. These αβ PC monomers usually form trimeric APC ($(\alpha\beta)_3$). As for PC, the fluorescence of APC has been attributed to the covalent linkage of phycocyanobilin to cysteine-84 of the α-subunit as well as to cysteine-84 and cysteine-155 residues of β-subunit. The APC core (Fig. 1a) is formed by four APC trimers in *Synechocystis* sp. PCC6803 [54] and has a maximum fluorescence emission at ~660 nm, and the molar extinction coefficient at $\varepsilon_{650}$ is $0.7 \times 10^6\ M^{-1}\ cm^{-1}$ for the 104 kDa APC trimer [55].

The two subunits of *PE* named α-PE (20 kDa) and β-PE (22 kDa) are reported to coordinate from 2–6 chromophores via thioether bonds (i.e. 2–6. PEB, PUB or PVB or a combination thereof; Fig. 1) per αβ monomer $(\alpha\beta)_1$ [56]. These αβ-PE monomers are generally organised into disc-shaped trimers $(\alpha\beta)_3$ or hexamers $(\alpha\beta)_6$. As an example, PE in *Gloeobacter violaceus* (PDB: 2VJH) is reported to form hexamers

coordinating 4 PEB and 1 PUB per αβ monomer. The maximum fluorescence emission occurs at ~578 nm and the molar extinction coefficient at $\varepsilon_{578}$ is $2 \times 10^6\ M^{-1}\ cm^{-1}$ for a 240 kDa R-PE hexamer [52].

PBPs emit an intense autofluorescence which results from their strong light absorption and intense fluorescence emission within the visible spectrum when not coupled into the photosystems [57]. Wynam et al. (1985) [57] reported that a proportion of the light energy is absorbed by PE in PBS of *Synechococcus* sp. DC2 when cultivated under excess nitrate. As a result the cells exhibited high autofluorescence as the PE granules accumulated (as a form of stored nitrogen) and were uncoupled from PBS in the photosystems. Efficient excitation energy coupling among the chromophores in the PBP trimers and hexamers in the PBS contributes to high autofluorescence.

*Biological functions:* PE, PC and APC absorb radiation in regions of the visible spectrum in which Chl has a low absorptivity (Fig. 2, 470–620 nm). Photosynthetic organisms typically have antenna systems that are tuned to their environmental conditions to best capture the light energy that they require. For example at the illuminated surface of a water column (euphotic zone) PAR in the 400–700 nm range is abundant, while below this (disphotic zone) less red, yellow and green light is available, resulting in dim blue illumination [58]. Consequently, organisms have evolved antenna systems best adapted to capture differing wavelengths of light under a range of light intensities to support optimal light to chemical energy conversion [35, 59]. Phycoerythrin is adapted to capture high energy wavelengths ($\lambda_{max}$ ~ 565 nm), phycocyanin intermediate energy wavelengths ($\lambda_{max}$ ~ 620 nm) and allophycocyanin low energy wavelengths ($\lambda_{max}$ ~ 650 nm) [60]. Their major biological function is to increase the energy absorbed from light and its transfer to the redox active reaction centres and the special pair chlorophylls (i.e. $P_{680}$ in PSII and $P_{700}$ in PSI). In cyanobacteria, they also offer protection against photodamage [61].

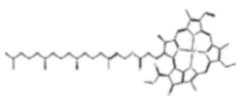
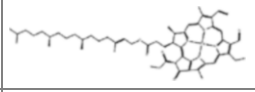
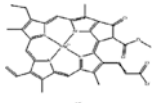
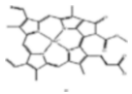
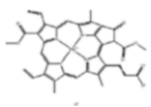
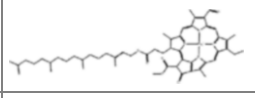
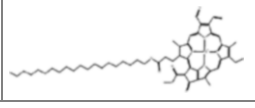
## 2.2 Chlorophylls

*Definition:* Chlorophylls are tetrapyrrole based chromophores that are generally green in colour.

*Classes:* Chlorophylls are classified as Chl *a*, *b*, $c_1$, $c_2$, $c_3$, *d* and *f* in the order that they were discovered [62] (Table 2).

*Sources:* Chlorophylls are abundant in the photosynthetic machinery of cyanobacteria, algae and plants where they are coordinated within specific light harvesting antenna proteins and the redox active reaction centres of PSI and PSII. In cyanobacteria, green plants and green microalgae, Chl *a* is the predominant form of chlorophyll with other chlorophylls usually considered to be accessory chlorophylls. Chl *b* is common in land plants and microalgae while Chl *c* has been reported in marine algae including diatoms, brown algae and dinoflagellates [63].

**Table 2** Chlorophyll structure (ChemDraw 20.1.0) and spectral properties. ($\lambda_{exc}$ – excitation wavelength; NA – Not available)

| Chlorophyll pigments | Chemical structure | Chemical formula | Colour | Absorption maxima (nm) | Fluorescence emission maxima (nm) |
|---|---|---|---|---|---|
| Chl *a* |  | $C_{55}H_{72}O_5N_4Mg$ | Blue/green | 430,664 | 668 ($\lambda_{exc} = 430$) |
| Chl *b* |  | $C_{55}H_{70}MgN_4O_6$ | Green/yellow | 460,647 | 652 ($\lambda_{exc} = 453$) |
| Chl *c1* |  | $C_{35}H_{28}MgN_4O_5$ | Green/ yellow | 442,630 | 633,694 ($\lambda_{exc} = 450$) |
| Chl *c2* |  | $C_{35}H_{28}MgN_4O_5$ | Green/yellow | 444,630 | 635,696 ($\lambda_{exc} = 453$) |
| Chl *c3* |  | $C_{35}H_{28}MgN_4O_5$ | Green/yellow | 452,627 | 635,690 ($\lambda_{exc} = 452$) |
| Chl *d* |  | $C_{54}H_{70}MgO_6N_4$ | Green | 401,696 | NA |
| Chl *f* |  | $C_{55}H_{70}MgO_6N_4$ | Green/yellow | 700 | 720 ($\lambda_{exc} = 425$) |

Chl *d* has been reported in certain cyanobacteria, for example in the cyanobacterium *Acaryochloris marina* it makes up 99% of the chlorophyll [64]. Chl *f* was found in extracts from stromatolytes, layered sedimentary formations which are rich in cyanobacteria [65].

Chlorophyll synthesis (Fig. 2a) involves the reduction of protochlorophyllide. Two pathways exist for chlorophyll biosynthesis, one taking place in darkness (using the enzyme dark-operative protochlorophyllide oxidoreductase) and the other requiring continuous light (light-dependent protochlorophyllide oxidoreductase).

*Structures & Properties:* Chlorophylls *a, b,* $c_1$, $c_2$, $c_3$, *d* and *f* consist of a large aromatic tetrapyrrole macrocycle with a fifth modified cyclopentane, responsible for their light absorption and redox chemistry [66, 67]. A central Mg ion maximises excited state lifetime and the interactions of Chls with their proteins, and in many cases a hydrophobic phytyl tail is present (Chl *a, b, d & f*) although this tail is absent *in* Chl *c1, c2* and *c3* [68]. Chlorophylls differ in their chemical formulae at their C2, C3, C7, C8, C17 positions and in their C17-C18 bonds (Table 2). The only difference between Chl *a* and Chl *b* is that at the C-7 position on the pyrrole ring B, there is a methyl group (–$CH_3$) in Chl *a*, while in Chl *b* there is a formyl group (–CHO) at the same position. In Chl *d* a formyl group (–CHO) replaces the vinyl group (–CH=$CH_2$) at the C-3 position of the pyrrole ring A of Chl *a* (Table 2). In Chl *f* a formyl group (–CHO) instead replaces the methyl group (–$CH_3$) at the C-2 position of the pyrrole ring A of Chl *a* (Table 2).

Although most chlorophylls absorb in the red (660–665 nm) and blue (~430 nm) regions of the spectrum, these structural differences result in subtle shifts in their respective absorption and fluorescence spectra. Consequently, chlorophylls differ somewhat in their colour: Chl *a* is blue-green (absorbs predominantly violet-blue and orange-red light), Chl *b* is yellow-green, Chl *c*'s are blue-green, *Chl d* is green and absorbs in the far-red region of the spectrum (710 nm, outside of the visible range) as does Chl *f* (yellow-green). The phytyl chains of Chl *a, b, d* and *f* make these chlorophylls oil soluble and give them a wax like consistency as solids [69].

*Biological functions:* Collectively chlorophylls have four major biological functions including light capture, excitation energy transfer, acting as electron donors, and energy dissipation (Fig. 1a).

*Light capture:* The first function is to capture light. Different chlorophylls have different absorption spectra. Consequently, by coordinating different combinations of chlorophylls within the antenna systems (e.g. Chl *a* and *b* in the light harvesting systems of microalgae and higher plants) photosynthetic organisms can use chlorophylls to optimise their absorption spectra to capture the light that they require. The broader the absorption spectra and the larger the cross-sectional area of a given antenna, the more light can theoretically be captured [37]. Interestingly in Chl *d* and f the typical red peaks of Chl *a* and *b* are shifted towards the far red (which enables capture of the infra-red portion of the spectrum). Consistent with this it was recently suggested that Chl *f* may function solely as an antenna chromophore [70], but in *Acaryochloris marina*, Chl *d* makes up 99% of the chlorophyll (~80% of total lipid soluble pigment and >2% cell dry weight) suggesting that it also has a role in

primary light harvesting in certain organisms [64, 71]. Chl d assists in the capture of far-red light (FRL) and is thus thought to be responsible for remodelling PSI under FRL-induced photoacclimation (FaRLiP) [64].

*Excitation energy transfer:* The second function of chlorophylls is to support the transfer of excitation energy from the antenna to the redox active Chl *a* dimer ($P_{680}$ and $P_{700}$) in PSII and PSI reaction centres, respectively. Chlorophylls can support long-lived excited states, making them powerful photosensitisers that play an important role in excitation energy transfer. The safe transduction of this excited state into chemical energy is the basis of photosynthesis. Typically, the absorption spectra shift from blue (shorter/higher energy wavelength) towards the red (longer/lower energy wavelength) towards the reaction centres to facilitate energy transfer.

*Electron donor:* The third biological function of chlorophylls is to drive $P_{680}$ and $P_{700}$-mediated redox chemistry. Chlorophylls and chlorophyll derivatives (e.g. pheophytin) can act as primary electron donors and acceptors, transporting electrons within a few picoseconds across half the thylakoid membrane [72]. Here again the ability to support long-lived excited states is important.

*Energy dissipation:* The fourth function of chlorophylls is photoprotection. Under conditions of excess light, the photosystems and particularly PSII are subject to photodamage due to the formation of reactive oxygen species. To prevent this, certain photosynthetic organisms including higher plants and microalgae have evolved mechanisms to dissipate excess light (up to 85–90%) derived energy from chlorophyll-containing proteins [73].

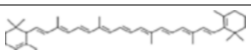
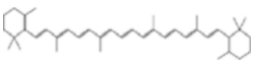
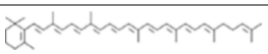
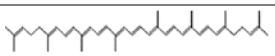
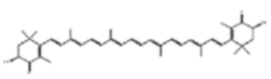
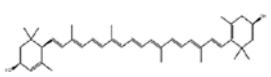
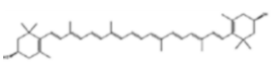
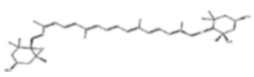
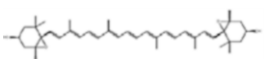
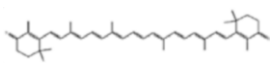
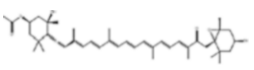
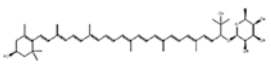
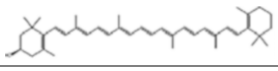
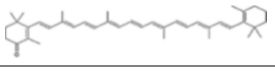
## 2.3 Carotenoids

*Definition:* Carotenoids are lipophilic tetraterpene derivatives which consist of eight isoprene molecules and typically contain 40 carbon atoms [74, 75].

*Classes:* Approximately 1,100 carotenoids [76] have been reported and these have been categorised into carotenes (hydrocarbons) and xanthophylls, which additionally contain oxygen. The structure and properties of some of the most industrially relevant carotenoids are summarised in Table 3. Of these, the *carotenes* include α-carotene, β-carotene, γ-carotene and lycopene. The *xanthophylls* include lutein, zeaxanthin, neoxanthin, violaxanthin, canthaxanthin, fucoxanthin, antheraxanthin, myxoxanthophyll, β-cryptoxanthin and echinenone.

*Sources:* Carotenoids are produced by bacteria, fungi, cyanobacteria, algae, plants and animals, where they fulfil a plethora of different roles, but they are most abundant in photosynthetic organisms. Of these 1,100 carotenoids about 30 are reported to have a function in photosynthesis [77]. Consequently, in photosynthetic organisms, these hydrophobic molecules are often enriched in the thylakoid membrane [74]. In higher plants certain xanthophylls (i.e. zeaxanthin, antheraxanthin and violaxanthin) that are involved in the photoprotective xanthophyll cycle and so are located in the light harvesting complexes in the thylakoid membranes. In cyanobacteria, xanthophylls have been reported to be located in the

**Table 3** Major carotenoid structures (ChemDraw 20.1.0) and spectral properties

| Carotenoid pigments | Chemical structure | Chemical formula | Colour | Absorption maxima (nm) |
|---|---|---|---|---|
| *Carotenes* | | | | |
| α-Carotene |  | $C_{40}H_{56}$ | Light-yellow | 378, 400 and 425 |
| β-Carotene |  | $C_{40}H_{56}$ | Orange | 425, 450 and 480 |
| γ-Carotene |  | $C_{40}H_{56}$ | Yellowish-orange | 437, 462 and 492 |
| Lycopene |  | $C_{40}H_{56}$ | Red | 443, 471 and 502 |
| *Xanthophylls* | | | | |
| Astaxanthin |  | $C_{40}H_{52}O_{24}$ | Red | 482 |
| Lutein |  | $C_{40}H_{56}O_2$ | Yellowish-red | 425, 448 and 476 |
| Zeaxanthin |  | $C_{40}H_{56}O_2$ | Yellow | 428, 454 and 481 |
| Neoxanthin |  | $C_{40}H_{56}O_5$ | Yellow | 486,495 |
| Violaxanthin |  | $C_{40}H_{56}O_5$ | Orange | 417, 440 and 470 |
| Canthaxanthin |  | $C_{40}H_{56}O_2$ | Yellowish-orange | 450, 475 and 506 |
| Fucoxanthin |  | $C_{40}H_{56}O_6$ | Orange | 423 and 445 |
| Myxoxanthophyll |  | $C_{46}H_{66}O_8$ | Bright red | 450, 475 and 506 |
| β-Cryptoxanthin |  | $C_{40}H_{56}O$ | Yellowish-orange | 425, 449 and 476 |
| Echinenone |  | $C_{40}H_{54}O$ | Brownish-red | 452 |

hydrophobic part of the cytoplasmic membranes [78] but they may also be present in the thylakoids [79].

The carotenoids are typically synthesised from isopentenyl pyrophosphate (IPP) via the methylerythritol-4-phosphate (MEP) pathway in cyanobacteria and in chloroplasts of microalgae and higher plants (Fig. 2a) and via the mevalonic acid (MVA)

pathway in the cytosol of bacteria and fungi [77]. Two important enzymes which regulate the first committed steps towards carotene biosynthesis are phytoene synthase and phytoene desaturase. Silencing the genes encoding these enzymes is reported to completely eliminate carotenoid production [80, 81].

*Structures & Properties:* Carotenoids are unsaturated hydrocarbons with extended conjugated double bond networks that are an essential component of their light absorbing (chromophore) [82] and antioxidant properties [77]. Carotenoids generally absorb light in the violet to green (400–550 nm) region of the spectrum and so tend to be yellow, orange and red in colour [83]. Carotenoids which capture light from shorter wavelengths (e.g. 400 nm) are redder. Their individual colours depend on the length of the polyene component (3–13 conjugate double bond systems) which influences the delocalisation of electrons along the entire length of the polyene chain [72, 77]. The longer the conjugated bond system, the more delocalised the electrons within and the lower the energy required to change state. The range of the light energy captured reduces as the length of the conjugated bond system increases [72, 77]. Xanthophylls, which additionally contain oxygen, may possess hydroxyl groups (e.g. hydroxycarotenoids such as zeaxanthin and lutein), keto groups (canthaxanthin and echinenone) and epoxy groups (violaxanthin and diadinoxanthin) [77]. The structures of some xanthophylls are even more complex, combining several functional groups, for example astaxanthin (keto-hydroxy groups), dinoxanthin and fucoxanthin (epoxy-acetylated groups and allene linkages) and monadoxanthin (acetylene linkages) [21].

*Biological functions:* Carotenoids are indispensable components of chlorophyll/carotenoid binding photosystems (Fig. 2a) of photoautotrophs (e.g. cyanobacteria, eukaryotic algae and plants) but also have other roles including the protection of membranes from oxidation [79, 84]. In photosynthesis carotenoids have three key roles: *Structural stabilisation* of the photosystems [85], regulation of *light capture* [86] and supporting *energy dissipation* and *photoprotection*, for example through the process of Non-Photochemical Quenching (NPQ) which dissipates excess energy as heat [86].

*Structural stabilisation*: β-carotene is the only carotenoid reported in the atomic resolution structure of the cyanobacterial PSII complex [84]. For example, *Synechococcus* sp. PCC7335 was reported to have 11–12 β-carotene molecules [87, 88] in PSI (19 β-carotene molecules per monomer of the PSI trimer) when cultivated under far-red light [89]. Carotenoids are reported to assist in maintaining the stability of the PSII structure [90]. For example, the *Synechocystis* sp. PCC 6803, the △*crtB* mutant (deletion of the *crtB* gene coding for phytoene synthase) exhibited limited carotenoid biosynthesis and the absence of xanthophylls. Yet although cyanobacterial phycobilisomes, PSII and PSI reportedly lack xanthophyll, these mutants produced intact phycobilisomes while displaying reduced PSI and PSII oligomerisation. Interestingly, xanthophylls reportedly rigidify the fluid phase of the membranes and limit oxygen penetration to the hydrophobic membrane core (susceptible to oxidative degradation) [78]. This is due to the presence of lipid acyl chains in xanthophyll molecules that are responsible for van-der-Waals interactions [78]. In thylakoids, therefore, this may be important for the correct assembly of PSI,

PSII and their antenna systems [79]. It may also be important for the protection of other membranes against oxidative damage.

*Light capture:* Carotenoids can capture violet-green light. Excited β-carotene molecules that are excitonically coupled to chlorophylls within a light harvesting antenna system can transfer the derived excitation energy to a neighbouring chlorophyll molecule (usually Chl *a*), thereby broadening the absorption spectrum or antenna size of the photosystem [75]. Carotenoids can account for ~20–30% of all light harvested [4, 91].

*Energy dissipation and photoprotection:* In cyanobacteria, the water-soluble Orange Carotenoid Proteins (OCP) which bind a single carotenoid (3′-hydroxy-echinenone; chromophore) can act as photosensors that can trigger light-activation [92, 93] and quenching of excess light energy in the PBS through the release of excess heat. This can prevent oxidative damage to proteins, DNA and lipids [94]. Absorption of blue-green light induces structural changes in both the protein and carotenoid, which triggers NPQ induction, although the NPQ mechanism is still under active investigation [93]. Under low light or in darkness, OCP converts back to the inactive state. This process has been shown to be mediated by another protein called the Fluorescence Recovery Protein (FRP) that interacts with the active form of OCP and accelerates the reconversion of active OCP to the inactive form [95]. Carotenoids also serve as sacrificial molecules to neutralise reactive species (e.g. oxygen free radicals) [4, 96, 97]. Here, β-carotene helps to quench excess light in the chlorophyll triplet state by releasing it as heat [77]. It is the only carotenoid bound to the core reaction centre complex of photosystem II and offers protection against UV radiation [4, 98]. Zeaxanthin and echinenone are reported to protect the repair stage of the PSII recovery cycle from photoinhibition in cyanobacteria by decreasing the level of singlet oxygen that inhibits protein synthesis [99].

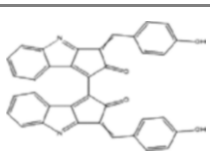
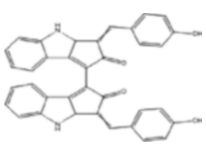
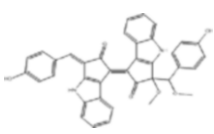
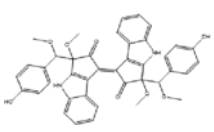
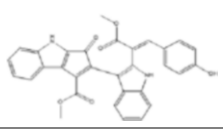
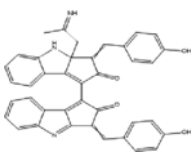
## 2.4 Scytonemin

*Definition:* Scytonemin is an aromatic indole alkaloid (Table 4).

*Sources:* Scytonemin has been reported to accumulate in the extracellular matrix of a broad range of cyanobacteria [100] including species of the genera *Scytonema*, *Aulosira* (*A. fertilissima*), *Nostoc* (*N. linckia*, *N. spongiaeforme, N. punctiforme*), *Schizothrix* (*S. coriacea*), *Lyngbya* (*L. majuscule, L. aestuarii*), *Leptolyngbya* (*L. boryana*), *Laspinema* (*L. thermale*) and *Chlorogloeopsis* (*C. fritschii*). It has been reported that an 18-gene cluster responsible for scytonemin synthesis in *N. punctiforme* is upregulated upon exposure to UV-A radiation and co-transcribed as a single operon [101].

*Structures & Properties:* Scytonemin is a secondary metabolite that absorbs UV-C (100–280 nm), UV-B (280–315 nm) and UV-A (315–400 nm) radiation but has a low absorbance in the PAR (400–700 nm) range. It is generally insoluble in water and moderately soluble in organic solvents. Derivatives of scytonemin include scytonine, dimethoxy-scytonemin, tetramethoxy-scytonemin and scytonemin-imine (Table 4) [101, 102].

**Table 4** Scytonemin-derivatives structure (ChemDraw 20.1.0) and spectral properties

| Scytonemin derivatives | Chemical structure | Chemical formula | Colour | Absorption maxima (nm) |
|---|---|---|---|---|
| *Scytonemin* |  | $C_{36}H_{20}N_2O_4$ | Yellowish brown | 252,278,300,386 |
| *Reduced scytonemin* |  | $C_{36}H_{24}N_2O_4$ | Bright red | 246,276,314,378 |
| *Dimethoxy scytonemin* |  | $C_{38}H_{28}N_2O_6$ | Red | 215,316,422 |
| *Tetramethoxy scytonemin* |  | $C_{40}H_{36}N_2O_8$ | Purple | 212,562 |
| *Scytonine* |  | $C_{31}H_{22}N_2O_6$ | Reddish pink | 207,224,270 |
| *Scytonemin-3a-imine* |  | $C_{38}H_{25}N_3O_4$ | Reddish brown | 237, 366, 437, 564 |

*Biological functions:* The location of scytonemin in the extracellular matrix and its UV absorbing and PAR light transmitting properties likely provide cyanobacterial cells with UV protection while allowing PAR light (400–700 nm) into the cell to drive photosynthesis. The energy captured in the UV range is thought to be released as heat [103]. Scytonemin synthesis is induced by high irradiance and most effectively by UV-A and UV-B radiation (~85%) [104]. Cells surrounded by a scytonemin containing sheath [105] exhibited resistance to UV-A induced photobleaching of Chl *a*. In *Chlorogloeopsis* sp., photosynthesis was inhibited and growth delayed until substantial amounts of scytonemin had been deposited in the sheaths [105].

## 3 Applications

This diverse array of pigments derived from cyanobacteria, i.e. phycobiliproteins (blue and red, Table 1), chlorophylls (green, Table 2), carotenoids (red, orange and yellow, Table 3) and scytonemin (Table 4), can be translated into advanced technical and commercial products [9, 10]. Indeed, cyanobacterial pigments already have a wide range of industrial applications (Fig. 3) especially in the food, cosmetics, nutraceutical and pharmaceutical sectors [17, 106]. Besides their use as colourants and dyes, they are used as food additives, nutraceuticals, putative pharmaceuticals, cosmetics, molecular assays, aquaculture feeds and textiles. One of the first potential


**Fig. 3** Applications of cyanobacterial pigments. Cyanobacterial pigments have been reportedly used as fluorescence probes (Single-Cell Imaging – e.g. Supernova 428 dye), food colourants, food additives, nutraceuticals, putative pharmaceuticals, cosmetics, molecular assays, aquaculture feed and textiles

industrial uses for chlorophyll was during experiments in early colour photography by Becquerel (1874) [107] by employing chlorophyll as a photosensitiser of collodion (a flammable, viscous solution of nitrocellulose in ether and alcohol) and silver bromide. Chlorophylls were also used in surgical dressings and as chelators (carriers of micronutrients like cobalt, zinc, manganese, iron and molybdenum) in hydroponics [11, 16, 21].

### *3.1 Food and Nutraceuticals*

Commercially, phycobiliproteins (PBP) are broadly classified into two categories – phycocyanin and phycoerythrin, based on their colour. Phycocyanin has a bright blue colour and is considered versatile, although it is heat and light sensitive. Phycoerythrin is a bright red water-soluble pigment used as a natural food colourant. Both are non-toxic and have been reported to provide antioxidant [108], anti-cancer [109], anti-inflammatory [110], anti-obesity [111], anti-angiogenic [112], neuroprotective [113] and anti-ageing properties [51, 114], though in many cases this may require further study to verify these claims. Phycocyanin is widely used as a natural colourant in ice cream, soft drinks, candies, chewing gum, desserts, cake decorations, icings and frostings, milk shakes as well as lipsticks and eyeliners [51]. Although PBP-rich *Spirulina* extracts are FDA approved (2013) food colourants and additives, they are susceptible to heavy metal contamination and therefore, human use is tightly regulated [115]. Stable isotope labelled metabolites with phycoerythrin have gained attention as fluorescent probes for cytometry and immunodiagnostics [116, 117].

Cyanobacteria can be produced to contain high levels of carotenoids [118]. The global carotenoid market in 2016 was valued at approximately USD 1.24 billion and forecast to increase to USD 1.74 billion by 2025 at a 4.3% CAGR [119]. The market share of the major carotenoids in this sector, anticipated in 2021 is in the order of β-carotene (26%), astaxanthin (25%), lutein (18%), fucoxanthin (15%), canthaxanthin (10%) and lycopene (6%) [120]. The global chlorophyll market was valued to be USD 279.5 million in 2018 and is anticipated to reach USD 463.7 million by 2025 with a 7.5% CAGR from 2018 to 2025 [121]. In Europe, both carotenoids (yellow, orange and red colour) and chlorophyllins (90% of green colour in food) are widely used as food-colouring agents (approved as Group II food additives; authorised by the European Commission).

Carotenoids play an important role in the global food industry as food additives. Of the many known carotenoids, only ~40 are produced commercially. These include β-carotene and astaxanthin, and, to a lesser extent, lutein, zeaxanthin and lycopene. The major carotenoids produced commercially today are β-carotene and astaxanthin, which are currently produced from the commercial strains *Dunaliella salina* (14% β-carotene of dry weight) [122] and *Haematococcus pluvialis* (3% astaxanthin of dry weight), respectively [123]. The largest astaxanthin consumer is the salmon feed industry (FDA approved in 1987) [124]. Astaxanthin is widely used

in aquaculture feeds [106] as a colourant for fish and shrimp; the reddish pink pigmentation of salmon is considered an important consumer criterion of quality [125]. The annual aquaculture market of this pigment is estimated at USD 200 million, with an average price of USD 2,500 $kg^{-1}$ [123]. Astaxanthin is also known as 'super vitamin E' as it exhibits the highest antioxidant property (500× more potent than α-tocopherol). Natural carotenoids from cyanobacteria have potential to replace commonly used synthetic colourants such as Erythrosine (pinkish red; E127), Sunset Yellow FCF (yellowish orange; E110), Tartrazine (lemon yellow; E102) and Allura red (red; E129). β-Carotene is used as a food-colouring agent with the E number E160. Lutein (bright yellow) cannot be synthesised by humans and has a protective role against macular degeneration of the eye. It is therefore an important dietary supplement (E161b in the European Union) [126, 127]. Hammond et al. (2014) studied the effect of daily uptake of lutein (10 mg) and zeaxanthin (2 mg) supplement in 100 healthy adults over a period of 1 year and regularly recorded their contrast sensitivity and glare tolerance. The study concluded good improvement in both the parameters and thus suggested lutein and zeaxanthin good for ocular health. Carotenoids are also used in nutraceuticals (e.g. astaxanthin approved by FDA as a human nutraceutical ingredient in 2004 [128]). Carotenoids extracted from *Spirulina* sp. are used to treat vitamin A deficiency, β-carotene and cryptoxanthin being precursors of vitamin A [30, 129].

## *3.2 Cosmetics*

The global pigment-based cosmetic market was valued at USD $10 billion in 2020 and is anticipated to increase to USD $17 billion by 2028 at a ~7% CAGR [130]. The demand for natural pigments in the cosmetic industry has significant traction due to the increasing safety concerns associated with synthetic sunscreen compounds that exhibit cytotoxicity [20, 131]. The interest in cyanobacterial pigments in cosmetics (e.g. sunscreens, creams, lotions) is mainly due to their reported photoprotective property (see biological functions in Sect. 2.4) that prevents skin cancer and suppresses ageing-related skin issues (demonstrated through increased cell viability in keratinocyte cell line HaCat, fibroblast cell line 3T3L1 and endothelial cell line hCMEC/D3 exposed to 10 μg $mL^{-1}$ aqueous cyanobacterial extract containing high levels of phycocyanin) [132]. Scytonemin is a yellow to brown lipophilic pigment that is exclusively found in cyanobacteria and is employed in sunscreens due to their promising effect on protection from UV radiation [104, 105]. Scytonemin is extracted from the cell wall of cyanobacteria cultivated under harsh conditions (e.g. exposure to high solar radiation; desiccation). The UV radiation trigger for natural scytonemin production prevented ~92% of radiation from entering the cell, making it a promising ingredient for cosmetics [110, 133]. Further, the cyanobacterial carotenoids, including β-carotene, fucoxanthin, zeaxanthin, lutein, echinenone, astaxanthin and canthaxanthin also exhibit strong antioxidative properties which help in the reduction of UV-induced oxidative damage [123, 134]. Darvin

et al. [135] performed in-vivo carotenoid assays on human skin from healthy normal skin volunteers (20–70 years old) at multiple points over a year and also studied differences in absorption capacity based on the application. They concluded that carotenoids are crucial components of the antioxidative protective system of the human skin and ideally supplied as a topical application. Scarmo et al. [136] demonstrated the effect of carotenoids on skin health by performing dermal biopsies and analysing blood samples to generate a correlation of individual and total carotenoid content in human skin. Carotenoids absorbed in the gut are transported to the epidermis and the two abundant carotenoids found in skin were beta-carotene and lycopene which suggested their role in photoprotection. Lutein and zeaxanthin are marketed as nutraceutical tablets to be ingested and then deposited in lipophilic tissues in humans. Phycobiliproteins have an already established market in the cosmetic sector and are mainly derived from *Arthrospira platensis* (commonly known as *Spirulina platensis*) [51, 137]. Similarly, phycocyanin and phycoerythrin are widely incorporated into hair conditioners, anti-ageing, skin-whitening and anti-wrinkle skin creams and moisturisers, colourant in eye shadow, eye liners, soaps, nail polish and lipsticks [138]. Given the potential of scytonemin in UV screening and free radical scavenging, together with its non-toxic properties [139], this highly stable pigment [133] offers biotechnological opportunities for exploitation by the cosmetics industry [104]. Examples of companies that use cyanobacterial pigments in their cosmetic products today include Lush Cosmetics Pty. Ltd., L'Oreal Pty. Ltd. and Aubrey Organics Inc.

### *3.3 Pharmaceuticals and Diagnostics*

PC is commonly used in immunoassays such as flow cytometry and high-throughput screening [35, 51, 59]. PE is considered one of the world's brightest fluorophores and is widely employed in Time Resolved Laser Induced Fluorescence (TR-LIF), flow cytometry and immunofluorescent staining [140]. Similarly, fluorescent phycobiliproteins are used in fluorescent microscopy, flow cytometry, fluorescence-activated cell sorting, diagnostics, immunolabelling, Fluorescence Resonance Energy Transfer (FRET) assays and immunohistochemistry [59, 60, 137]. Phycobiliproteins are also reported to possess therapeutic properties such as anti-inflammatory and anti-tumour activities [138, 141]. Czerwonka et al. 2018 [142] demonstrated anti-tumour activity of phycocyanin extracts from *Spirulina* sp. Using A549 lung adenocarcinoma cells, and recording cell viability, proliferation and morphology, the cell viability and proliferation of A549 tumour cells were found to be significantly reduced (cell cycle inhibited in G1 phase). The tumour cells were also much more sensitive to PC than the normal skin fibroblasts. Lopes et al. [118] reported the effective treatment of psoriasis using carotenoid extracts from five different cyanobacterial strains from the genera *Alkalinema*, *Cyanobium*, *Nodosilinea*, *Cuspidothrix* and *Leptolyngbya*. HPLC analysis of acetone carotenoid extracts showed high levels of $\beta$-carotene, zeaxanthin, echinenone and lutein.

Lutein also has applications in maintaining ocular health, reportedly acting as a photoprotective agent for macular cells [126]. Reynoso-Camacho et al. [15] demonstrated the efficacy of lutein to treat colon cancer in rat models, by investigating the protein expression levels of K-ras (coded by Kirsten rat sarcoma virus gene, responsible for delivering signals to the cell's nucleus), PKB (Protein Kinase-B, regulates cell survival and apoptosis), and β-catenin (regulates cell–cell adhesion and signal transduction) in rats. Lutein treatment reduced these levels by 25%, 32% and 28% in the prevention phase and by 39%, 26% and 26% in the treatment phase. In another study, FloraGLO® Lutein was found to increase the sensitivity/response of transformed and tumour cells to chemotherapy agents, inducing apoptosis in MCF-7 tumour cells [143]. Scytonemin has antioxidant activity and functions as a radical scavenger to prevent cellular damage resulting from reactive oxygen species produced upon UV radiation exposure and thus has potential applications in biomedical products [104]. Scytonemin is reported to repress proliferation of T-cell leukaemia Jurkat cells ($IC_{50} = 7.8$ μM) in humans [61] and to act as an inhibitor of human polo-like kinase 1 (PLK1), the enzyme involved in regulating the G2/M transition in the cell cycle. Zhang et al. (2013) [144] demonstrated the antiproliferative activity of scytonemin (3–4 μmol/l) against multiple myeloma (anti-tumour activity) targeting PLK1 on three different myeloma cell lines (U266, RPMI8226 and NCI-H929). The study concluded that scytonemin significantly decreased cell proliferation. Thus scytonemin could be used as a therapeutic agent for the management of chronic disorders involving inflammation and proliferation (such as Alzheimer's, arthritis and cystic fibrosis) [145]. Consequently, cyanobacterial pigments offer a broad array of opportunities for further evaluation and industrial scale-up to supply existing markets and realise new opportunities.

## 4 Pigment Production in Cyanobacteria

Cyanobacteria can be used as renewable microbial cell factories [146]. Their optimisation for pigment production requires augmentation of both biomass productivity and pigment yield [11, 17, 147]. The interdependence of these two variables depends on pigment type, and whether the pigments are primary or secondary metabolites. Understanding pigment synthesis pathways and the growth characteristics of production strains are therefore both important.

Cyanobacterial biomass and pigment yields rely on strain-specific characteristics and their alignment with cultivation parameters, such as light intensity and spectral quality [34], the availability of macro and micronutrients [148–150], $CO_2$ supply [150, 151], temperature [152, 153] and mixing rates [151, 154].

## 4.1 Cultivation Parameters and Their Impact on Biomass and Pigment Yields

### 4.1.1 Carbon and Energy Supply

The industrial production modes for microbes differ in their supply strategy for carbon (e.g. hetero- and mixotrophic) and energy (e.g. photo-, chemotrophic). *Chemo-heterotrophic organisms* have a metabolic strategy that derives both energy and carbon from organic compounds (chemosynthesis) to enable growth. Thus, the production processes applying chemo-heterotrophs are essentially depending on the organic carbon source, typically sugars, which can add cost (both media costs and the cost of maintaining sterile cultures) and limit viable options for specific-applications. That said *photo-autotrophic* cultures have added costs due to the need for light and $CO_2$ delivery. Economic and environmental feasibility is thus product-, process and location-specific and can be assessed using techno-economic and life-cycle analysis tools [172].

However, many cyanobacteria are neither completely photo-autotrophic nor completely chemo-heterotrophic; they can perform both photosynthesis and chemo-synthesis in a mixed mode of growth called mixotrophy, which has advantages for commercial production. *Photo-heterotrophic* growth is a specific type of mixotrophy, where light is an essential energy source for the cells but can be supplemented with energy derived from the metabolisation of organic carbon compounds, e.g. when growing under light limiting conditions. Under *facultative mixotrophic* growth light is not essential anymore and the organisms can be grown either heterotrophically or autotrophically, and modes can be changed throughout the production process [173]. Under *obligate mixotrophic* growth, the organism utilises both, organic and inorganic carbon ($CO_2$), simultaneously to support growth and maintenance.

Several studies found that mixotrophic and particularly photo-heterotrophic cultivation modes resulted in higher biomass yields compared to chemo-heterotrophic cultivation [174–178] (Table 5). Schwarz et al. (2020) [179] studied the influence of different growth modes (using different carbon sources; mixotrophic and heterotrophic) on two xenic cyanobacterial strains – *Trichocoleus sociatus* and *Nostoc muscorum*. Mixotrophic cultivation at a light intensity of 100 μmol photons $m^{-2}$ $s^{-1}$ led to the highest biomass concentrations. Glucose was identified as the best organic carbon source for *N. muscorum* (2.46 g $L^{-1}$) while raffinose was best for *T. sociatus* (3.77 g $L^{-1}$) [179]. The uptake of complex sugars such as raffinose in cyanobacteria is believed to be mediated through sugar transporters such as the GlcP transporter (fructose/glucose transport system) which was identified in the model organism *Synechocystis* sp. PCC6803 [180] and the ABC fructose transporter which was identified in *Nostoc punctiforme* [181]. *Synechococcus elongatus* PCC7942 was identified to have three different sugar transporters, including galP (glucose), cscB

**Table 5** Reported biomass and pigment yields achieved in cyanobacteria

| Cyanobacteria strain | Biomass productivity ($g\ L^{-1}\ day^{-1}$) | Pigment productivity ($mg\ L^{-1}\ day^{-1}$) | Reactor type/ scale | Growth condition if different from BG11 (photo-autotroph) | Illumination intensity ($\mu\ mol\ m^{-2}\ s^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| *Phycocyanin (PC)* | | | | | | |
| *Spirulina platensis* M2 | 0.18 | 15.00 | 300 L Raceway Pond | Zarrouk | Sunlight (Italy) | [155] |
| *Spirulina platensis* | 0.32 | 24.00 | 282 L Raceway Pond | Zarrouk | Sunlight (Italy) | [155] |
| *Spirulina platensis* | 0.05 | 3.00 | 135000 L Raceway Pond | Zarrouk | Sunlight (Spain) | [26] |
| *Anabaena sp. ATCC 33047* | 0.24 | 13.00 | Raceway Pond | Custom | 200 | [156] |
| *Spirulina platensis* M2 | 1.32 | 92.00 | 11 L Tubular PBR | Zarrouk | Sunlight (Italy) | [157] |
| *Spirulina platensis* | 0.12 | 14.00 | 500 mL flask | Zarrouk | 140 | [158] |
| *Spirulina platensis* | 0.06 | 10.00 | Open tank | Zarrouk | 30 | [159] |
| *Synechocystis sp.* | – | 12.00 | 100 mL flask | – | 75 (16 h light) | [160] |
| *Spirulina platensis* | 0.33 | 50.00 | Tubular PBR | Zarrouk | 200 (14 h light) | [161] |
| *Spirulina platensis* TISTR 8172 | 0.03 | 1.3[a] | 9 L tank | Zarrouk | Sunlight; white filter | [162] |
| *Spirulina platensis* TISTR 8172 | 0.038 | 4.3[a] | 9 L tank | Zarrouk +16.8 $g\ L^{-1}\ NaHCO_3$ | Sunlight; white filter | |

(continued)

**Table 5** (continued)

| Cyanobacteria strain | Biomass productivity ($g\ L^{-1}\ day^{-1}$) | Pigment productivity ($mg\ L^{-1}\ day^{-1}$) | Reactor type/ scale | Growth condition if different from BG11 (photo-autotroph) | Illumination intensity ($\mu\ mol\ m^{-2}\ s^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| *Spirulina platensis* TISTR 8172 | 0.034 | 6.4[a] | 9 L tank | | Sunlight; yellow filter | |
| *Spirulina platensis* | 0.74 | 13.00 | 1 L Flat panel PBR | Spirul | 50 | [163] |
| *Spirulina platensis* WH879 | 0.436 | 94.00 | 1 L Flat panel PBR | Zarrouk | 450 | [164] |
| Anabaena oryzae SOS13 | N/A | 0.12 | 250 mL flask | BG11-$N_0$ | 30 | [165] |
| *Nostoc* sp. LAUN0015 | 0.057 | 0.01 | 500 mL flask | – | 156 (12 h light) | [166] |
| *Nostoc* sp. UAM206 | 0.064 | 0.01 | | | | |
| *Anabaena* sp. 1 | 0.141 | 0.08 | | | | |
| *Anabaena* sp. 2 | 0.115 | 0.06 | | | | |
| *Nostoc* sp. NK | 0.32 | 57.00 | 1 L Column PBR | BG11-$N_0$ | 100, Red light | [167] |
| *Spirulina sp. S1* | 0.108 | 0.07 | 300 mL flask | – | 100 | [168] |
| *Spirulina sp. S2* | 0.057 | 0.03 | | BG11 + 0.3% glucose | | |
| *Anabaena sp. C2* | 0.068 | 0.02 | | BG11 + 0.15% glycerol | | |
| *Anabaena sp. C5* | 0.059 | 0.09 | | BG11 + 0.3% glucose | | |
| *Nostoc sp. 2S7B* | 0.071 | 0.01 | | BG11 + 0.3% glycerol | | |
| *Nostoc sp. 2S9B* | 0.024 | 0.03 | | BG11 + 0.3% glycerol | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Synechocystis* sp. PCC 7338 | 0.07 | 0.0006 | 250 mL flask | ASN-III + 1.2 M NaCl | 30 | [169] |
| *Nostoc* sp. NK | 0.32 | 0.057 | 1 L column PBR | BG11-$N_0$ | 100 | [167] |
| *Synechocystis salina* LEGE 06,155 | N/A | 7[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Phycoerythrin (PE)* | | | | | | |
| *Anabaena oryzae* SOS15 | N/A | 0.49 | 250 mL flask | BG11-$N_0$ | 30 | [165] |
| *Nostoc* sp. LAUN0015 | 0.057 | 0.0005 | 500 mL flask | – | 156 (12 h light) | [166] |
| *Nostoc* sp. UAM206 | 0.064 | 0.0003 | | | | |
| *Anabaena* sp. 1 | 0.141 | 0.10 | | | | |
| *Anabaena* sp. 2 | 0.115 | 0.08 | | | | |
| *Synechocystis* sp. PCC 7338 | 0.07 | 0.10 | 250 mL flask | ASN-III + 1.2 M NaCl | 30 | [169] |
| *Synechocystis salina* LEGE 06,155 | N/A | 4.3[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Allophycocyanin (APC)* | | | | | | |
| *Anabaena oryzae* SOS14 | N/A | 0.28 | 250 mL flask | BG11-$N_0$ | 30 | [165] |
| *Spirulina sp. S1* | 0.108 | 0.01 | 300 mL flask | – | 100 | [168] |
| *Spirulina sp. S2* | 0.057 | 0.0004 | | BG11 + 0.3% glucose | | |
| *Anabaena sp. C2* | 0.068 | 0.0009 | | BG11 + 0.15% glycerol | | |
| *Anabaena sp. C5* | 0.059 | 0.05 | | BG11 + 0.3% glucose | | |
| *Nostoc sp. 2S7B* | 0.071 | 0.03 | | BG11 + 0.3% glycerol | | |

(continued)

**Table 5** (continued)

| Cyanobacteria strain | Biomass productivity ($g\ L^{-1}\ day^{-1}$) | Pigment productivity ($mg\ L^{-1}\ day^{-1}$) | Reactor type/ scale | Growth condition if different from BG11 (photo-autotroph) | Illumination intensity ($\mu\ mol\ m^{-2}\ s^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| *Nostoc sp. 2S9B* | 0.024 | 0.02 | | BG11 + 0.3% glycerol | | |
| Synechocystis sp. PCC 7338 | 0.07 | 0.30 | 250 mL flask | ASN-III + 1.2 M NaCl | 30 | [169] |
| Synechocystis salina LEGE 06,155 | N/A | 8.7[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *β-Carotene* | | | | | | |
| *Synechococcus elongatus* PCC 7942 | 0.13 | 0.70 | 250 mL flask | – | 120 | [171] |
| *Synechococcus elongatus* R48 | 0.12 | 0.60 | | – | | |
| *Synechococcus elongatus* RG48 | 0.91 | 0.50 | | – | | |
| *Synechocystis salina* LEGE 06,155 | N/A | 0.11[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Zeaxanthin* | | | | | | |
| *Synechococcus elongatus* PCC 7942 | 0.13 | 0.50 | 250 mL flask | – | 120 | [171] |
| *Synechococcus elongatus* R48 | 0.12 | 0.80 | 250 mL flask | – | | |
| *Synechococcus elongatus* RG48 | 0.91 | 1.10 | 250 mL flask | – | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Synechocystis salina* LEGE 06,155 | N/A | 0.05[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Lutein* | | | | | | |
| *Synechocystis salina* LEGE 06,155 | N/A | 0.14[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Echinenone* | | | | | | |
| *Synechocystis salina* LEGE 06,155 | N/A | 0.48[a] | 5 L flask | Z8 + 25 g $L^{-1}$ NaCl | 100 (16 h light) | [170] |
| *Chlorophyll a* | | | | | | |
| *Spirulina platensis* TISTR 8172 | 0.031 | 0.41[a] | 9 L tank | Zarrouk | Sunlight; white filter | [162] |
| | 0.017 | 0.44[a] | | | Sunlight; blue filter | |
| | 0.027 | 0.48[a] | | | Sunlight; red filter | |
| | 0.038 | 0.47[a] | | Zarrouk +16.8 g $L^{-1}$ $NaHCO_3$ | Sunlight; white filter | |
| | 0.024 | 0.49[a] | | | Sunlight; blue filter | |
| | 0.031 | 0.52[a] | | | Sunlight; red filter | |
| *Spirulina platensis* | 0.12 | 0.24 | 500 mL flask | Zarrouk | 140 | [158] |
| *Synechocystis* sp. PCC 7338 | 0.07 | 0.30 | 250 mL flask | ASN-III + 1.2 M NaCl | 30 | [169] |

[a] Denotes pigment yields in mg $g_{DW}^{-1}$ $day^{-1}$

(sucrose) and xylEAB (xylose) [182]. The variability in the carbohydrate uptake rates between strains were attributed to their metabolic activity and the varying membrane permeability to different organic substrates [183]. The mixotrophic cultivation of *Spirulina platensis* using glucose as a carbon source under continuous light yielded the highest biomass (2x that obtained in phototrophic and heterotrophic cultures). This led to the suggestion that photo-driven and oxidative glucose metabolism function efficiently and independently. The photosynthetic pigment content was also found to be 1.5–2× higher in mixotrophic cultures [162, 184, 185].

### 4.1.2 Key Macro- and Micronutrients Optimisation

Given the diversity of cyanobacteria and their ability to thrive in diverse habitats, it is not surprising that high-efficiency cyanobacterial production requires the optimisation of all species-specific production parameters. In addition to light, $CO_2$ and water, cyanobacteria also need other macro- and microelements, to enable growth. Strain-specific optimisation of chemical media composition for commercial production is therefore one of the most important processes to increase not only biomass yields and product quality but also economic viability. This in turn reduces the cost and complexity of downstream processing and increases the economic sustainability of the cultivation system.

Collectively, there are 21 elements (C, O, H, N, P, Ca, Mg, K, Cu, Mn, Zn, Fe, Co, Mo, Se, Ni, V, B, Na, Cl and S) and several vitamins broadly needed for cyanobacterial growth [186]. However, bioavailability of each element depends significantly on various factors such as solubility, chemical speciation, pH, temperature, ionic strength, inorganic anions, chelates or interaction with other elements. The biological significance of each nutrient and examples of cultivation impacts on pigment synthesis are given in Table 6.

The *elemental stoichiometry* of phytoplankton (with cyanobacteria being a major constituent) has been reported to be 106C: 16N: 1P (molar ratio) [235], the so-called Redfield ratio. Subsequent studies [236, 237] expanded this ratio and have included trace elements to C(124): N(16): P(1): S(1.3): K(1.7): Mg(0.56): Ca(0.5): Fe (0.0075): Zn(0.0008): Cu(0.0038): Cd(0.00021): Co(0.00019). Many cyanobacterial media formulations (e.g. BG11, Zarrouk) are based on this Redfield ratio [238] assuming that this reflects the essential nutrient requirements of the organism. Such media are most successful in enabling the survival for a vast diversity of cyanobacteria strains, however, for a given species or a specific product target, such media are not necessarily perfectly optimal. Fine-tuning of cultivation medium composition for commercial production can significantly influence product concentration, yield, volumetric productivity as well as overall process economics. Nutrient optimisation is often a laborious, expensive, open-ended and time-consuming process that involves many steps and iterations.

The *selection of culture media component and growth conditions* involve target literature reviews on the selected strain and growth medium to optimise the yield of the final pigment product. Either simple or complex salts may be used. For example, the triple superphosphate ($Ca(H_2PO_4)2H_2O$; ingredient in *Spirulina* sp. growth

**Table 6** Elements important in cyanobacterial cultivation and pigment pathways

| Nutrient (~abundance in biomass, %w/w) | Biological role | Impact on pigment synthesis | Reference |
|---|---|---|---|
| *Macronutrients* | | | |
| Carbon (20–65%) | Basic component of biomass | Increasing ambient $CO_2$ supply accelerates growth. Supplementation with organic carbon sources can improve pigment yields<br>High concentration of glucose and glycerol exhibits increase in the production of PBPs in *Anabaena* and *Spirulina* strains | [187–189] |
| Nitrogen (1–14%) | Required for nucleic acid and protein synthesis | Ammonium toxicity reduces growth rates by disturbing the high inter-thylakoid pH and uncouples photosynthesis<br>*Fischerella* sp. produced more phycobiliproteins under high nitrogen (nitrate or ammonium) conditions | [46, 189] |
| Phosphorus (0.05–3.3%) | Significance in the production of phospholipids, and nucleic acids<br>Involved in regulatory phosphorylation events, critical for the synthesis of ATP and NADPH<br>Accumulates as polyphosphate granules (used in P-starvation) | Higher concentrations lead to precipitation<br>Phosphate optimisation in *Phormidium ceylanicum* cultures resulted in 2.3-fold increase in PC production | [190–192] |
| Calcium (0.2–8%) | Integral part of the water splitting manganese cluster in PSII<br>Involved in intracellular signalling and $CO_2$ fixation<br>Stabilises lipid bilayers<br>Critical to the abiotic and biotic stress related signalling cascades (blooms) | Calcium optimised cultures of *Anabaena fertilissima* PUPCCC 410.5 were reported to have 1.6-fold increase in phycocyanin and 4.5-fold increase in phycoerythrin<br>Calcium was reported to prevent the significant degradation of pigments during high cadmium uptake in *N. muscorum* | [193–197] |
| Magnesium (0.35–7.5%) | Central atom of all chlorophylls<br>Cofactor for the enzymes involved in Chl synthesis pathway (e.g. Mg-chelatase) | Magnesium starvation was reported to lead to chlorosis in *Synechocystis* sp. PCC6803 | [198–202] |
| *Micronutrients* | | | |
| Iron | Involved in DNA and RNA synthesis, N assimilation and Chl synthesis<br>Component of non-heme and | Increased C-PC (45 mg $g^{-1}$) was reported in *Euhalothece* sp. KZN with iron optimisation | [203–205] |

(continued)

**Table 6** (continued)

| Nutrient (~abundance in biomass, %w/w) | Biological role | Impact on pigment synthesis | Reference |
|---|---|---|---|
| | heme-containing proteins<br>Crucial part of iron-sulphur proteins (e.g. ferredoxin); necessary for cyclic and non-cyclic photophosphorylation events | | |
| Manganese | Assists proper functioning of malic dehydrogenases, superoxide dismutase and oxalosuccinate decarboxylases<br>It is a key component for water splitting (Mn-cluster of PS II) | Manganese is reported to support the growth of *Anabaena* sp. PCC 7120 under iron-starved conditions (oxidative stress) and showed increased Chl *a* and phycocyanin yields | [206–208] |
| Copper | Cofactor for enzymes involved in the elimination of superoxide radicals such as ammonia monooxidase, lysyl oxidase and amine oxidases<br>Copper limitation leads to a copper-sparing reorganisation of metabolism and photosynthetic complexes | Increased Cu concentrations reduced the pigment content in *Nostoc muscorum* | [209–214] |
| Zinc | Maintains membrane integrity<br>Offers protection to the phospholipid membrane bilayer from photodamage<br>Cofactor for a multitude of enzymes including RNA polymerase, carbonic anhydrase and proteases<br>Aids formation of carbohydrates and catalyses the oxidation processes | Zinc stress limited growth rates but increased phycocyanin content in *Spirulina platensis*<br>Higher pigment content was reported in zinc-adapted cells of *Synechococcus* sp. PCC 6803 | [209, 215–217] |
| Boron | Absence inhibits nitrogenase activity in *Nodularia sp*., *Chlorogloeopsis sp*. and *Nostoc sp*. cultures<br>Stimulates growth rates in the absence of combined nitrogen in *Nostoc muscorum* and *Anabaena cylindrica*<br>Boron deficiency in *Nostoc sp*. leads to chlorosis | Phycocyanin content increased in *Spirulina* sp. under boron-limitation | [218–221] |
| Cobalt | Cobalt is an integral part of cobalamin (vitamin $B_{12}$) and helps to convert ribonucleotides to deoxyribonucleotides required for RNA synthesis | *Spirulina sp.* grown in the presence of cobalt ($CoCl_2$) exhibited higher levels of phycocyanin and carotenoids, while showed a decrease in the content of chlorophylls | [222, 223] |

(continued)

**Table 6** (continued)

| Nutrient (~abundance in biomass, %w/w) | Biological role | Impact on pigment synthesis | Reference |
|---|---|---|---|
| Vanadium | Influences chlorophyll synthesis<br>Integral part of V-haloperoxidases | Presence of vanadium stimulated heterocyst formation and resulted in lower pigment content in *Anabaena cylindrica* | [224] |
| Molybdenum | Essential for nitrate assimilation and nitrate reduction<br>Cofactor for enzymes such as nitrate reductase, molybdopterin adenylyl transferase and xanthine oxidase | Pigment content and nitrogen-fixing activity were higher in cultures containing molybdenum in *Anabaena cylindrica* cultures | [207, 224, 225] |
| Selenium | Role of a cofactor in enzymes regulating the metabolic pathways<br>Essential for the formation of selenoproteins (oxidoreductases) | High-selenium concentration (450 mg $L^{-1}$) resulted in both high biomass and high pigment accumulation in *Spirulina platensis*<br>Formation of Se-PC (selenium bound phycocyanin) has higher superoxide and hydrogen peroxide radical scavenging activities than PC | [175, 226, 227] |
| *Counter ions* | | | |
| Potassium (1.2–7.5%) | Balances the charge in the cytoplasm; controlling the turgor pressure<br>Dominant counter ion ($K^+$) for the large excess of negative charge on proteins, nucleic acids and lipids | *Microcystis aeruginosa* buoyancy weakened with the increase in the $K^+$ concentration leading to cell death<br>High K concentrations also led to gas vacuole formation reducing pigment content | [200, 228, 229] |
| Sodium | Impacts salinity, osmotic stress and membrane transport<br>Essential for the translocation of pyruvate and promotes the biomass growth under K-limited conditions | Sodium glutamate stress in *Spirulina platensis* FACHB-314 resulted in phycocyanin hyperaccumulation | [200, 230, 231] |
| Chloride | Key role in osmoregulation<br>Balances electrical neutrality in the cells and aids in the uptake macronutrients (N and P) | Increased salinity was reported to increase the carotenoid and allophycocyanin content but decrease the phycocyanin and phycoerythrin content in *Spirulina platensis* | [232–234] |

recipe is a mixture of 20% total P (44–48% $P_2O_5$), 13–15% calcium (Ca) and about 4% residual phosphoric acid ($H_3PO_4$). The availability of certain elements is frequently hindered by precipitation (e.g. of magnesium salts, forming insoluble $Mg_3(PO_4)_2$) and further complicated by nutrient carryover (e.g. intracellular granules stored in vesicles or from the material of the reactor walls). Thus, understanding the effect of different elemental interactions is essential to determine their availability and perform nutrient optimisation. Additionally, the selection of nutrient components for commercial scale production also involves cost consideration. Commonly used N-sources include nitrate, ammonia and/or urea. To reduce cost, waste streams (e.g. non-toxic or non-pathogenic industrial waste) are sometimes employed to supply nutrients in large scale (depending on the reactor type and final product) [239, 240].

Both *media design and the optimisation strategy* (based on a suitable mathematical model) are pre-requisites to conduct media optimisation experiments. Strategies for media optimisation include component exchange (different sources for the same element), bioavailability controls and culture parameter modifications (e.g. temperature, pH). Media optimisation methods have significantly evolved in the past two decades, from using biomass elemental composition to the use of complete and incomplete factorial statistical approaches (e.g. using approaches such as Plackett-Burman or Box-Behnken designs) [149, 241]. The data analysis for a large dataset with many variables is usually performed using Response Surface Methodology (RSM) to select the best condition and Analysis Of Variance (ANOVA) to establish statistical significance [149, 241].

## 4.2 Mass Cultivation Systems and Process Management

Mass cultivation of cyanobacteria can be performed in open systems (mixed ponds), closed systems (photobioreactors), or hybrids thereof. Biomass (dry weight) productivities are reported to range from 35 to 70 T $ha^{-1}$ $year^{-1}$ in commercial systems [242–244]. In comparison, soybeans typically yield a harvest of up to ~3.5 T $ha^{-1}$ $year^{-1}$, corn ~10 T $ha^{-1}$ $year^{-1}$ and sugarcane ~70 T $ha^{-1}$ $year^{-1}$ [245].

### 4.2.1 Open Systems

Open cultivation systems are typically circular raceway ponds and offer simplicity of design, low capital cost and a relatively easy scalability. In commercial production, raceway systems are most common and consist of a circuit of parallel channels in which the microalgae culture is circulated (e.g. by paddle wheels or pumps) [246]. Disadvantages include higher evaporation rates, poor light distribution, dilute cultures which increase the cost of harvesting, nutrient and biomass dilution with rainfall and higher susceptibility to contamination. Advanced pond systems are often called High-Rate Ponds (HRP) and are relatively shallow, mixed by paddle wheels (or equivalent) and the cultivation solution circulates in a circuit leading to reduced

energy consumption and water usage, optimised water depths and increased algae biomass yields.

### 4.2.2 Closed Systems (Photobioreactors)

Closed cultivation systems were mainly designed to overcome the challenges associated with contamination, illumination, harvest efficiency and evaporative water loss in open ponds. Photobioreactors (PBRs) provide a closed (but rarely axenic) environment, which allows better control of culture parameters compared to High-Rate Ponds (HRPs). Different types of PBR (Fig. 4) have been employed to increase the biomass and bioproduct productivity. Closed systems include both indoor (artificial light) and outdoor cultivation (sunlight). Most importantly PBRs are selected based on the target product and the associated need for high quality control to attain regulatory approvals.

Many photobioreactors that differ in design and size have been evaluated at lab, pilot, or commercial scale. Examples include flat panel PBR (used at, e.g., Subitec GmbH Germany; Arizona State University, USA), tubular PBRs (used at, e.g., Roquette GmbH, Kloetze, Germany; University of Almeria, Spain; Microphyt, France) and submerged flat panel systems (used at, e.g., Proviron Inc., USA). PBRs can be further classified into horizontal, inclined, vertical or spiral designs based on the shape and inclination of the PBR. Biofilms or hybrid systems combine features of HRP and PBRs such as floating PBRs (used at, e.g., AlgaeStream SA, France). Each PBR design has its own characteristics, and each differs in mixing and fluid dynamics, light dilution properties, surface area to volume ratio, illumination per footprint area, gas exchange and mass transfer. The main drawbacks for most closed PBR designs compared to open cultivation systems are their high capital cost, high operating costs and scalability challenges. The major advantage of PBR systems is that they achieve higher product yields per unit volume due to the improved supply of light, whether the product is biomass, a secondary metabolite, or an overexpressed protein of interest (e.g. phycocyanin, phycoerythrin). Other advantages include higher culture density, light dilution (allows light to reach deeper areas of a culture via a larger surface area to volume ratio), reduced evaporation, lower contamination, the ability to filter out IR heat load and minimisation of stress which can reduce aggregation and increase product quality. Light dilution and larger surface area to volume ratios through vertical systems minimises photoinhibition (e.g. NPQ) and hence increases photosynthetic conversion efficiencies (PCE) (further discussion in Sect. 4.2.4). PBRs offer the advantage of reproducible cultivation, controlled illumination and spectral quality. Material properties (e.g. durability, spectral quality, UV and thermal resistance, sterilisation efficiency, brittleness) play an important role in production costs and require case-specific analysis.

Generally, to attract investment for cyanobacteria cultivation systems, they should be proven economically viable under operational field conditions, scalable and ideally have a low capital expenditure (CAPEX) and operational expenditure (OPEX).

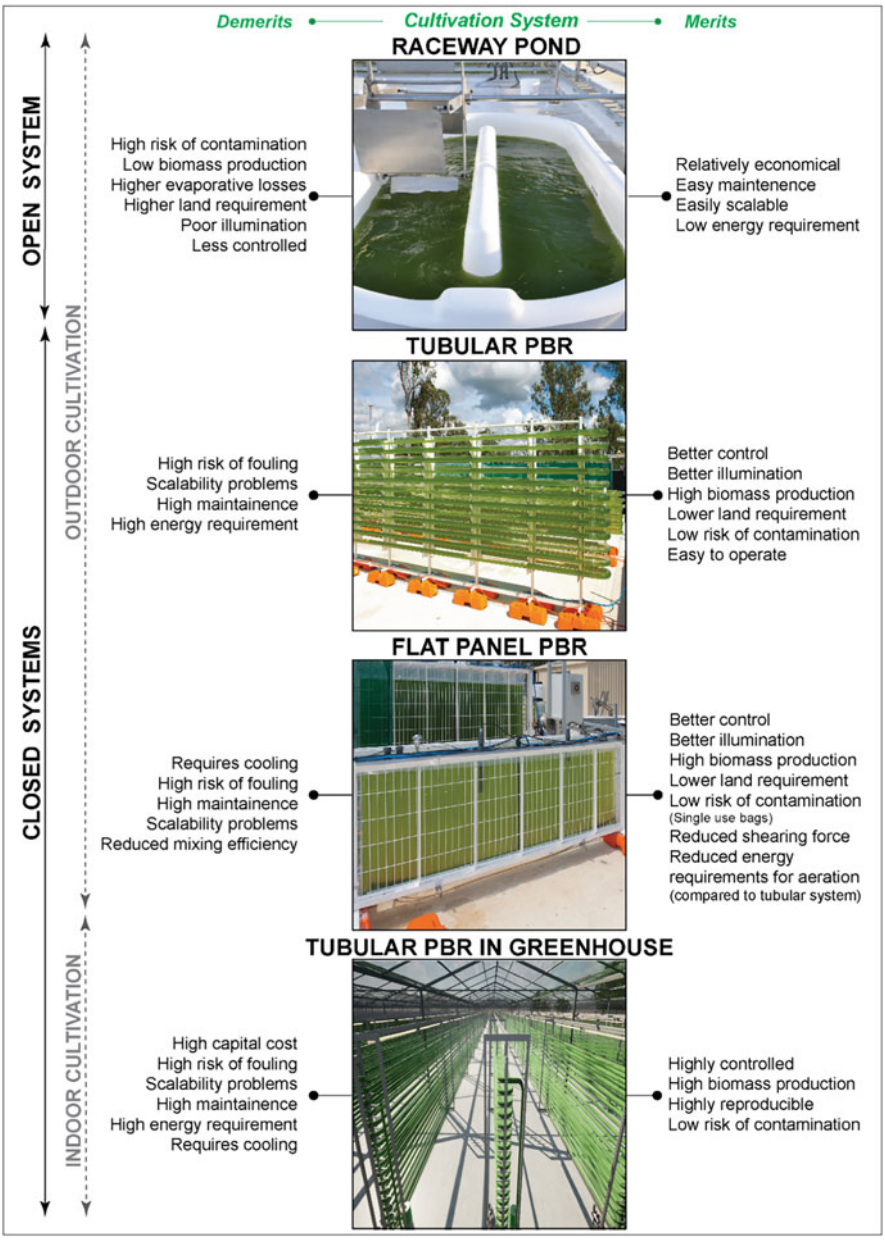



**Fig. 4** Cyanobacterial cultivation systems. The different types of cultivation system components are broadly classified into two categories – open/closed production systems and indoor and outdoor cultivation facilities. Open production systems include raceway ponds while closed systems include a range of photobioreactors (PBR) such as tubular and flat panel PBRs. More expensive production systems (e.g. tubular bioreactors) are used to provide higher yields and control, while cheaper systems (e.g. open ponds) tend to be used more for commodity products. Production systems can be used both in indoor and outdoor cultivation facilities depending on the final product requirements. The indoor or closed greenhouse facility installed with tubular PBRs offers a highly controlled

In parallel with economic assessment (Techno-Economic Analysis; TEA), environmental sustainability can be evaluated through comprehensive Life-Cycle Assessment (LCA) by accounting for all energy and material inputs and outputs associated with a particular product or process over all stages of its life cycle: extraction of raw materials, manufacturing, transport, use and recycling or disposition [247]. Life-Cycle Costing (LCC) assesses economic sustainability through similarly comprehensive financial accounting [248].

Photoautotrophic *Spirulina* cultivation in different PBR designs have achieved productivities of 0.40 g $L^{-1}$ $day^{-1}$ (bench-top helical tubular PBR [249]), 0.46 g $L^{-1}$ $day^{-1}$ (tubular PBR [250]), 0.021 g $L^{-1}$ $day^{-1}$ (air-lift PBR [251]) and 0.018 g $L^{-1}$ $day^{-1}$ (bubble-column PBR [251]) and 0.15 ± 0.005 g $L^{-1}$ $day^{-1}$ (low-cost a floating horizontal PBR without mixing [151]). Under photosynthetic conditions both the growth and product accumulation in cyanobacteria are highly light-dependent. Most commercial strains of cyanobacteria are filamentous strains which are often both shear sensitive and extremely adhesive due to their outer mucilaginous sheath, which can cause biofouling and increase the cleaning and sterilisation requirements particularly in tubular PBRs. For example, Zhang et al. (2021) [252] developed a miniature bubble-column PBR (50 L, 60 cm × 60 mm × 137 mm) for *Spirulina* sp. cultivation and achieved a biomass yield of 0.34 g $L^{-1}$ $day^{-1}$ during a 25-day cultivation. Even though globally cyanobacteria cultivation is currently largely conducted in open ponds, higher biomass productivities are achieved in PBRs. In Europe, a 2021 study on commercial microalgae production systems showed that 71% are produced in PBRs, 19% in open ponds and 10% in fermenters [253]. Further biomass and pigment yields in different closed bioreactors are summarised in Table 5.

### 4.2.3 Performance Comparison, Transfer of Scale and Process Control

*Photosynthetic performance* of cyanobacteria can be measured in terms of *energy conversion efficiency* (PCE) or *energy conversion rate* (productivity), both of which can be used to compare the performance of different cultivation system designs.

Cyanobacteria culture performance is often defined in terms of growth rate $\mu$ ($h^{-1}$ or $day^{-1}$) which measures the increase in biomass fraction per unit time. However, a high growth rate is not necessarily equivalent to a high productivity $P$ (g $m^{-2}$ $day^{-1}$). Productivity is the product of specific growth rate and the total biomass (typically expressed as biomass concentration $Y$, g $L^{-1}$). The productivity can be expressed as *volumetric biomass productivity* $P_{vol}$ (g $L^{-1}$ $day^{-1}$; biomass increase per unit reactor

**Fig. 4** (continued) environment. However, low-cost open pond systems can be operated in closed environments to enhance control. The advantages and disadvantages of each cultivation system are summarised. (Photographs were obtained from the Centre for Solar Biotechnology, University of Queensland Australia). The rendered image (bottom) provided courtesy of Dr. Fred Fialho Leandro Alves Teixeira (University of Queensland Australia)

volume), or as *areal biomass productivity,* either $P_{areal}$ (g $m^{-2}$ $day^{-1}$; biomass increase per unit reactor footprint) or $P_{SA}$ (g $m^{-2}$ $day^{-1}$; biomass per unit illuminated surface of reactor, based on surface area to volume ratio). The photosynthetic performance varies during the cultivation process of a batch regime due to self-shading of the cells or aggregated filaments experienced with high biomass density.

*Transfer of Scale:* Smaller-scale analyses in flasks or microwell plates help to determine the criteria for optimal productivity conditions while large-scale studies provide context and constraints for analyses at smaller scale systems and help to define criteria for the optimisation for high-efficiency systems. At larger scales, engineering parameters become more important and focussed on providing technical solutions for a more economically viable process. Traditionally, system designs and inoculum preparation are often scaled up stepwise in approximately 10-fold volume increases for cyanobacteria. Monitoring culture parameters (light, temperature, pH, $CO_2$) on a regular basis and logging them using suitable software offers significant benefits to achieve a target culture condition.

*Process control* aims to maintain the culture at optimal growth conditions to maximise productivity for a given bioreactor design. Growth rates and maximum biomass yields vary for different system designs due to differences in factors such as SA:V ratio and light supply. Successful process control requires suitable dimensioning and drivers of dosing equipment (e.g. nutrients, water, $CO_2$, base or acid, crop protection agents, anti-foam agent) to balance and maintain process parameters at adequately fast time scales and to attain high energy efficiency. The development of reactor-specific computer simulations may enhance process control reducing material wastage and time. Ideally, growth and production models and machine learning approaches can help to identify which of the 'easy-to-measure' parameters can be used and how they can be implemented to predict culture behaviour and hence optimise process control to reduce costs and increase cultivation robustness.

*Process regime:* In biotechnological processes, it is possible to maintain a culture at a target growth phase using a continuous cultivation regime (exponential/stationary phase to increase pigment accumulation). In laboratories this is achieved by simultaneously feeding fresh media (feed flow rate $F$) and harvesting (effluent) the culture at the same rate (inflow = outflow) to keep the culture volume ($V$) constant. The resulting dilution rate ($D$) equals the specific growth rate ($\mu$) and is defined by the quotient of the feed flow rate ($F$) to working volume ($V$). For a batch regime cultivation, the dilution rate ($D$) equals zero. Cell aggregation (common in filamentous strains) and product accumulation in the cultivation media can disturb the accuracy of process control. For example, if optical density is used for monitoring culture density cell aggregates may interfere with accuracy. The closer the dilution rate of a steady state is kept to the maximum specific growth rate ($\mu_{max}$), the more difficult it is to maintain a robust cultivation.

In cyanobacteria cultivation platforms, the energy source (solar energy or artificial light) and the carbon source ($CO_2$) are interdependent, and their supply must be matched to one another. Light serves as the main energy source, being supplied depending on weather conditions, while $CO_2$ as the main C-source is supplied with the air flow rate ideally in response to available light. Nutrients such as N and P are

supplied via the media feed flow ($F$) (Dilution rate, $D = \mu = F/V$) with the aim of maintaining sufficiency. The energy supply is indirectly controlled by the degree of light dilution depending on biomass concentration which makes the process control more difficult compared to heterotrophic cultivation regimes. The biomass concentration varies between different cultivation system designs as the optical properties and hence light energy received by the culture are also influenced by cultivation system optical path length (PBR thickness) or light dilution effects due to the spacing between vertical PBR modules. As a result of periodic fluctuations in the irradiance in outdoor systems (day/night), light availability is often synchronised with the cell division time (circadian rhythm), which makes the prediction models less accurate. Growth models dealing with light and nutrient limitation [254] assist with the development of new concepts to maintain high productivity levels and robust process control during dynamically changing weather conditions. Real-time experimental data can provide feedback to specifically developed models for cyanobacterial pigment production platforms with a selected strain and reactor at a selected geographical location.

### 4.2.4 Light Supply and Optimisation

In dense cultures, light intensity decreases dramatically with the distance from the illuminated surface, due to self-shading of the cells and light absorption by intracellular pigments. In a well-mixed culture this creates cycles of light and dark phases for each cell, which can be observed in an air-lift reactor, in which the light seems to form a gradient as it penetrates the reactor [255]. Antenna engineering in cyanobacteria, for example through the reduction of the light harvesting antenna size, has the potential to increase the productivity of cyanobacteria cultivation systems at a commercial scale [256].

The illumination intensity determines the amount of light energy available for photosynthesis and thus directly affects the rate of pigment production [148]. As photosynthetic pigments are directly related to and influenced by the composition of the light provided to the culture, optimisation of light intensity and quality is critical for higher pigment yields [257–259]. Light harvesting in cyanobacteria is carried out primarily by phycobilisomes (PBS). The functioning of PBS is continuously modulated to enable adaptation to variations in light (intensity and spectral quality). During high light stress, PBS rapidly saturate the photosynthetic electron transport chain (ETC), which leads to the accumulation of over-excited Chl molecules within the RC, which in turn increases the generation of Reactive Oxygen Species (ROS) which damage the photosynthetic apparatus.

Strategies employed by cyanobacteria under high light stress include:

- Orange Carotenoid Protein (OCP)-dependent NPQ: NPQ of PBS fluorescence occurs in a process mediated by the OCP, which is induced by blue light [260–262].

- State transitions: These regulate the distribution of excitation energy between PSII and PSI [263, 264].
- Quenching of PSI chlorophylls by P700 cation radical or triplet state (based on P700 redox state) [265–267].
- Excitonic delocalisation of the antenna complexes from the RC [268].

Tamary et al. (2012) [269] studied the structural and functional alterations (energetic coupling, stability and membrane association) of PBS induced by high light stress in *Synechocystis* sp. PCC 6803. They identified that high light intensity with white light leads to electronic decoupling of the PBSs due to over-excitation of PBP-chromophores and Chl molecules.

It has been shown that both light intensity and spectral quality affect the phycocyanin content in cyanobacteria [159, 270]. Interestingly, *Spirulina platensis* possesses a very low energy Chl *a* in PSI and only PC in their PBS for energy capture, so PE cannot be produced using this species [271]. High light conditions were found to favour PC accumulation in *Spirulina platensis* [159] (Table 5). Chaiklahan et al. (2022) [272] reported that light optimisation as a cultivation management strategy of a 10 L PBR increased the biomass concentration of *Spirulina* sp. from 0.67 to 1.23 g $L^{-1}$ and the PC content from 16% to 24% by increasing the illumination intensity from 140 to 2,300 μmol $m^{-2}$ $s^{-1}$ demonstrating that cyanobacterial pigment production is highly dependent on the illumination intensity and exposure time (12:12 light:dark cycle).

### 4.2.5 Salinity and pH

The availability of saline, brackish or wastewater streams at a cultivation site can significantly reduce the 'freshwater' consumption of a cyanobacterial system and improve its competitiveness. In large-scale continuous production systems salinity levels must be maintained within prescribed limits, therefore blowdown of water is required to remove excess salts. The vast amount of counter ions (e.g. Na, Cl) from supplied nutrients (if applied as salts) remain in the water as the nutrients are taken up by the microbes (e.g. N, P, Mg, Ca). Their concentration is further increased by evaporative water losses. The use of closed bioreactor systems offers the potential to increase efficiency, minimise evaporation and enable water and nutrient recycling. The challenge is to do so cost effectively.

Salinity levels play a significant role both in biomass and pigment productivity in cyanobacteria [231, 233, 273]. Strain-specific optimisation of salinity is crucial for proper cell function, filament elongation, metabolic activity, ion regulation (membrane potential) and osmotic balance (turgor pressure in gas vacuoles) [274]. Increases in salinity have been reported to have adverse effects on non-tolerant cyanobacteria and are indicated to cause inhibition of electron transport [233]. For example, it is thought that high levels of salinity lead to a higher influx of $Na^+$ ions which in turn induce PBS detachment from the PSI/PSII in the thylakoid membrane, reducing photosynthetic activity and thus lowering growth rates [233].

Strategies employed by cyanobacteria to survive salt stress include:

1. $Na^+/H^+$ antiport – Reduces the uptake of $Na^+$ ions and promotes an active efflux [275].
2. Enhanced antioxidative defence system – Triggers the expression of salt-induced and osmotic-induced proteins to tolerate salt stress [276].
3. Active extrusion of toxic inorganic ions and the accumulation of compatible solutes (to compensate the difference in water potential) [277] which are low-molecular mass organic compounds (e.g. sucrose, trehalose and glycine betaine), that do not have a net charge and can be accumulated in high (molar) amounts without negatively interfering with cellular metabolism [278].

Salt stress modulates the composition of phycobilisomes (PBS; PE:PC ratio). *Anabaena* sp. NCCU-9 cultivated under low salinity levels (~10 mM) was reported to have increased PBP content [279]. Abd El-Baky et al. [280] reported that C-PC productivity and the antioxidant capacity were higher in *Spirulina maxima* cultures cultivated under high salinity levels (Zarrouk medium supplemented with 0.1 M NaCl). Lee et al. [169, 202] studied the effect of salt stress on *Synechocystis* sp. PCC 7338 cultivated in ASN-III medium supplemented with 1.2 M NaCl (high salinity) and achieved an increased yield of *Chl a* (4.18 mg $L^{-1}$), PE (1.70 mg $L^{-1}$) and APC (4.08 mg $L^{-1}$).

Similar to salinity, the pH of a culture medium affects cyanobacteria growth and is altered during the cultivation process by the supply and uptake of $CO_2$ and nutrients. Many studies have reported the effect of pH on the growth of cyanobacteria and identified that the optimum pH for mostly used strains to date generally ranged between 7.4 and 9 [153, 281, 282]. However, some cyanobacteria are extremophiles that prefer highly alkaline or more acidic conditions, which can be used as a competitive advantage in the cultivation regime for contamination control.

### 4.2.6 Temperature

Cyanobacteria, with the ability to perform adaptive cell differentiation, are known to survive in a diverse range of temperatures (−20–70°C). These temperature-tolerant cyanobacteria are classified into 4 groups – psychrophilic (−20–10°C), psychrotrophic (>20°C), mesophilic (50°C) and thermophilic (>80°C). The fatty acid composition, fluidity and integrity of the membrane changes, based on the temperature. High temperature stress inhibits photosynthetic machinery and results in uncoupling of PBS [283]. The heat shock proteins (Hsps; $Hsp_{100}$ in unicellular cyanobacteria, e.g. *Synechocystis* sp. and $Hsp_{60}$ in filamentous cyanobacteria, e.g. *Anabaena* sp.) function as chaperones and assist in protein refolding required for high-temperature tolerance [284]. The HtpG protein from the $Hsp_{90}$ family protects the photosynthetic apparatus by interacting with PBS, preventing PBP aggregation [284]. At low temperatures, cyanobacteria were observed to desaturate membrane fatty acids and induce enzymes that improve transcription and translational efficiency. Tiwari et al. (2016) [285] reported that heat stress (45°C) reduced

the pigment content in *Anabaena* sp. PCC7120, but this effect was countered by the addition of calcium to the cultures (0.25 mM Ca supplementation in BG11; increased PC, Chl *a* and carotenoid levels).

### 4.2.7 Mixing and Shear Sensitivity

In most PBR systems, mixing is coupled to aeration and degassing to balance aerobic conditions and inhibiting oxygen concentrations in the culture. Mixing is also needed for the optimal nutrient distribution and, in contrast to heterotrophic cultivations, for optimal light penetration as it avoids sedimentation and self-shading of cells [286]. The sensitivity to mixing is highly strain-specific for cyanobacteria due to their range of morphologies (unicellular, colonial and filamentous). Ravelonardo et al. [154] examined the effect of agitation on biomass growth of *Spirulina platensis*, comparing air-lift systems, pumping and mechanical stirring methods for mixing. They conclude that filamentous cells were highly fragile and achieved the highest biomass productivity (1.8 g $L^{-1}$) in the mixing regime with lowest shearing force, a bubble-column reactor without additional mixing. Xiao et al. [287] reported that both unicellular (*Microcystis flos-aquae*) and filamentous (*Anabaena flos-aquae*) cyanobacteria can modulate their growth rates in response to the mixing rates via asynchronous cellular stoichiometry of C, N and P, for better nutrient uptake. Further research in association with shear regime and growth rate-dependent sensitivity to turbulence would improve the understanding and optimisation of mixing in commercial-scale ponds and PBRs.

## 5 Downstream Processing

Pigment extraction requires biomass dewatering to harvest cells, cell disruption to release the pigment followed by pigment extraction and purification. These steps are further elucidated below.

### *5.1 Biomass Harvesting*

The first step of biomass harvesting (dewatering) describes the separation of solids (cells) which are mixed in a dilute suspension, from the liquid phase (media). Dewatering efficiency depends on several factors including viscosity, particle size and density, specific gravity of the particle compared to the medium. The choice of technique depends on the properties of the cyanobacterial species and the final product requirements. The dewatering strategy of an industrial-scale process impacts both economic viability and product quality, while it must be aligned with the other processing steps, such as lysis, extraction and refinement.

Cyanobacterial cells in culture can generally be considered to be particles whose stability is due to surface charge (electronegative; *p*H of 2.5–11.5 [273, 288]), steric effects (due to water molecules bound to the microalgal surface) and adsorbed macromolecules or extracellular organic matter. When compared to other particles in suspension, cyanobacteria species differ in characteristics such as size, shape and motility that each influences their harvesting behaviour. The main techniques currently employed in microalgae harvesting include flocculation, gravity sedimentation, flotation, electrophoresis techniques, filtration and screening as well as centrifugation. The performance of each dewatering technique can be quantitatively evaluated by the rate of water removal, the solid content of the recovered cyanobacteria-water slurry and the efficiency/yield of the dewatering technique.

*Sedimentation* can be applied as the first step of dewatering (Fig. 5a). During sedimentation different materials are separated from one another based on their density and/or particle size [289]. Gravity sedimentation naturally separates a feed suspension into a concentrated slurry and clear liquid. Harvesting by sedimentation at natural gravity can be accomplished via lamella separators (plates installed to increase settling area) and sedimentation tanks. In these systems the highest energy demand is related to pumping the slurry. Typically higher biomass concentrations result in improved sedimentation rates and 95% biomass recovery has been reported after 24 h of settling for *Spirulina platensis* [290]. However, the settling rate is low compared to other dewatering techniques, due to the small difference in density between water (freshwater = 1,000 kg $m^{-3}$ or saltwater = 1,025 kg $m^{-3}$) and cyanobacteria (1,040–1,140 kg $m^{-3}$) [291]. Collectively these properties make sedimentation a low-cost but time-consuming process.

*Flocculation* is used to increase the efficiency of sedimentation or flotation-based dewatering (Fig. 5b). Here, a particle in a solution forms an aggregate with other particles to form flocs [292–294]. Flocculation occurs when the solute particles interact and adhere to each other. Chemical flocculation can be induced by inorganic flocculants (e.g. alum, ferric sulphate, lime) [294] or organic polymer and polyelectrolyte flocculants (e.g. Purifloc, Zetac 51, Dow 21M, Dow C-31, Chitosan [295]) which are usually positively charged [293]. The stability of the flocs is dependent on the forces that interact between the particles themselves and the particles and water. Electroflocculation is induced by the passage of electric current passed between the two electrodes (anode and cathode) immersed in the culture. The negatively charged cells tend to move towards the positive electrode (anode) leading to neutralisation and formation of cell flocs/aggregates [295]. Certain cyanobacterial species have the ability to self-flocculate in response to a change in their environment or stress. This phenomenon is known as auto-flocculation [289]. Flocculation can also be induced by adjusting $CO_2$ supply in the cultivation system [296]. Typically, while flocculation increases the efficiency of flotation or sedimentation, the dewatered biomass likely contains the flocculant, which may lead to the requirement of further refinement processes and increases cost.

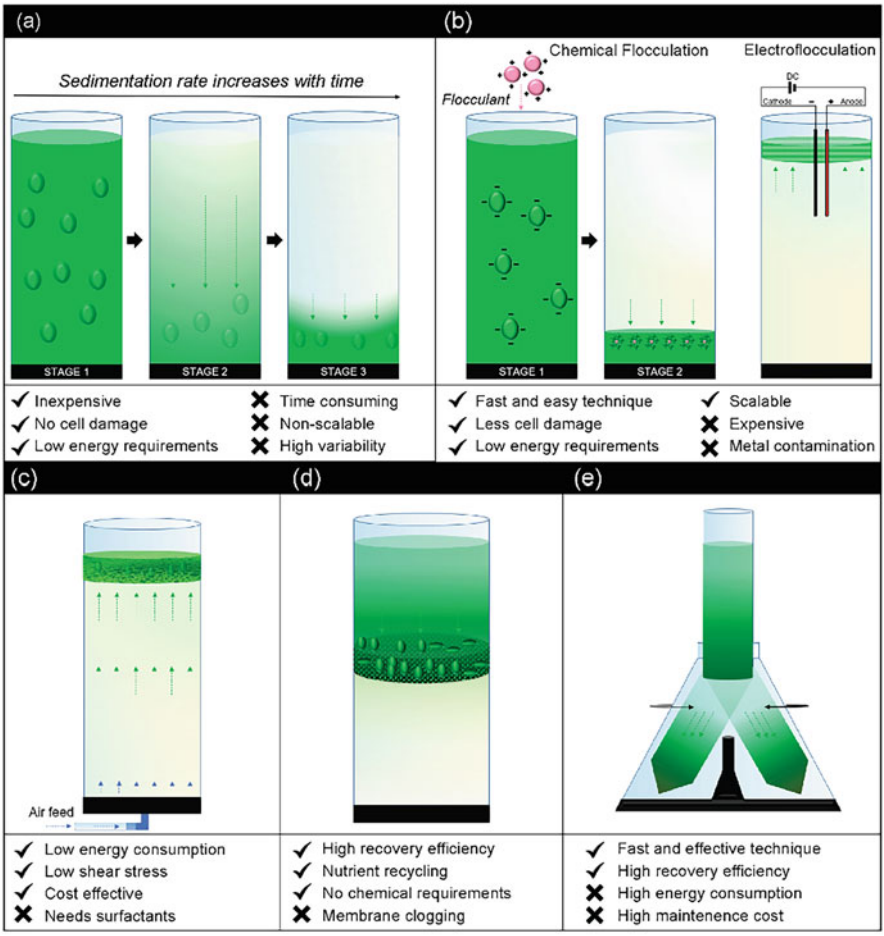



**Fig. 5** Cyanobacterial biomass harvesting techniques. (**a**) Sedimentation, (**b**) Flocculation, (**c**) Filtration, (**d**) Froth flotation and (**e**) Centrifugation. The advantages (✓) and disadvantages (**X**) of each techniques mentioned

*Froth flotation* is a physiochemical gravity separation technique based on density differences between the cells and the aqueous phase [297–299]. Air is pumped into the flotation unit with or without an additional organic/inorganic chemical, and the resultant bubbling causes biomass accumulation along with the froth of bubbles at the top phase (Fig. 5c) [300]. This froth layer is separated and treated to harvest the biomass [301]. The flotation process can be subdivided according to the methods used for the bubble formation (e.g. dispersed air flotation, dissolved air flotation, microbubble generation and electrolytic flotation [300]). Flotation can also be combined with flocculation technique to separate a floating floc layer [300]. The advantages and disadvantages of froth flotation are summarised in Fig. 5c.

*Filtration* utilises a permeable size-exclusion based material through which a suspension is passed to separate smaller (e.g. aqueous phase) from larger molecules

or particles (e.g. cells). Membrane filtration (tangential flow/cross-flow filtration) is the most commonly used harvest technique in *Spirulina* sp. farms [302, 303] (Fig. 5d). Filtration requires a pressure difference across the filter which can be driven by gravity, applied pressure or the use of a vacuum [303, 304]. Membrane filters are classified based on the pore size into macro- (greater than 10 μm), micro- (0.1–10 μm) and ultrafiltration (0.02–0.20 μm) as well as reverse osmosis (<0.001 μm) [305]. Filtration is widely used as a secondary dewatering step because it is reasonably cheap, fast and effective. The main drawbacks are increased energy requirements and the cost of downtime associated with washing membranes or replacing membranes due to fouling [306].

*Centrifugation* applies centrifugal force to enhance the dewatering efficiency (Fig. 5e) [307, 308]. Spinning the cell suspension creates the differential pressure necessary for a particle to separate from the liquid. The efficiency of the recovery process is dependent on the centrifugal force, particle size and density [308]. The two common types of centrifuges used for microalgae are disc stack and decanter centrifuges [307]. The major drawback of this technique is its high energy requirement; decanter centrifuges have been estimated to consume 3,000 kWh per ton of dry biomass [309]. Centrifugation for *Spirulina* sp. biomass harvest has been reported by many studies over the years from 1980 to 2001 and was later mostly replaced by membrane filtration or flocculation-flotation techniques due to more cost-effective scale-up. Centrifugation is often employed for small-scale laboratory harvests or very high-value products. De Souza Sossella et al. (2020) [310] compared the effect of harvesting techniques (centrifugation, chemical flocculation and froth flotation) on enzyme hydrolysis for *Spirulina platensis* biomass, where chemical flocculation yielded the highest harvesting and hydrolysis efficiency [310].

## 5.2 Product Release via Cell Disruption or Pre-Treatment

To extract intracellular products such as pigments, a suitable cell disruption step is a crucial part of downstream processing. Typically, cell disruption is performed after the first dewatering step to reduce treatment volume and save costs. An ideal cell disruption method would be one that lyses the cells and selectively releases the target product without damaging it, while using the least possible energy. The major challenge is to obtain high disruption efficiency while maintaining the functionality of the cyanobacteria pigments during cell disruption [311]. The major barrier to cell disruption is the sturdy cell wall and the thylakoid membrane, which encloses the pigments [311].

Typically, cyanobacterial cell walls consist of six layers. A rigid peptidoglycan layer overlays the inner cell membrane, and this is tightly connected to the outer membrane of the wall and contains muramic acid on the upper surface. The microfibrillar framework and an amorphous outer mucilaginous sheath are composed of polysaccharides, lipids and proteins (Fig. 6). Cell disruption methods can be classified into mechanical (e.g. bead milling, homogenisation, ultrasonication), physical (e.g. drying, pulsed electric field) or chemical/biological (e.g. acid, base,

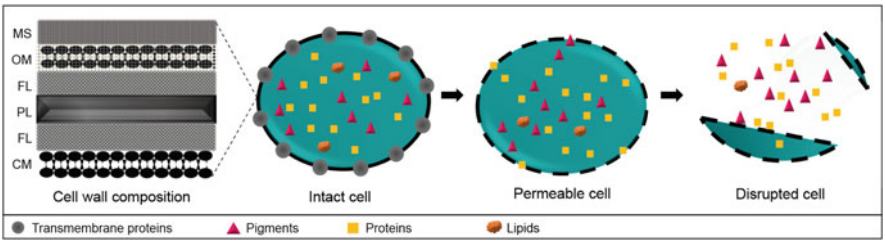



**Fig. 6** Different stages of cell disruption. Schematic view of cell wall composition of commercial pigment producing cyanobacteria (*Spirulina* sp.) – MS – Mucilaginous Sheath, OM – Outer Membrane, FL – Fibrillar Layer, PL – Peptidoglycan Layer, CM – Cell membrane. Pigment extraction proceeds from left to right, as the cells are lysed. The cyanobacterial filaments typically quickly break into single cells. Intact cells are usually permeabilised more slowly and once broken typically release their intracellular contents quickly

enzymes) and are optimised depending on strain-specific parameters, including cell wall structure, cell size, product location, product solubility and energy requirements. The merits and demerits of some commonly used cell disruption methods are summarised in Table 7.

### 5.2.1 Mechanical Pre-Treatment Methods

The most commonly used mechanical methods for microalgal cell disruption are bead milling, high-pressure homogenisation and ultrasonication. To date, for soluble proteins such as PC and hydrophobic pigments such as carotenoids the most used cell disruption technique in large scale is bead milling.

*Bead milling* is a high-intensity cell lysis method which uses kinetic energy to force small beads (glass, ceramic, plastic, or steel) to collide with each other and the cells [320, 322]. The factors that affect the efficiency of the bead milling process include cell size, bead size and type of material, cell density, feed flow rate and chamber volume. For cyanobacterial species such as *Nodularia* sp., *Anabaena* sp., *Nostoc* sp., and *Spirulina* sp., smaller bead sizes of about ~0.1–0.3 mm are reported to be optimal [167, 326, 327]. The bead milling method has been reported to be more effective than ultrasonication or pulsed electric field treatment, achieving extraction efficiencies of up to 95% for total proteins at low to moderate energy consumption rates [304].

*High-Pressure Homogenisation* (HPH) is a mechanical cell lysis method during which the cells are subjected to high pressure that forces them to pass through a narrow opening, causing a rapid pressure release that breaks open the cell wall. The combination of intense shear force, cavitation and turbulent flow induces rapid cell disruption (that leads to emulsion formation) and is suitable even for strains with highly stable cell wall structures. One of the highest yields of C-phycocyanin extraction from *Cyanobacterium aponinum* PCC10605 [328] was achieved by applying three to six passes of HPH (1,000–1,500 bar) to a 1–2% biomass suspensions resulting in a total protein release of 70–90% [329]. HPH can achieve a high

**Table 7** Comparison of different cyanobacterial cell disruption methods

| Cell disruption technique | Merits | Demerits | Reference |
|---|---|---|---|
| *Mechanical methods* | | | |
| Freeze-drying | • Gentle extraction of fragile compounds | • High energy requirement and time-consuming<br>• High maintenance cost<br>• Difficult to scale up | [312] |
| Bead milling | • Simple equipment<br>• Rapid process<br>• High disruption efficiency<br>• Easy scale-up<br>• High degree of automation | • High energy requirements<br>• High cooling requirements for thermolabile compounds | [313] |
| High-pressure homogenisation | • High disruption efficiency<br>• No biomass drying required<br>• Easy scale-up | • High energy requirement<br>• Temperature rise may lead to degradation of thermolabile compounds<br>• Rigid cell wall may hinder product release | [314, 315] |
| Ultrasonication | • Simple<br>• Rapid process<br>• High reproducibility<br>• High disruption efficiency | • Moderate energetic costs<br>• Temperature rise<br>• Rigid cell wall hinders product release<br>• Risk of reactive hydroxyl radicals<br>• Difficult scale-up | [315] |
| *Physical methods* | | | |
| Pulsed electric field (PEF) treatment | • Simple operation<br>• High energy efficiency<br>• Rapid process<br>• Easy scale-up | • High maintenance costs<br>• Temperature rise affects product stability<br>• Dependence on medium composition<br>• Degradation of fragile compounds | [314, 316–318] |
| Microwave-assisted cell disruption | • Simple operation<br>• Rapid process<br>• High disruption efficiency<br>• Easy scale-up<br>• Eliminates the requirement or dewatering of algal biomass | • High energy requirements<br>• High maintenance costs<br>• Requires extensive cooling for thermolabile compounds<br>• Lipid degradation and protein aggregation, denaturation, and formation of free radicals | [319] |
| *Chemical methods* | | | |
| Acid/alkali treatment | • No special equipment required<br>• Rapid process<br>• High disruption | • Hazardous<br>• Possibility of damaging desired product<br>• Difficulties in purification | [320–322] |

(continued)

**Table 7** (continued)

| Cell disruption technique | Merits | Demerits | Reference |
|---|---|---|---|
| | efficiency<br>• Low product contamination risk | • Extreme pH changes can cause protein denaturation | |
| Osmotic lysis | • Lower energy requirement<br>• Easy scale-up<br>• Low cost | • Time consuming<br>• Generates high salinity of wastewater<br>• Low efficiency | [320, 322] |
| Enzymatic treatment | • Highly species-specific<br>• No special equipment required | • High cost at scale-up<br>• Enzyme stability and incubation time is critical | [313, 323–325] |

disruption efficiency and protein extraction yield, is relatively energy efficient [329], but has drawbacks in potential pigment degradation due to temperature elevation up to 84°C [330].

*Ultrasonication* is a mechanical treatment based on the use of ultrasound to generate cavitation bubbles (i.e. vapour bubbles formed from a flowing liquid in a region where the pressure of the liquid falls below its vapour pressure). These promote a non-specific cell-surface barrier disruption as bursting of the cavitation bubbles exerts a pressure that bursts the cyanobacterial cells. Sonication permeabilises both the cell wall and the membrane, a key difference from pulsed electric field (PEF) treatment (see below), which permeabilises only cell membranes [331]. In certain cases, sonication can be enhanced by the application of a secondary cell disruption method, such as high-shear mixing, enzymatic cell wall hydrolysis or chemical treatment. Such dual approaches can increase the release of soluble proteins (e.g. phycobiliproteins) from *Spirulina platensis* but in turn increases the cost of cell disruption [332–334].

### 5.2.2 Physical Pre-Treatment Methods

*Pulsed Electric Fields (PEF)* can provide a non-thermal approach to disrupt lipid cell membranes and allow low-molecular-weight molecules to enter into and diffuse out of the cells. The application of a high voltage PEF treatment for $10^{-4}$–$10^{-8}$ s typically disrupts cyanobacteria cells through electromechanical stress that causes irreversible permeabilisation [335]. As the phycobiliproteins are soluble rather than in the membrane fraction, PEF treatment is highly efficient at releasing them into the suspension. Jaeschke et al. (2019) [336] compared bead milling and PEF treatment on *Spirulina platensis* for C-PC extraction and recorded the highest C-PC yield of $85.2 \pm 5.7$ mg $g^{-1}$ biomass using a PEF at 122 J $mL^{-1}$ specific energy input. It was also noted that the antioxidant capacity of C-PC extracted with PEF was higher than that extracted with bead milling. Thus, PEF treatment may offer both an economic and functional benefit for pigment extraction. Furthermore, Akaberia et al. [337]

analysed the interaction of PEF (1 μs pulses; 40 kV $cm^{-1}$ field strength; energy input, 56–114 kJ $kg^{-1}$) and pH on C-phycocyanin extraction and found extraction yield and PC stability to be highest at pH 8 [337].

*Microwave-assisted cell disruption* is a rapid and efficient physical pre-treatment technique. The transmission of microwave energy into a cell suspension results in rapid vibration of the water molecules and rapid heating of the cell matrix [338]. The microwaves heat the solvent and increase the partition between the cell matrix and the solvent [319, 338]. The increase in intracellular kinetic energy exerts pressure on the cell wall and leads to cell rupture [120]. Similar to high-pressure homogenisation, the use of microwave-assisted pre-treatment leads to significant emulsion formation that impedes solvent recovery [329].

### 5.2.3 Chemical Pre-Treatment Methods

Chemical disruption is achieved through the addition of a chemical additive such as a detergent, solvent, acid, chaotrope or a chelator to the biomass to degrade the cell membrane.

*Acid or alkali treatment* is a highly effective chemical pre-treatment method in which the cell wall of cyanobacteria is hydrolysed [339]. Chemical treatments work by attacking certain linkages in the cell wall, hydrolysing the phosphodiester bonds and ultimately dissolving the entire cell wall or permeabilising the cell wall layers [340]. Pigments extracted using ethanol require stronger acid concentrations than those extracted using methanol or acetone, to lower pH to an acceptable range for pigment determination (pH causes spectral shift). It was reported that the spectral shifts in Chl *a* absorbance persisted for about 30 min after acidification (0.005 mol HCl $L^{-1}$) [341]. The major advantage of chemical over mechanical pre-treatment is the lower energy requirement and elimination of emulsion formation (as long chain polysaccharides, proteins and phospholipids are hydrolysed into their constituent units) [342, 343]. However the pigments can be damaged, costs can be higher and neutralisation is required.

*Osmotic lysis* (osmotic shock) involves exposing cyanobacterial cells to a low-salt or hypotonic extracellular environment that results in a net flow of water into the cells, which can ultimately result in them bursting [322]. However, due to its low efficiency, it is one of the less commonly used methods of cell disruption [344].

*Enzymatic treatment* is generally performed under mild conditions and is a relatively eco-friendly, non-hazardous, and a low energy alternative to mechanical and chemical techniques. In some instances, enzymatic disruption can result in more efficient protein extraction than mechanical and chemical cell disruption [345]. Cyanobacteria have rigid cell walls to protect the cell from the environment and thus increase their survival capacity. The cyanobacterial cell wall comprises tri-layered structures of cellulose and proteins with other components such as mannose, xylan, algenan and glycoproteins, with minerals (Fig. 6) [321]. Thus, the selection of enzymes is typically biomass-specific and based on composition and cell wall structure for the target species. Cyanobacterial cell disruption employing lytic

enzymes is quite popular in product and application-based scenarios (e.g. phycocyanin-based pharmaceutical production), owing to their ability to avoid the harsh conditions that phycobiliproteins are subjected to in other techniques. Several lytic enzymes can be used, such as cellulase which can effectively hydrolyse the cellulosic structure of the cell walls, and lysozyme which can hydrolyse the linkage between peptidoglycan residues [345]. Enzymatic treatment is most commonly used to improve extraction yields rather than for cell disruption alone and normally requires a preceding cell permeabilisation or disruption step [338]. However, the use of lytic enzymes typically increases extraction costs. The exploitation of biological mechanisms for in situ production of a lytic enzyme that can activate to cleave the cell wall is a promising solution to overcome high purified enzyme prices [36, 346].

## *5.3 Product Recovery via Pigment Extraction*

After cell rupture, released intracellular pigments are recovered from the ruptured cell sample using an extraction solvent. For water-soluble pigments (such as phycobiliproteins), the extraction solvent can be water, the culture medium itself or an aqueous buffer. For water-insoluble pigments such as chlorophylls and carotenoids, one of the following extraction systems is typically used: organic solvent extraction, pressurised solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, pulsed-electric field extraction, ionic liquid extraction and supercritical carbon dioxide extraction [129]. Commercially, extraction processes with lower solvent consumptions, shorter extraction times and higher environment sustainability are preferred, as over the lifetime of a production plant the associated operational costs can exceed initial capital expenditure on infrastructure. The cell disruption and pigment extraction methods used in previous studies investigating pigment recovery from commercial pigment production strains are summarised in Table 8.

### 5.3.1 Conventional Organic Solvent Extraction

Water-soluble pigment binding proteins such as phycocyanin need to be extracted in an aqueous medium (using solvents such as water, sodium phosphate buffer or phosphate buffer saline) following cell lysis. In contrast, chlorophylls and carotenoids with high partition coefficients in organic solvents migrate out of the biomass into the solvent phase during organic solvent extraction, with a rate and extent of extraction limited primarily by the cell wall and the solubility of the target pigment. Solvents used for the extraction of chlorophylls and carotenoids include acetone, chloroform/methanol mixtures, DMSO, dodecane, ethanol, ethyl acetate, hexane, methanol, methylene chloride, vegetable oil and a mixture of one or more of the above solvents [354]. As global bio-economies expand and the importance of

**Table 8** Summary of cell disruption methods and their pigment yields

| Pigment – production species | Biomass state | Cell disruption | Pigment extraction | Max. pigment recovery | Key findings | Reference |
|---|---|---|---|---|---|---|
| Phycocyanin *S. platensis* | Oven dried (60° C) | Ultrasound-assisted protic ionic liquid extraction – 25 kHz, 25°C | Solvent extraction: 2-HEAA or 2-HEAF and 0.1 M sodium phosphate buffer | 6.34 mg APC $g_{BDW}^{-1}$ 5.95 mg PC $g_{BDW}^{-1}$ 2.62 mg PE $g_{BDW}^{-1}$ | Highly effective in extracting all PBP due to their greater diffusional power and interaction with the pigments | [347] |
| | Freeze dried biomass | Ultrasonication | Suspended in 0.1 M phosphate buffer, sonicated at 80 W, 20 kHz at 10°C | 60 mg PC $g_{BDW}^{-1}$ dry biomass), purity of 80% | Other biological compounds such as chlorophyll were also released during the ultrasonic disruption | [348] |
| B-Phycoerythrin *Porphyridium cruentum* | Direct wet biomass | Low pressure homogenisation (~90 MPa) | Resuspended in distilled water and HPH (~270 MPa) | 3.6 mg B-PE $g_{BDW}^{-1}$, 0.79 purity ratio | Intracellular proteins are extracted more effectively at low HPH pressure than B-PE | [349] |
| *Astaxanthin H. pluvialis* | Freeze dried | – | Pressurised solvent extraction, methylene chloride: methanol (1:3 v/v) at 1500 psi, 40°C | 10.9 mg astaxanthin $g_{BDW}^{-1}$ | Increasing temperature degraded pigments. | [350] |
| | Powder | – | Supercritical carbon dioxide extraction with ethanol as co-solvent | 77.9 wt% astaxanthin at 55 MPa, 343 K and 3 mL $min^{-1}$ $CO_2$ | Astaxanthin content increased with increasing temperature and pressure | [351] |
| β-Carotene *D. salina* | Direct culture from cultivation | – | Centrifugal partition chromatography with ethyl oleate and dichloromethane | 65% β-carotene recovery | Solvent biocompatibility is critical | [352] |
| | Spray dried | Bead milling (glass beads) | Supercritical carbon dioxide extraction | 115.43 μg β-carotene/g biomass at 400 bar, 55°C | At optimum condition, the carotenoid recovery of $SCCO_2$ extraction was half that of solvent extraction | [353] |

*2-HEAA* 2-hydroxy ethylammonium acetate, *2-HEAF* 2-hydroxy ethylammonium formate

process sustainability increases, use of bio-based solvents (e.g. acetone and ethyl lactate) is increasing. The solvent selected for industrial-scale pigment extraction should also be relatively volatile to support recovery [355, 356]. If the extracted pigments will be used for nutraceutical or food applications, it is also preferable to use non-toxic solvents for the extraction (e.g. acetone and ethanol instead of chloroform and methanol) [354].

### 5.3.2 Accelerated Organic Solvent Extraction

Accelerated Solvent Extraction (ASE) or Pressurised Liquid Extraction (PLE) is an organic solvent extraction performed at elevated temperature or pressure. The solvent is maintained in its liquid state throughout the extraction process [329]. Increased temperature and pressure aids in cyanobacterial cell wall disintegration, accelerating the mass transfer kinetics. Thus, PLE uses less solvent and can complete the extraction process in a shorter timeframe compared to a conventional organic solvent extraction but has higher energy requirements. High temperature (~200°C) introduces the risk of degradation of temperature sensitive pigments. PLE used during astaxanthin production from *H. pluvialis* improved apparent extraction yield but reduced the actual yield of intact astaxanthin, as assessed by the antioxidant activity of the extract [350] (Table 8).

### 5.3.3 Ionic Liquid Extraction

Ionic liquids are salts of weakly held anions and cations that remain in a liquid state over a wide range of temperatures. For pigment extraction, ionic liquids with a melting point less than 100°C are typically selected to eliminate the need for additional energy inputs to bring them into the liquid phase. Ionic liquids are broadly classified into two categories – protic and aprotic (based on their ability to transfer protons from acid to the base). Protic ionic liquids are generally molten salts (synthesised by transferring protons from a Bronsted-Lowry acid to a Bronsted-Lowry base). Many of their physical properties, such as polarity, hydrophobicity and viscosity, are adjustable and can be controlled by the exchange or combination of ions [129, 357] so that their solvating power can be specifically tailored to the target compound to enhance solvent–solute interaction and increase extraction efficiency [358]. Their high extraction efficiency is attributed to the liquid's high diffusivity and strong interaction with the pigment molecules. Indeed ionic extraction has been efficiently used for lipid extraction [359]. Chang et al. [360] reported the use of an Aqueous Two-Phase System (ATPS) with ionic liquids and achieved an extraction efficiency of 99% with the separation factor being 5.8 [360]. Sanchez-Laso et al. [361] reported the use of 1-ethyl-3-methylimidazolium ethyl sulphate for the extraction of phycobiliproteins from *Spirulina platensis* and achieved a recovery of 67.2 mg $g_{BDW}{}^{-1}$ PC, 20.9 mg $g_{BDW}{}^{-1}$ APC and 5.3 mg $g_{BDW}{}^{-1}$ PE, the overall extraction efficiency was ~80% which was slightly lower than the ATPS approach reported previously (99%) [360].

### 5.3.4 Supercritical Carbon Dioxide Extraction

When carbon dioxide gas is subjected to temperature and pressure beyond its critical values (Critical Temperature – $T_c$ at 31.1°C and Critical Pressure – $P_c$ at 73.86 bar), it is transformed into a supercritical fluid that exhibits physical properties intermediate between a liquid and a gas. Supercritical carbon dioxide ($SCCO_2$) is a highly effective extraction solvent because of its transitional properties, such as high diffusivity and adjustable solvating power, that can be varied by pressure. The high diffusivity of $SCCO_2$ enables it to rapidly penetrate the cellular matrix and complete extraction within a shorter timeframe than less diffuse solvents [362, 363]. 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. In addition to being a powerful extraction solvent, $SCCO_2$ is non-toxic, produces a solvent-free pigment extract (no additional solvent recovery step) and operates at a moderate temperature range which minimises degradation of thermally sensitive pigments [364, 365]. A major disadvantage of $SCCO_2$ extraction is the higher capital cost of the extraction apparatus but this can potentially be mitigated by lower longer term operating costs.

## *5.4 Pigment Purification*

Crude cyanobacterial pigment extracts often contain other impurities that interfere with their stability and function. These impurities include chlorophylls and lipids in carotenoid extracts and proteins in phycobiliprotein extracts and can be separated from the desired pigments using chemical and/or chromatographic techniques. Selection of a purification method is highly affected by the final product application as PC with a purity ($A_{620}/A_{280}$) $\geq$0.7 is considered as food grade, $>$3.9 as reagent grade and $>$4.0 as analytical grade. Thin layer chromatography (Chl and carotenoids), liquid chromatography and spectrophotometric analysis are widely used for the analysis of the purified pigments [325, 366]. Calcium hydroxide precipitation, acid precipitation and column chromatography have previously been used to remove chlorophylls from astaxanthin and β-carotene extracts [367–369]. Phycobiliprotein purification generally involves an initial lysis step (e.g. freeze-thaw, sonication) followed by subjecting the lysate supernatant to one or more of the following steps: ammonium sulphate precipitation, activated carbon and chitosan precipitation, aqueous two-phase purification with polyethylene glycol, gel permeation chromatography, for example, with a Sephadex G-150 column (Fig. 7a) and anionic chromatography with diethylaminoethyl cellulose (DEAE) [370, 371], anion exchange chromatography with a Q-Sepharose column (Fig. 7b) and concentration by ultrafiltration (Fig. 7c) or tangential flow ultrafiltration (30–50 kDa). Different stages of PC extraction from cyanobacterial biomass are shown in Fig. 7d. Halim et al. [30] described the extraction of PC from *Galderis sulphuraria* in which

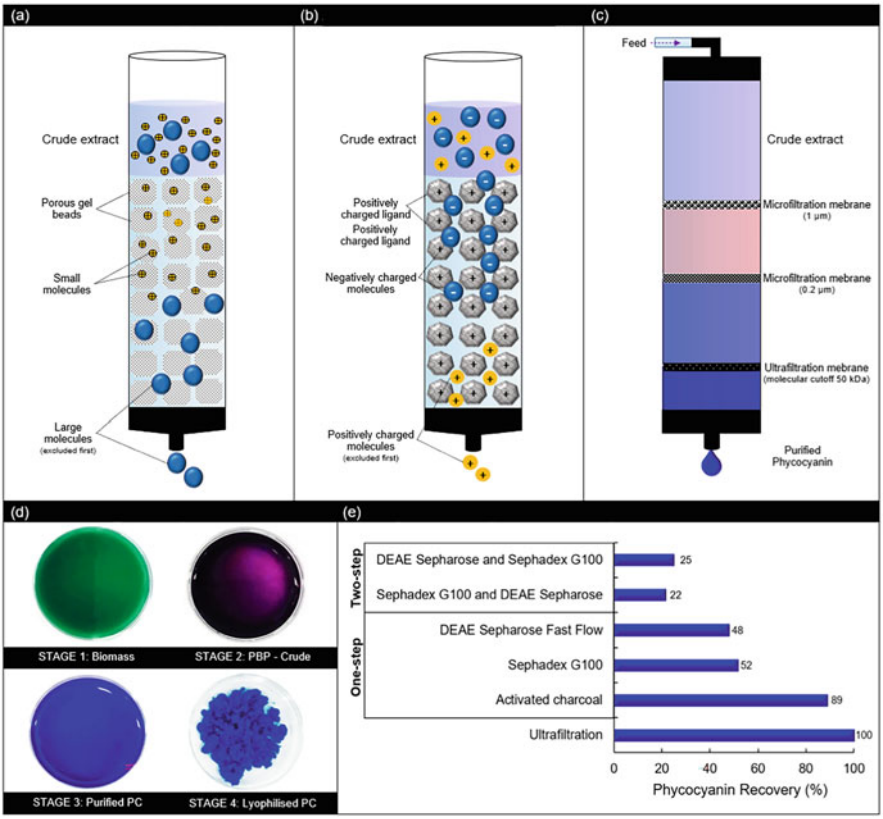



**Fig. 7** Cyanobacterial pigment purification. Schematic of the most commonly employed PC purification techniques – (**a**) Gel filtration/permeation chromatography, (**b**) Anion exchange chromatography and (**c**) Ultrafiltration. (**d**) Different stages in PC production – harvested cyanobacterial biomass, PBP aqueous crude extract (contains PE, APC and other soluble proteins), purified PC and lyophilised PC powder. (**e**) Comparison of different PC purification techniques based on PC recovery (%). Ultrafiltration method using microfiltration membranes (1 µm, 0.2 µm) and ultrafiltration membrane with molecular cut-off of 50 kDa has recorded among the highest recovery rates but achieved comparatively low purity [372]

ammonium sulphate precipitation with aqueous two-phase extraction and ultrafiltration resulted in both the highest PC yield (42 wt% of PC in the crude extract) and the highest product purity ($A_{620}/A_{280} = 4.5$). Chaiklan et al. [372] investigated stepwise extraction of PC and economic feasibility analyses by comparing different PC purification techniques from *Spirulina* sp. which included ultrafiltration, one-step and two-step chromatography techniques using three different matrixes: activated charcoal, Sephadex G100 and DEAE Sepharose Fast Flow (Fig. 7e). The highest PC recovery rate was recorded using ultrafiltration (Yield: 6.43 mg/mL) but the purity achieved was comparatively low ($A_{260}/A_{280}$- = 1.22;Fig. 7e) [372].

## 6 Pigment Bioprocessing Challenges

The development of more cost-effective cyanobacterial pigment production processes requires improved production (Sect. 4.2), disruption (Sect. 5.2) and extraction techniques (Sect. 5.3) to drive down the costs and enhance quality and value. The main challenges of natural pigments production include optimising species selection, cost of production as well as the product, quality and stability.

*Increasing pigment yield:* As cyanobacteria can be relatively slow growing, biomass and pigment yields can be low compared to other microbes (e.g. microalgae; growth rate of *Chlorella* sp. ~0.047 $day^{-1}$ [241] while *Spirulina* sp. is only 0.0027 $day^{-1}$ [162]). This explains why the first pigments commercially produced (e.g. phycocyanin) were unique to cyanobacteria, of high value and expressed at high levels. Recent technological advances in photobioreactor development and process optimisation parameters are overcoming scale-up associated challenges [373–375]. Bio-process optimisation and genetic engineering of the strain are two-key ways to increase biomass and pigment accumulation.

*Disruption and extraction techniques:* Cost and efficiency require optimisation for each target product. For example, microwave-assisted cell disruption is an efficient method to disrupt biomass, but the use of high temperatures can also result in pigment degradation. During traditional solvent-extraction of chlorophylls and carotenoids, the choice of solvent and biomass-solvent ratio is critical to achieve high final pigment yields. The choice of solvent is often also influenced by regulatory policies. For example, although hexane is an excellent solvent for carotenoid extraction, it must be completely removed to comply with regulations for human consumption. This hurdle can technically be overcome by replacing hexane with green solvents such as ethanol, ethyl acetate or critical $CO_2$ extraction, but this can compromise pigment yields. To date, lead disruption processes for pigments are based on bead milling for both phycobiliproteins and carotenoids.

*Enhancing product stability:* Natural pigments such as carotenoids and chlorophylls are generally sensitive to light, pH, UV, temperature and oxygen as oxidation of their conjugated bond systems results in fading (e.g. in β-carotene and astaxanthin) and a reduced shelf life. Other natural pigments such as phycobiliproteins and chlorophylls are sensitive to other ambient conditions like metal ion concentrations, heat or organic solvents that can denature proteins. C-phycocyanin (C-PC) has been approved as a food additive and blue colourant and it is typically used in the αβ-monomeric and trimeric forms which coordinate the Phycocyanobilin (PCB) chromophore. The hexamer may, however, offer improved stability and colour properties [337, 376, 377]. C-PC has been reported to retain its hexameric form (Fig. 8) in the pH 5–7 range and to be more stable below 46°C [377]. Therefore, PC application in the food sector is mainly limited due to its sensitivity to external factors. The use of effective encapsulation techniques or stabilising agents such as glucose, alginate, pectin, whey protein and carrageenan would help overcome this challenge.

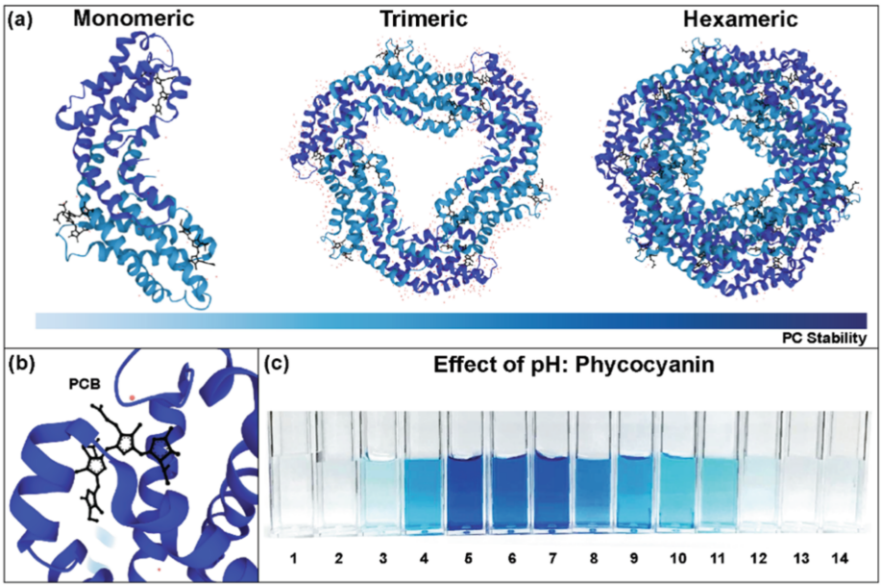



**Fig. 8** Stability of C-Phycocyanin. (**a**) Crystal structure of monomeric, trimeric and hexameric forms of C-phycocyanin (from *Thermosynechococcus vulcanus*; acquired from PDB) – monomeric (least stable; 1ON7), trimeric (3O2C) and hexameric (most stable; 1I7Y). (**b**) Phycocyanobilin (PCB), the chromophore responsible for the blue colour of PC. (**c**) Effect of pH on PC. The PC extracts were derived from *Spirulina platensis* wet biomass using the freeze-thaw method with water as solvent. The pH of the extracts was adjusted using 0.1 N HCl/NaOH

To be economically and environmentally beneficial, pigment production (as a single product or as co-product) in biorefineries requires strong process intensification strategies. The final pigment product should be stable under environmental factors such as light, pH, temperature, UV and food matrices. Development of novel encapsulation techniques based on the market value of pigments will thus assist in the production of more stable natural pigments with a higher shelf life (expanding their applications). Understanding the biosynthetic pathways of cyanobacterial pigments is an important starting point, followed by identifying genes and the gene cascades responsible for pigment production, which supports metabolic engineering approaches for pigment accumulation.

## 7 Commercial Pigment Production Technologies

Currently commercial production of cyanobacteria strains is confined to phycocyanin production but has the opportunity to be expanded for the production of other pigments including chlorophyll and carotenoids. Cyanobacteria produce most of the major carotenoids present in microalgae. With expansion of strain phytoprospecting

and cultivation optimisation, they are promising candidates for industrial production for many pigments. Cyanobacteria strains reported to produce different pigments of commercial interest (serve as alternatives) and their corresponding production strains are summarised in Fig. 9.

## *7.1 Patents and Technology Transfer*

Patents are public documents and effectively part of the open access literature that document recent technical developments that have commercial potential [381]. A patent search on Patent Lens (Lens.org), a patent database with an integrated framework that serves nearly all the patent documents in the world, for the pigment 'Phycocyanin' showcases an example for current cyanobacterial pigments in the market and is represented in Fig. 10.

Technological developments and transfer can help to address existing scalability challenges and increase the economic feasibility of production platforms [382, 383]. The selection of production technology and process optimisation is highly application-specific in the case of pigments. For example, phycocyanin marketed as a food colourant (blue Spirulina powder with 2–6% PC – selling price USD \$160 $kg^{-1}$) is produced in open ponds with a low number of extraction steps while the pure phycocyanin marketed for flow cytometry applications (~98% pure; selling price USD \$217,000 $g^{-1}$) is produced under highly controlled environments with a series of purification (chromatography) steps. Examples of some recent patents that focus on cyanobacterial pigment-based technological innovations include:

- Method for separating and purifying high-purity phycobiliprotein from nitrogen-fixing cyanobacteria (080-530-697-056-493; August 2021; Pending).
- Phycocyanin-casein/porous starch microgel as well as preparation method and application thereof (091-869-437-651-829; June 2021; Pending).
- Supercritical cracking process of phycocyanin (002-379-984-590-359; April 2021; Pending).
- Method for extracting phycocyanin from *Spirulina* sp. through low-salt flocculation method (051-541-487-645-566; Jan 2021; Pending).
- Mixing temperature tank for phycocyanin (055-912-539-763-114; Nov 2020; Active).
- Spray drying device applied to phycocyanin production (067-605-942-811-388; Nov 2020; Active).

The increasing number of natural pigment-based patents (related to cyanobacteria and microalgae) is considered as evidence to consolidate the growth of cyanobacterial pigments market, which is expected to grow further in the upcoming years (increasing the likelihood of replacing synthetic pigments). Most of the published patents are reported to be technological patents in association with novel cultivation and extraction techniques [384].

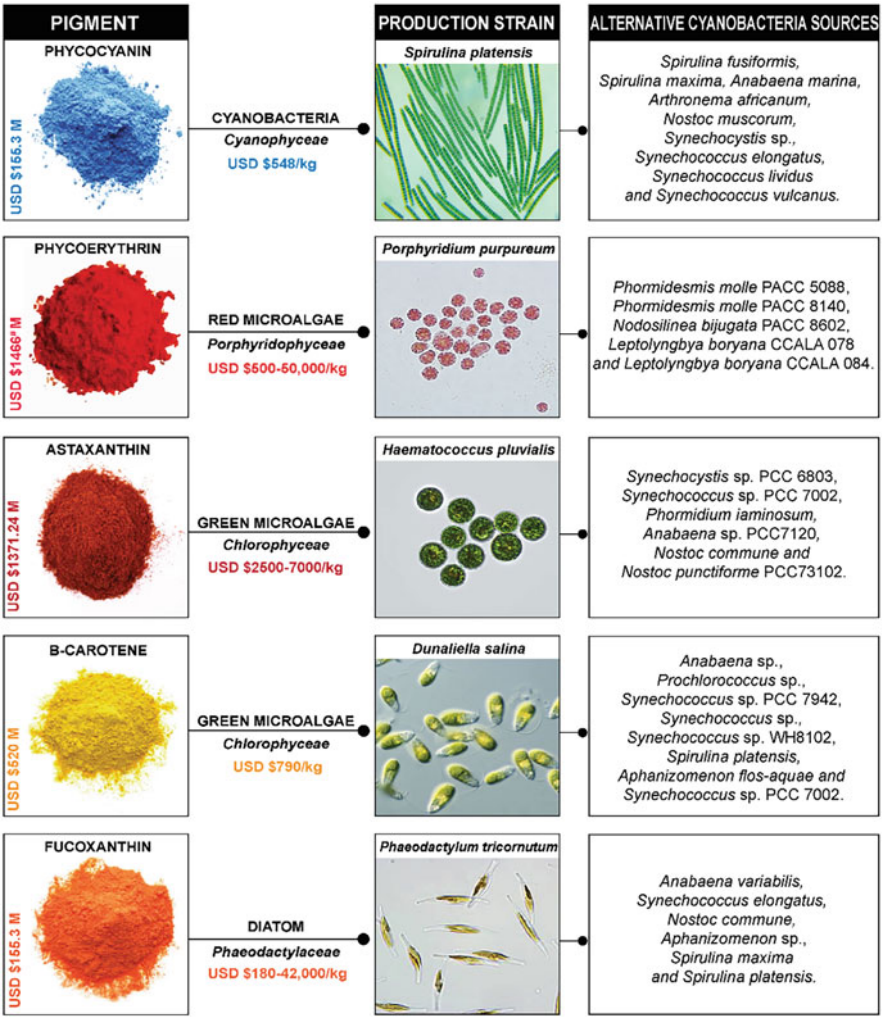



**Fig. 9** Current commercial algae and cyanobacterial-based pigments. The pigments (left), the commercial strain (source, middle) and potential cyanobacterial strains with high pigment content. The micrographs of the commercial strains were obtained from the 'Microalgae Strain Catalogue' [378]. The reported strains from the literature are listed as potential candidates to replace or supplement the current production strains. The market size of the pigments in 2020 (USD millions) are denoted for each of the pigments (according to BCC research – https://www.bccresearch.com/). The selling price for each pigment (per kg) is also provided [379, 380]. #indicates the market size in 2019. (Lutein and chlorophyll are not listed as they are commercially produced only from plant sources, marigold flowers and alfalfa, respectively)

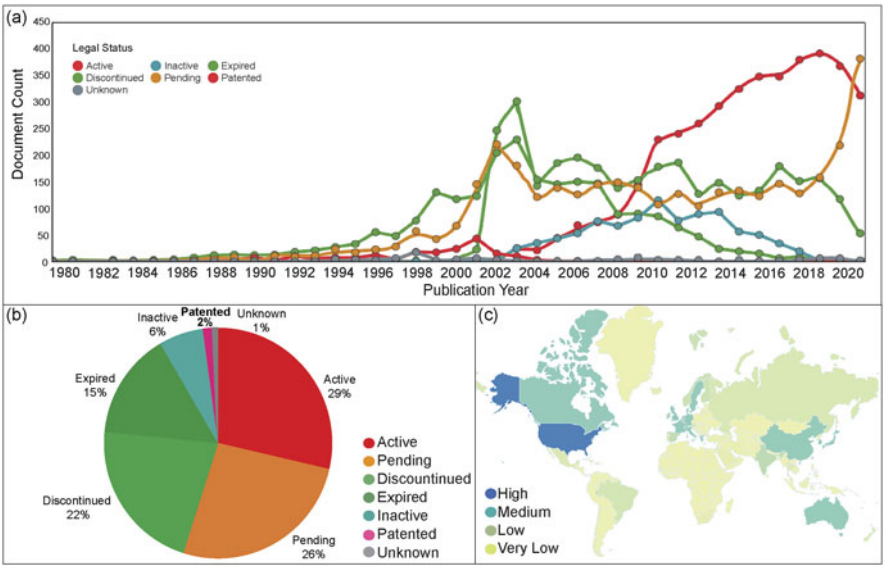



**Fig. 10** Phycocyanin patent analysis. (**a**) Phycocyanin-based patent document count vs publication year (with legal status). The number of active patents significantly increased after 2004 but saw a general drop after about 2018 (**b**) Legal status of the patents vs document count. There are many patent applications pending (latency period) and discontinued categories which still provide useful literature for competitor analysis. (**c**) Patent performance by jurisdiction (country). Currently, USA holds the highest number of PC patents ($n = 9{,}875$)

## 7.2 *Techno-Economic Analysis and Life-Cycle Analysis: CAPEX/OPEX and Price Points*

Cyanobacteria provide the basis for a range of light-driven biotechnologies and exhibit promising characteristics such as high biomass yields (30–33 T dry weight $ha^{-1}$ $year^{-1}$ [26, 385]), utilisation of non-arable land and ocean water, and integrate $CO_2$ utilisation and capture opportunities [386].

The global production of *Spirulina* sp. comprises about 10,000 tons of dry biomass per annum [387]. The focused attention on the improvement of production and processing steps for microalgae is used to derive both low volume, high-value products and high volume, low value commodities [388, 389]. Techno-Economic Assessments (TEA) and Life-Cycle Assessments (LCA) are important foundational tools to evaluate the economic, social and environmental benefits of specific cyanobacteria processes. TEA is used to analyse and optimise the economics of the process (e.g. production systems, dewatering, cell disruption, purification) by calculating, comparing and simulating the Capital expenditure (CAPEX), operational expenditure (OPEX) and product sales which provide the income stream. TEA analysis has been widely used to evaluate and optimise the efficiency and economic performance of various production processes [172, 390]. TEA includes analysis of

cost parameters such as energy inputs and outputs which accounts for delivered energy and energy losses associated with the production. It enables the calculation of Energy Return on Energy Invested (EROEI) based on operating conditions, total capital investment, production cost and payback period [9]. LCA, on the other hand, is a method to perform environmental analysis of the complete production process cycle and includes parameters such as GHG emissions, cumulative energy demands, eutrophication potential and waste management. Individually, TEA evaluates economic efficiency and LCA evaluates the environmental efficiency and can also be used to assess social benefit (e.g. jobs and eco-system services) [172]. Integrated TEA/LCA allows simultaneous analysis of economic, social and environmental factors and is a powerful tool that enables model guided design to fast-track triple bottom line system optimisation, de-risk scale-up and enable the development of robust business models [172].

TEA/LCA has been used to evaluate a wide variety of cultivation technologies (which include open pond systems and different types of photobioreactors [172, 390]) to evaluate their product yield and quality and ultimately commercial viability. The open pond system is among the simplest in terms of construction and operation, leading to lower capital and operational costs compared to photobioreactors (PBRs) [255, 391]. However, PBRs have advantages in terms of maintaining strain purity, biomass productivity, optimising light delivery, $CO_2$ supply and use efficiency, and controllability. TEA/LCA is also used to simulate different downstream processes (e.g. cell disruption, product recovery/extraction, purification, formulation) and to compare, evaluate, integrate and optimise different process components as well as the complete process [305, 392, 393].

Biorefinery strategies designed to produce multiple products can offer economic benefits, but this is not always the case. Chaiklan et al. (2018) [372] performed an economic feasibility study on extracting multiple products (phycocyanin produced with lipids and polysaccharides) from *Spirulina platensis*. They concluded that single-product production of phycocyanin was economically feasible, but the multiple-product approach (coproduced with lipids and polysaccharides) was not feasible. The estimated production cost of phycocyanin was USD \$250 $kg^{-1}$ which is an encouraging figure for large-scale production.

In summary, the use of TEA, LCA or integrated TEA/LCA (TELCA) is very important to fast-track systems optimisation, de-risk scale-up and establish robust business models [172, 390]. In particular, our international community is faced with the urgent challenge of reducing $CO_2$ emissions by almost 100% by 2050. This will require an investment of about USD \$40 Trillion, and so robust system optimisation is critical as the scale-up cost is equivalent to approximately 31% of the Worlds ~\$127 Trillion 2019 Global GDP [394].

At the current cost of USD \$3 – 9 $kg^{-1}$ (biomass dry weight), cyanobacteria are already accessible for the production of a range of high-value products in industries. Rapid advancements in high-throughput production strain selection [241, 395], photosynthetic machinery (antenna engineering), product biosynthesis, process optimisation (light, macro and micronutrients, $CO_2$, pH, temperature), reactor design and scale-up [255], harvesting and purification techniques [396], location selection

(climate, land costs, regional jobs), automation (to reduce operational cost), biorefinery (multi-product approach), cryopreservation [397, 398], scale-up (laboratory, pilot scale, and industrial) [255], as well as TEA/LCA [172, 389] and policy adaptations [172] are collectively contributing to improved production systems which in turn are the areas for future development in the cyanobacteria-based industries [399]. It is anticipated that biomass prices can be reduced towards USD ~$1 $kg^{-1}$ allowing the industry to expand from high-value products down to commodity products [172].

## 8 Global Pigment Market Analysis: Opportunities and Challenges

*Opportunities:* Growing awareness about the health benefits of natural pigments is supporting the growth in demand. The World Health Organisation (WHO) developed a global action plan for prevention and control of chronic diseases, encouraging a diet with essential nutrients, enriched with bioactive components (e.g. Ω-3 PUFAs (Poly-Unsaturated Fatty Acids) and Polyphenols) [400] and thus further increasing the overall demand.

Cyanobacterial and microalgal biomass are already in the market and have recently gained attention as alternatives to produce nutrient-rich foods. They are known to have a high nutritional value being rich in phycocyanin, chlorophylls, essential fatty acids (e.g. gamma linoleic acid), carbohydrates and trace minerals supporting consumer acceptance and marketing of natural pigments from microalgae and cyanobacteria. The colour and bioactive properties of cyanobacteria pigments are a dual benefit for multiple industrial sectors (e.g. Phycocyanin – blue protein pigment from *Spirulina* sp., termed a 'Diamond Food' in the food sector and also used widely in cosmetics and pharmaceuticals) [139, 401, 402].

In the past few decades there has been a transition to the development and use of natural food products and additives to replace chemically produced additives. The global carotenoid market was estimated to be USD $0.76 billion in 2007 (β-carotene held the largest share). In 10 years, the carotenoid market doubled to USD $1.5 billion (astaxanthin held the largest share) and is anticipated to rise further to USD $2.0 billion by 2022 with a CAGR of 5.7% [403]. This shift from β-carotene to astaxanthin was mainly due to the use of astaxanthin in animal and aquaculture feed (USD $300 million) and in nutraceuticals (as an antioxidant agent; USD $30 million) in 2009. Astaxanthin is still known as the most powerful antioxidant (6,000× stronger than Vitamin C [404]). The astaxanthin market demand is expected to increase to $800 million and $300 million by 2020 for animal feed and for nutraceuticals, respectively [405]. Carotenoid pigments such as astaxanthin, β-carotene, fucoxanthin and lutein from microalgae are attracting attention as yields are much higher compared to their conventional sources (e.g. lutein yields from microalgae is 6x higher than from marigold flowers; yield of astaxanthin from

microalgae is ~300× higher than from salmon or krill). Additionally, natural pigment production from cyanobacteria and microalgae is much faster with lower cultivation costs (compared to plants) and can be produced throughout the year around the world.

*Market and Competitive landscape:* The market value of astaxanthin produced from microalgae is reported to be USD $2,500 $kg^{-1}$ with the production cost of microalgae feedstock of USD $5 – USD $20 $kg^{-1}$ dry weight [406]. Commercially, *Haematococcus pluvialis* and *Dunaliella salina* are widely used production strains for astaxanthin and β-carotene production, respectively. The production of *H. pluvialis* is about 300 tons per year primarily from the USA, Israel, and India [10, 123, 407]. AstaReal, Inc. is the pioneer company that commercialised astaxanthin (1994). They marketed natural astaxanthin in 4 forms AstaReal® L10 oleoresin (10% extract), AstaReal® EL25 (2.5% powder), AstaReal® A1010 (astaxanthin-rich dry algae biomass) and Novasta (animal nutrition). Based on the global carotenoid market analysis, Europe has a strong and potential market due to the increasing demand for animal feed, health supplements and cosmetics. Involvement of leading cosmetic industries such as Unilever, L'Oreal, Henkel and Beiersdorf is expected to underpin the growth of the carotenoid market value in the European market. A number of key vendors are playing a major role in producing carotenoid pigments across the globe such as Lycored, Divis Laboratories, Naturex SA, BASF Corporation, FMC Corporation, and ExcelVite SDN BHD. Some of the top companies for cyanobacteria and microalgae-based pigments (already in the market) are listed in Table 9.

*Challenges:* There is considerable research and commercial interest to develop reliable natural colourants and to improve their stability. Most pigment-based patents are technological patents that claim efficient and gentle extraction techniques that offer final pigment stability (Sect. 7.1). Meeting the current challenges in the natural pigment market would further help their use and commercialisation.

- Synthetic colourants have already been in use for the past few decades and offer strong pigmentation, stability, easier processing, lower cost, and availability in unlimited quantities.
- The pigments produced from other microbial sources such as fungi, bacteria and yeast (by genetic engineering approaches) are exploited for different commercial applications [16, 17, 21, 147] and can increase market competition.
- Some of the major challenges reported when employing natural pigments in food industries include higher cost of production (e.g. carotenoids require solvent extraction), limited application (non-compatible with some foods), complexity of the process (thermal sensitivity) and inconsistent quality (degradation/fading).

**Table 9** Examples of cyanobacteria and microalgae-based pigment production companies

| Pigment | Current production strain | Companies | Location |
|---|---|---|---|
| Phycocyanin | *Spirulina* sp. | Earthrise Nutritionals, LLC | USA |
| | | Cyanotech Corporation | USA |
| | | Qingdao ZolanBio Co., Ltd. | China |
| | | Yunnan Green A Biological Project Co., Ltd. | China |
| | | Parry Nutraceuticals | India |
| | | Tianjin Norland Biotech Co., Ltd. | China |
| | | Zhejiang Binmei Biotechnology Co., Ltd. | China |
| | | Fuqing King Dnarmsa Spirulina Co. Ltd. | China |
| | | Japan Algae Co., Ltd. | Japan |
| | | Bluetec Naturals Co., Ltd. | China |
| | | Dongtai City Spirulina Bio-engineering Co., Ltd. | China |
| | | BlueBioTech Int. GmbH | Germany |
| | | AlgoSource Pvt Ltd. | France |
| | | D.D. Williamson & Co., Inc. | USA |
| | | Chr Hansen Holding A/S | Denmark |
| | | Sensient Technologies Corporation | USA |
| | | Naturex Inc. | France |
| | | GNT Group B.V. | Netherlands |
| | | Phyco-Biotech Laboratories | France |
| | | Sigma-Aldrich Corporation | USA |
| Astaxanthin | *Haematococcus pluvialis* | Cyanotech Corporation | USA |
| | | Parry Nutraceuticals | India |
| | | BlueBioTech International GmbH | Germany |
| | | Algatechnologies Ltd. | Israel |
| | | AlgaeCan Biotech Ltd. | Canada |
| | | AstaReal AB | Japan |
| | | Algae Health Sciences – A BGG company | USA |
| | | Algalif Iceland ehf. | Iceland |
| | | Algamo s.r.o. | Chile |
| | | Piveg, Inc. | USA |
| β-carotene | *Dunaliella salina* | Algalimento SL | Spain |
| | | Seagrass Tech Private Limited | India |
| | | Plankton Australia Pty Ltd | Australia |
| | | Hangzhou OuQi Food co., Ltd. | China |
| | | Shaanxi Rebecca Bio-Tech Co., LTD | China |
| | | Nutragreenlife Biotechnology Co. Ltd. | China |
| | | Israeli Biotechnology Research (IBR) Ltd | Israel |
| | | Xi'an Fengzu Biological Technology Co., Ltd. | China |
| | | Fuqing King Dnarmsa Spirulina Co., Ltd. | China |
| | | Monzón Biotech S.L | Spain |

## 9 Future Perspectives

Cyanobacterial pigments offer significant potential in multiple industrial sectors, including food and pharmaceuticals. The multidisciplinary aspect considered in natural pigment production for the food sector is that the colourants are used both as dyes and additives providing nutritional benefits. Advancements in phytoprospecting and bioprocess engineering have been useful for enhancement of biomass yields by optimising cultivation and extraction strategies (e.g. biomass harvest, solvent selection, extraction, purification and final formulation) and allow higher pigment yields and easy scalability. The combined identification of both biomass productivity and pigment concentration will enable the development of economically feasible pigment production scenarios with enhanced pigment yields and quality. Development of high-throughput screens helps to fast-track the optimisation of production conditions for the chosen target strains and guides the understanding of differences in strain-specific and pigment-specific production scenarios. Further analysis and understanding of the metabolomics will provide significant insights in developing the strategies for in vitro pigment accumulation. A completely different challenge for cyanobacterial pigments is associated with the regulatory bodies. Their approval depends on whether the pigment is a pure extract or dry biomass powder and the pigment concentration (e.g. Spirulina blue powder is marketed as crude/impure PC). Another challenge involves the effect of pigments on taste (consumer acceptance) and their stability, which can be improved through encapsulation or refinement techniques.

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