Journal of Applied Phycology (2020) 32:3831–3852
https://doi.org/10.1007/s10811-020-02231-z

Biotechnology applied to *Haematococcus pluvialis* Fotow: challenges and prospects for the enhancement of astaxanthin accumulation

Regis Le-Feuvre[1] · Priscila Moraga-Suazo[2] · Javiera Gonzalez[1] · Sergio San Martin[1] · Vitalia Henríquez[3] · Andrea Donoso[1] · Cristian Agurto-Muñoz[1,4]

Received: 22 April 2020 / Revised and accepted: 13 August 2020 / Published online: 22 September 2020
© Springer Nature B.V. 2020

Abstract

Among natural sources, the green alga *Haematococcus pluvialis* is the major producer of the potent antioxidant pigment astaxanthin, a high-value compound whose demand is still not sufficiently covered by the current industrial production. Despite the availability of low-cost synthetic astaxanthin, natural astaxanthin is more potent and accepted for human consumption and food additive uses. This review highlights the use of different biotechnological approaches aiming to increase astaxanthin production yields and discusses the advantages and drawbacks of traditional alternatives widely used on other microorganisms. These traditional approaches span from the easy to perform but not devoid of problems random mutagenesis, to advanced methods like microalgae genetic engineering, which has great potential for enhancement, despite being highly restricted in several countries by genetically modified organism legislation. In addition, we propose the underexplored approach of artificial polyploidization for the obtention of strains with increased cell size, which have the advantage of being considered as non-genetically modified organisms that do not require modification of the current industrial production procedures.

Keywords *Haematococcus pluvialis* · *Haematococcus lacustris* · Antioxidant · Genetic engineering · Classical mutagenesis · Polyploidy

Introduction

Microalgae are photosynthetic microorganisms comprising a diverse group of eukaryotic and cyanobacteria members present in a wide range of environments, being highly abundant in oceans, lakes and rivers, as well as extreme environments including desert and polar regions (Lee 2008). Microalgae also have a huge ecological role, accounting for approximately 50% of the world's organic carbon fixation (Field et al. 1998; León and Fernández 2007). Their huge diversity is only compared with their biotechnological potential. Current research is focused on exploiting diverse bioproducts made by these microorganisms, including polyunsaturated fatty acids (PUFAs, like DHA and EPA), proteins, vitamins, polysaccharides, and carotenoids with high biotechnological impact, mainly in food products (Pulz and Gross 2004; Sousa et al. 2008), biodiesel production (Dickinson et al. 2017), bioremediation (Muñoz and Guieysse 2006), and cosmetics (Enzing et al. 2012). Nowadays, the microalgae market produces

Regis Le-Feuvre and Cristian Agurto-Muñoz contributed equally to this work.

✉ Regis Le-Feuvre
rlefeuvre@udec.cl

✉ Cristian Agurto-Muñoz
cagurto@udec.cl

[1] Grupo Interdisciplinario de Biotecnología Marina (GIBMAR), Centro de Biotecnología, Universidad de Concepción, Concepción, Chile

[2] Departamento de Ecosistemas y Medio Ambiente, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Santiago, Chile

[3] Laboratorio de Genética e Inmunología Molecular. Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

[4] Departamento de Ciencia y Tecnología de los Alimentos CyTA, Facultad de Farmacia, Universidad de Concepción, Concepción, Chile

nearly 9000 t of dry matter per year, with a global market size of nearly US$ 6.5 billion (Mobin and Alam 2017).

Among high-value microalgal compounds, the red pigment astaxanthin has the highest demand in the market due to its antioxidant properties. It belongs to the xanthophyll family and can be found in some microalgae, bacteria, yeasts, and marine animals (Lorenz and Cysewski 2000; Ambati et al. 2014). The unicellular freshwater microalgae *Haematococcus pluvialis* Fotow 1844 (recently proposed to be a synonym of *Haematococcus lacustris* (Girod-Chantrans) Rostafinski 1875 by Nakada and Ota 2016) is the richest source of astaxanthin, a powerful antioxidant with nutraceutical and pharmacological applications. Also, astaxanthin is key for pigmentation in farmed salmon, trout, and poultry (Sommer et al. 1992; Gutiérrez et al. 2012). In humans, astaxanthin-based dietary supplements have demonstrated reduction of several disorders, such as neurodegenerative diseases (Guerin et al. 2003), cancer (Chew et al. 1999; Tanaka et al. 2012), ocular diseases (Cort et al. 2010), skin diseases (Tominaga et al. 2012), and on the other hand, immune response enhancement (Park et al. 2010). Also, it has been reported that it acts as an anti-inflammatory agent (Speranza et al. 2012) and an inhibitor of LDL cholesterol (Iwamoto et al. 2000; Choi et al. 2011). Despite that the pigment is mostly used for aquaculture, the market of astaxanthin-based products for human consumption is constantly growing, being estimated at US$ 35–60 million in 2008 (Frost 2008). From 2011 to 2016, the global production of natural astaxanthin increased from 43,279 to 73,717 kg, with a rate of 11.24%. This shows an expected US$1.5 billion market for natural astaxanthin by 2020, mainly through the market of capsules, ingredients for cosmetics, drinks, and functional food (Nguyen 2013).

The market price of natural astaxanthin is mainly determined by its concentration and purity. Regarding *H. pluvialis* dry biomass, prices may range from US$2500 to 7000 kg^{-1}, depending on its astaxanthin concentration (Bauer and Minceva 2019; Harith et al. 2020). Meanwhile, the price of astaxanthin oleoresin (usually obtained by supercritical CO_2 extraction) may range from US$8000 to $15,000 kg^{-1}, being usually commercialized with astaxanthin concentrations of 5, 7, 10, or 15% (personal communication from Astax Chile SPA). Global production is mainly concentrated in Europe, North America, China, and other Asian countries.

Despite the importance of the existing market for natural astaxanthin, over 95% of the available pigment is produced by chemical synthesis. Even though several studies have reported that the optimization of different culture parameters could reduce production costs, making the production of natural astaxanthin competitive, or even cheaper, than the synthetic product is still not possible (Li et al. 2011; Koller et al. 2014). The astaxanthin molecule has two asymmetric carbon atoms in positions 3 and 3′. Consequently, there are different optical isomers, or enantiomers: (3*S*,3′*S*); (3*R*,3′*R*) and (3*R*,3′*S*). Synthetic astaxanthin comprises a mixture of the three isomeric forms, while natural astaxanthin has optically pure (3*S*, 3′*S*)-chirality (Renstrøm et al. 1981; Grung et al. 1992), which provides stronger pigmentation in aquaculture (Østerlie et al. 1999) and also a much higher oxygen radical absorbance capacity (ORAC) when compared with synthetic astaxanthin (Naguib 1998) and the rest of the carotenoids (Chew and Park 2004; Palozza et al. 2009; Capelli et al. 2013). Moreover, synthetic astaxanthin is not approved for human consumption (Capelli and Cysewski 2012; Shah et al. 2016), nor associated with any other natural stabilizing compounds (e.g., β-carotene, lutein, and PUFAs) as found together in natural astaxanthin (Holtin et al. 2009; Schmidt et al. 2011). Striking differences between natural and artificial astaxanthin have already been reported by Capelli et al. (2013), showing that in terms of anti-oxidant properties, the synthetic compound is 20 times less effective than its natural counterpart. It also raises serious concerns about safety for human consumption, since artificial astaxanthin is considered a different chemical compound.

Given the above-mentioned background and its reported properties, consumers have already become aware of the benefits of natural astaxanthin. Therefore, optimizing the commercial production of natural astaxanthin is critical in order to supply its increasing global demand (Lorenz and Cysewski 2000; Liu et al. 2014; Shah et al. 2016).

Haematococcus pluvialis as a source of astaxanthin

Compared with other microorganisms that naturally produce astaxanthin, such as the red yeast *Xanthophyllomyces dendrorhous* and the microalga *Chromochloris zofingiensis*, *H. pluvialis* has the capacity to produce and accumulate significantly higher amounts of the pigment (Rise et al. 1994; Boussiba et al. 1999; Borowitzka 1999; Liu et al. 2014; Curtain 2000). Hence, *H. pluvialis* is considered the most promising microalgae for astaxanthin production, accumulating up to 4% of cell dry weight (DW), the highest content registered on nature (Boussiba 2000). However, these concentrations have only been reported under laboratory-controlled conditions, or by some commercial producers that report up to 6% DW (Table 1). *Haematococcus pluvialis* astaxanthin is currently offered as a dietary supplement, mainly in the USA and Japan (Sarada et al. 2002; Liu 2010), but it is also available in many other countries. Since this microalga has been generally recognized as safe (according to GRAS status), its commercialization should be guaranteed worldwide.

Haematococcus pluvialis is a common, single cell chlorophyte, distributed worldwide in temperate regions. It undergoes important morphological changes from green active cells to red immobile cells (Sun et al. 2015). When green vegetative cells are submitted to stressful environmental factors, such as high light intensity, high temperatures, external

Table 1 Some industrial producers of astaxanthin derived from Haematococcus. Mean culture yields on % DW were provided when the information was available from the industries website. NS, not specified

Company	Website	Type	Culture system	Typical yields (% DW)	Country	Main market product
AstaReal Holdings Co., Ltd.	www.astareal.com	Closed System	Stainless steel photobioreactor	6.0%	Japan	AstaReal
ALGA Technologies Ltd	www.algatech.com	Closed System	Outdoor Enclosed glass-tube photobioreactor	NS	Israel	AstaPure
Cyanotech Corporation	www.cyanotech.com	Open system	Outdoor raceway open pond	1.5-2.0%	USA	Bioastin
Supreme Biotechnologies Ltd.	www.astasupreme.co.nz	Closed System	Enclosed plastic bags photobioreactors	NS	New Zealand	Asta Supreme
Parry Nutraceuticals	www.alimtec.cl	Open system	Outdoor raceway open pond	2.0%	India	Zanthin
Alimtec S. A	https://valensa.com/vproducts/astaxanthin/				Chile	
Algae Health a BGG company	www.algaehealthsciences.com	Closed System	Outdoor Enclosed glass-tube photobioreactor	5.0%	China	AstaZine
Heliae *	www.truazta.com	Open system	Indoor raceway open pond	NS	USA	TruAzta
Algalif	www.algalif.com	Closed system	Indoor enclosed glass-tube photobioreactor	5.0%	Iceland	Algalif
Algamo	www.algamo.cz	Closed system	Indoor enclosed plastic bags photobioreactors	3.0-5.0%	Czech Republic	Algastin
Astax Chile SpA. (Former Pigmentos Naturales S.A.)	www.astaxchile.cl	Open system	Outdoor raceway open pond	2.0%	Chile	Dry algal biomass
Atacama Bio Natural S.A.	www.atacamabionatural.com	Open system	Outdoor raceway open pond	2.0%	Chile	Supreme Asta

*Currently Heliae is not producing Astaxanthin

pH changes, high salt concentration, and nutritional stress, the induction of large quantities of astaxanthin occurs, changing the cellular morphology to aplanospores, a red non-motile encysted phase (Zhang et al. 2009; Shah et al. 2016). Despite its exceptional characteristics for astaxanthin production, *H. pluvialis* growth rates are relatively slow, compared with other microalgae. Also, it is sensitive to polluted environments and contamination by other microorganisms (Olaizola 2000).

This review is focused on the diverse biotechnological approaches made to enhance the production of astaxanthin from these microorganisms and supply its increasing global demand. For additional information about *H. pluvialis*'s biology and culture, an excellent general review focusing on several aspects of astaxanthin production, including current challenges for commercial scale production and global market perspectives, can be found in Shah et al. (2016).

Astaxanthin biosynthesis

Astaxanthin biosynthesis in *H. pluvialis* is a complex process, highly up-regulated under stress conditions, which coincides with the accumulation of triacylglycerols (TAGs). Both compounds are deposited in cytosolic lipid bodies during the "red" or encysted aplanospore stage of *H. pluvialis* culture.

Astaxanthin is a C_{40} tetraterpene, belonging to the carotenoid family. It is biosynthesized by head-to-tail condensation of two isoprene isomers: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP can be originated from two dissimilar pathways: the mevalonate pathway (MVA), located in the cytosol, and the non-mevalonate pathway (DOXP), located in the chloroplast (Lichtenthaler et al. 1997; Lichtenthaler 1999; Eisenreich et al. 2001). *Haematococcus pluvialis* lacks three key enzymes of the MVA pathway, mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate pyrophosphate decarboxylase (MVD) (Gwak et al. 2014), but instead, it has the full set of enzymes for the DOXP pathway located inside the chloroplast. This is required for the conversion of photosynthesis-derived products into IPP.

In the following step, IPP undergoes isomerization to DMAPP. It has been long assumed that this conversion is catalyzed exclusively by isopentenyl pyrophosphate isomerase (IPI; Lichtenthaler 1999; Sun et al. 1998). However, recent transcriptomic studies suggest that no *ipi* genes of *H. pluvialis* (IPI1 and IPI2) are up-regulated during cellular accumulation of astaxanthin (Gwak et al. 2014). Different reports have suggested that another enzyme of similar activity, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), would be

most likely responsible for catalyzing the interconversion between IPP and DMAPP (Hoeffler et al. 2002; Rohdich et al. 2002; Gwak et al. 2014). Further study is required to assess the contribution of these three enzymes for astaxanthin accumulation.

Elongation of the isoprenoid chain is initiated with a molecule of DMAPP and subsequent linear additions of three IPP molecules, catalyzed by geranylgeranyl pyrophosphate synthase (GGPS) (Young and Britton 1993; Cunningham and Gantt 1998). The final step of this process ends in a C_{20} compound, geranylgeranyl pyrophosphate (GGPP), a shared precursor among other isoprenoids. Then, phytoene synthase (PSY) catalyzes the head-to-tail condensation of two GGPP molecules to form the first tetraterpene carotenoid, phytoene (Cunningham and Gantt 1998). The PSY enzyme has been seen to be up-regulated in *H. pluvialis* cells stressed with high-light, undergoing transformation from green zoospores to red aplanospore stage (Steinbrenner and Linden 2001; Vidhyavathi et al. 2008; Gwak et al. 2014). Two classes of PSY have been found in green algae like *Ostreococcus* and *Micromonas*, while some other green algae like *Chlamydomonas reinhardtii* and *Chlorella vulgaris* only possess one class of PSY (Tran et al. 2009). Different PSY genes have been cloned and characterized from microalgae, including *H. pluvialis* (Steinbrenner and Linden 2001) and *Chromochloris zofingiensis* (Cordero et al. 2011). The *Haematococcus* PSY has a N-terminal extension similar to a chloroplast targeting sequence, indicating that PSY targets the chloroplast of *H. pluvialis* (Steinbrenner and Linden 2001).

Subsequently, four successive desaturation reactions of phytoene are catalyzed by phytoene desaturase (PDS) and a ζ-carotene desaturase (ZDS). In the first two reactions, PDS converts phytoene to phytofluene and ζ-carotene. ZDS catalyzes two further reactions converting ζ-carotene to neurosporene and lycopene (Cunningham and Gantt 1998). Plastid terminal oxidases PTOX1 and PTOX2 act as cofactors for electron transfer between the C_{40} carotenoid intermediates, plastoquinone, and the final electron acceptor, oxygen (Li et al. 2010; Nawrocki et al. 2015). The enzyme PTOX1 is co-regulated with the astaxanthin synthesis in *H. pluvialis* (Wang et al. 2009; Nawrocki et al. 2015). The reactions catalyzed by PDS are among the rate-limiting steps of the pathway (Chamovitz et al. 1993), and mutations in this gene may confer a higher astaxanthin synthesis capacity (Li et al. 2010). PDS activity is inhibited by the herbicide norflurazon (NF) that competes with the cofactor plastoquinone (PQ) (Breitenbach et al. 2001).

Both terminal ends of the lycopene molecule undergo cyclization by lycopene cyclases (LCY-e and LCY-b), yielding α-carotene (lutein precursor) and β-carotene (astaxanthin precursor). Cyclization is a branching point in the carotenoid biosynthesis in most organisms (Shah et al. 2016). In *H. pluvialis*, most carbon flux is directed to β-carotene synthesis (Borowitzka 1999), and high level of LCY-b transcripts has been observed under stress conditions (Lorenz and Cysewski 2000; Gwak et al. 2014). The final two-oxygenation steps, catalyzed by β-carotene ketolase (BKT) and β-carotene hydroxylase (CrtRb), are rate-limiting steps for astaxanthin synthesis (Vidhyavathi et al. 2008; Linden 1999; Steinbrenner and Linden 2001). Even though the reactions can proceed in any order, BKT shows a higher substrate specificity towards β-carotene when compared with zeaxanthin, favoring initial addition of a keto group before the enantio-selective hydroxylation from canthaxanthin to astaxanthin, catalyzed by CrtR-b (Lotan and Hirschberg 1995). Enantio-selectivity of astaxanthin synthesis is one of the main advantages of *H. pluvialis* astaxanthin over its synthetic counterpart, representing a crucial part for the nutraceutical market. Since astaxanthin has two identical chiral centers at positions 3 and 3′, it can exist in four different configurations, yielding three different isomers: (3*R*,3′*S*); (3*R*,3′*R*); (3*S*,3′*S*), depending on the spatial orientation of the hydroxyl groups in the chiral carbon. In the course of the chemical synthesis, these isomers are present in a ratio of 2:1:1, respectively, yielding only 25% of the naturally occurring (3*S*,3′*S*) isoform. *Haematococcus pluvialis* synthesizes the (3*S*,3′*S*) stereoisomer of astaxanthin (Grung et al. 1992), resulting in a much sought-after product in the nutraceutical market. Astaxanthin biosynthesis pathway is presented in Fig. 1.

Culture systems and harvesting process

Commercial production of astaxanthin from *H. pluvialis* is currently focused on a two-stage strategy. The first stage consists in the production of vegetative biomass under favorable conditions, achieving rapid growth at high cell densities. Subsequently, a second stage is carried out where astaxanthin biosynthesis is promoted by applying stressful media changes like salt addition, temperature rises, increases in incident light and CO_2 addition (Christian et al. 2018). Nitrate starvation is also an effective method to increase the accumulation of astaxanthin. Recent studies have focused on intracellular nitrate starvation by using nitrate reductase (NR) inhibitors like Na_2WO_4, instead of changing the C/N ratio to create a nitrogen deficient culture media (Li et al. 2019). Furthermore, the addition of oxidizing substances can slightly induce oxidative stress and increase cellular carotenoid content (Guedes et al. 2011a, 2011b). These changes lead to the formation of *H. pluvialis* cysts, astaxanthin-rich cells that are easily harvested by passive settling and centrifugation. Finally, cell biomass is mechanically disrupted to release astaxanthin which is additionally dried. The final product can be encapsulated or subjected to astaxanthin extraction for pure nutraceutical formulations (Lorenz and Cysewski 2000; Guerin et al. 2003). Alternatively, *Haematococcus* astaxanthin can be produced indoors under mixotrophical conditions adding organic carbon sources like acetate or glycerol for astaxanthin induction (He et al. 2018; Wen et al. 2020; Zhang et al. 2020), obtaining

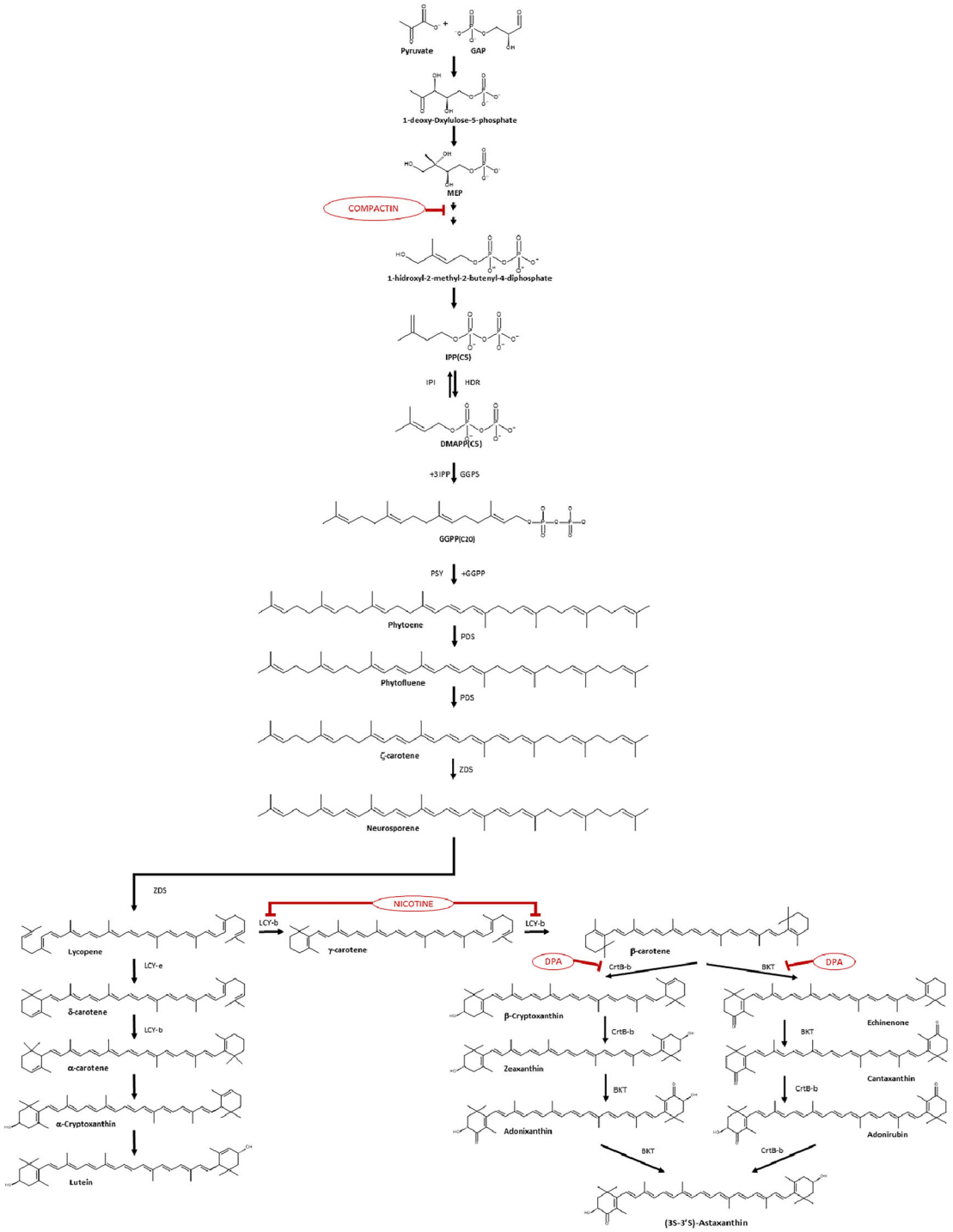

Fig. 1 Astaxanthin biosynthetic pathways on *H. pluvialis*. IPI, isopentenyl pyrophosphate isomerase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; BKT, β-carotene ketolase; CrtR-b, β-carotene 3,3′-hydroxylase

higher biomass concentration, and therefore higher astaxanthin yields. Nevertheless, production costs and contamination risks when applying these strategies are higher. Recently, new approaches like the addition of melatonin, specific phytohormones, or even electrical treatments to *H. pluvialis* culture have demonstrated to enhance astaxanthin accumulation; however, none of them have been validated at industrial scale (Ding et al. 2018, 2019; Kim et al. 2018).

Currently, the two most applied technologies for *H. pluvialis* astaxanthin production are open pond systems and closed photo-bioreactors (PBRs) (Gong and Bassi 2016). Cultivation in open systems requires less initial investment and maintenance, as set-up is cheaper. However, uncontrolled environmental conditions usually lead to low production and culture contamination. Closed systems have greater potential for optimization, higher mass transfer, higher production, and less contamination since they count with controlled operational conditions. All these advantages are associated to higher costs and complex set-ups (Singh and Sharma 2012; Borowitzka 2013; Acién et al. 2017; Moreno-Garcia et al. 2017). Currently, most PBR technologies are available for large-scale production by companies established in Europe and all over the world (Acién et al. 2017). Accordingly, the current industrial production relies only on open or closed systems as shown in Table 1.

Zhang et al. (2014) developed an alternative cultivation approach using an immobilized film method. However, the cultivation was not as efficient as the suspended algal cultures in PBRs due to poor light penetration (Zhang et al. 2016). Recently, Kiperstok et al. (2017) reported the possibility of continuous co-production of biomass and astaxanthin in a one-phase process at high light intensities up to 1015 μmol photons m^{-2} s^{-2}. This method is still under research and development, and currently is not used at industrial scale.

Biotechnological alternatives for astaxanthin production improvement

Since the major natural source of astaxanthin is *H. pluvialis*, a variety of strains have been isolated around the globe. The maximum yield reached in photobioreactors at industrial scale production is 5–6% DW and a maximum of 2% DW in 200,000-L open pond raceway systems, as informed by some companies (Table 1). Nevertheless, there is wide variation among different natural strains, with some of them reported as hyperproducers. For example, Chekanov et al. (2014) described an artic strain able to accumulate up to 3–5.5% astaxanthin under prolonged stress conditions. Later, Wang et al. (2019) described a newly discovered strain (JNU35), which hyperaccumulated up to 5.6% DW astaxanthin after complex culture optimization. Notwithstanding the existence of natural strains, the need for higher yields has motivated research aimed to enhance *H. pluvialis* natural capacity to produce astaxanthin.

Classical Mutagenesis

This is the simplest method regularly used to obtain different varieties of microorganisms, enhancing particular characteristics. This technique induces random changes on the genetic material by applying chemical mutagen agents or UV radiation. Additionally, plasma exposure has been successfully used on this microalga. Classic mutagenesis can be broadly divided into two main steps: (i) exposure of cells to mutagenic agents and (ii) isolation of mutant cells with increased astaxanthin contents.

Methods for obtaining astaxanthin-overproducer mutants

In most studies, variables such as the time of exposure and concentration of different mutagen agents are adjusted in order to obtain high mortality rates, in the range of 85–95%, originating potentially mutant individuals that are selected later using several approaches.

Among chemical mutagens, most of *H. pluvialis* available literature describes the application of ethyl methanesulfonate (EMS), either in isolation or in combination with UV radiation exposure in order to obtain mutants with enhanced astaxanthin production (Tjahjono et al. 1994; Sun et al. 1998; Wang et al. 2016). The effectiveness of this compound to generate overproducers varies largely. The first report, described by Tjahjono et al. (1994), resulted only in a modest increase of 6% with respect to the untreated strain. Since then, several overproducers have reached more than twice the astaxanthin yield of their original strains (data summarized on Table 2).

The mutagens N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Sandesh Kamath et al. 2008; Hu et al. 2008) or diethyl sulfate (DES) (Wang et al. 2016) are also employed with different dosages and exposure periods. UV radiation is also very effective at inducing changes in DNA, even when there is an intrinsic capacity of the microalgae to limit this damage (Shah et al. 2016). Revertant mutants can be easily avoided by including a period of dark incubation in order to evade the photoreactivation driven by light (Essen and Klar 2006). This method has been successfully applied by several authors (Chumpolkulwong et al. 1997; Sandesh Kamath et al. 2008) in order to obtain astaxanthin-enhanced strains. The combination of UV and chemical treatment has also been explored, with better astaxanthin yields obtained by subjecting the previously UV mutated cells to EMS treatment, reaching 2.5% DW (Chen et al. 2003), while more recent research have reported 4.7% DW (Wang et al. 2016). A third alternative consists in the exposure of cells to low-temperature plasma by atmospheric pressure dielectric barrier discharge plasma (DBD), a technique that produces energetic electrons and ions, reactive species, and UV radiation, used to induce gene mutations. Using this method, 3.3% DW astaxanthin yield has been achieved, with nearly 40% higher growth rates under high light irradiation (Liu et al. 2016). Later, the same group demonstrated that this mutant has higher astaxanthin accumulation due to higher photosynthetic activity, increased non-photosynthetic pigments protecting photooxidative stress, and better efficiency of CO_2 utilization (Chen et al. 2020).

Table 2 Astaxanthin yields reported after the modification of *Haematococcus pluvialis* using different mutagenesis strategies. Yields (in mg/g DW) of the base strain and the best mutant strain(s) are reported when available

Reference	Mutagen agent	Selective agents and conditions	Base strain and astaxanthin yield (mg g^{-1} DW)	Mutant strain best yield (mg g^{-1} DW)	Astaxanthin yield increase[a]	Growth media and condition[b]	Observations
Tjahjono et al. (1994)	EMS 0.135 M	Norflurazon 3 μM Fluoridone 3 μM Nicotine 100 μM Red colonies present on high irradiation (10 K lux).	NIES 144. National Institute for Environmental Studies, Tsukuba, Japan. 11.2–12.6	NZ 2.5.23 13.4 FL 1.8.6 12.4 NC 7 10.9	6.35% − 1.59% − 2.68%	Kobayashi et al. (1991). Mixotrophic	Authors obtained polyploid fusants with better yields based on mutant strains (described later on polyploidy section).
Chumpolkulwong et al. (1997)	EMS 0.135 M or UV 7 min	Compactin 2 mM Mutants grow and form red colonies	NIES 144. 50 pg $cell^{-1}$	M15 98 pg $cell^{-1}$ Mb 70 pg $cell^{-1}$	96% 40%	Kobayashi et al. (1993). Mixotrophic	No information available to calculate astaxanthin yield based on DW.
Sun et al. (1998)	EMS 0.2 M	Mutants form brown colonies under low light	NS strain from Göttingen Culture collection NS	Car-3 NS	NS	Sager and Granick (1953) Mixotrophic	Mutant duplicate carotenoid yield of base strain under high light on 24 h period. No information available to calculate astaxanthin yield.
Tripathi et al. (2001)	EMS 0.01 M or UV 15 min	DPA 15 μM Nicotine 220 μM Compactin 1.5 μM Resistant colonies selected on herbicides	Unidentified strain from Sammlung Algenkulturen, Pflanzen Physiologisches Institut, Universität Göttingen Germany 4.3–5.1	DPA 10.6 Nicotine 14.0 Compactin 9.1	145% 225% 112%	Kanz and Bold (1969) (BBM). Autotrophic Kobayashi et al. (1991). Mixotrophic	Very high increases on astaxanthin yields (2.2–3.2 fold) but based on a modestly producing base strain with 0.43% astaxanthin accumulation. Best astaxanthin yield reached with addition of Sodium Acetate
Chen et al. (2003)	UV 16 min EMS 0.1 M Each agent alone, or EMS and then UV treatment	Nicotine 200 mM DPA 15 μM Norfluorazon 3 μM Fluridone 3 μM Largest red colonies selected under high irradiation	F712 from Freshwater algae culture collection, China. 12	NC17 20 NC5 19 EU3 25	67% 58% 108%	Sanger & Granick (1953) (SM) Kobayashi et al. (1991) Mixotrophic	Successive application of EMS and UV originated the mutant with best yield. Best astaxanthin yield reached with addition of Sodium Acetate and Fe.
Hu et al. (2008)	MNNG 0.34 M	Colonies screened for astaxanthin yield by light microscopy and pigment analysis by RP-HPLC	NIES-144. 20	Mt 2877 39	95%	Kobayashi et al. (1991) Mixotrophic	Best astaxanthin yield reached with addition of SodiumaAcetate and Fe. High tolerance to luminous stress.
Sandesh Kamath et al. (2008)	UV 15 min EMS 0.2 M MNNG 0.1 mM	Glufosinate 28 μM Surviving colonies selected on herbicide	Unidentified strain from Universität Göttingen, Germany 21	U1 26 E5 12 N5 39.5	24% − 43% 88%	Kanz and Bold (1969) (BBM). Autotrophic	UV mutant produce more astaxanthin per cell but reach lower biomass than base strain. EMS mutant reached higher biomass but lower astaxanthin production.
Hong et al. (2012)	EMS 16 mM	Differential growth and color under different light intensities and	NIES 144. 45.2 (HLI)[c] 27.8 (MLI)[c]	PP-PS #160 57.6 (HLI) 42.8 (MLI)	27% 54%	Hata et al. (2001) (NIES) Mixotrophic	Highly photoinducible mutants obtained with higher capacity for astaxanthin production under moderate and high light conditions.

Table 2 (continued)

Reference	Mutagen agent	Selective agents and conditions	Base strain and astaxanthin yield (mg g^{-1} DW)	Mutant strain best yield (mg g^{-1} DW)	Astaxanthin yield increase[a]	Growth media and condition[b]	Observations
		phototropic and autotrophic conditions.				NIES-C modified without acetate. NIES-N modified without acetate and nitrogen	
Gomez et al. (2013)	EMS 0.08 M	DPA 25 μM	Strain 114, pigmentos naturales 20.3	B24 26.4	30%	Starr and Zeikus (1987) (Bristol) autotrophic	Astaxanthin yield varied between laboratory level and open ponds of 120 m^3
Wang et al. (2016)	UV EMS 0.16 M DES 8 mM	DPA 71 μM Stress conditions (30 °C, 7500 lx)	Strain 712, Bioengineering lab, College of Food Science and Engineering, Ocean University of China. 27.7	DPA12–1 47.21	70%	Wang et al. (2016) autotrophic.	Three stage mutagenesis for high biomass, high activity and finally high astaxanthin yield.
Liu et al. (2016)	DBD exposure	High-growth-rate strains	FACHB-712 Freshwater algae culture collection, Chinese Academy of Sciences. 21.1	M3 33.5	59%	Kanz & Bold (1969) (BBM) autotrophic	DPA used for photosynthetic activity determination not strain selection.
Cheng et al. (2016)	γ irradiation 4000 Gy	Surviving large red colonies under continuous low light	FACH-872 Freshwater algae culture collection, Chinese Academy of Sciences. 12.5	NS Mutant 19.5	56%	Dominguez-Bocanegra et al. (2004) (BBM) Autotrophic with high CO_2 and light	Fast growing mutant yields higher biomass, lipid and 2,4 times more astaxanthin content under high CO_2 concentration (6%) and high light conditions.

[a] Astaxanthin percentages calculated for *H. pluvialis* mutants grown in absence of mutagens or inhibitors as compared with base strain. Negative values denote a decrease on astaxanthin yield

[b] Mixotrophic conditions denote the inclusion of sodium acetate as an additional carbon source. Autotrophic conditions include only atmospheric or added CO_2 as carbon source

[c] Values determined under moderate light intensity (MLI, 70 μmol photons m^{-2} s^{-1}) or high light intensity (HLI, 150 μmol photons m^{-2} s^{-1})

UV ultraviolet radiation (254 nm), *DBD* atmospheric pressure dielectric barrier discharge, *EMS* ethyl methane sulfonate, *MNNG* N-methyl-N-nitro-N-nitrosoguanidine, *DES* diethyl sulfate, *NS* non-specified

The last alternative to induce genetic mutations is to exploit the high energy and penetration capabilities of γ rays. Using this technique, 2.4 times more astaxanthin has been demonstrated when applying γ-rays to an *H. pluvialis* strain, which also showed higher growth rates under high CO_2 stress when compared with the unmodified strain (Cheng et al. 2016).

Methods for screening overproducing strains

After exposure to mutagenic agents, successful isolation of mutants relies on their capacity to survive and grow in the presence of compounds at inhibitory concentrations, either by color changes of the mutants, or capacity to survive under different light conditions. It is expected that surviving strains will have an increased capacity to synthesize carotenoids and, consequently, produce more astaxanthin.

Herbicide-based mutant selection

On many cases, diphenylamine (DPA) has been used as an indicator for mutant selection. DPA is an herbicide that interferes with the carotenogenic pathway by inhibiting β-carotene oxygenase and β-carotene hydroxylase, blocking cell capacity to produce astaxanthin under stress conditions (Fig. 2). Colonies capable of synthesizing astaxanthin will stay as red colonies. They represent candidate pigment overproducers or DPA tolerant strains, as opposed to wild-type cells, which will remain green under these conditions (Tripathi et al. 2001; Chen et al. 2003; Gómez et al. 2013; Wang et al. 2016).

The concentration required for the selection varies depending on each experiment and the original strain used. Tripathi et al. (2001) used 15 μM DPA and a 1-stage mutagenesis scheme, yielding a strain with nearly 1% astaxanthin (DW), 2.46 times higher than the wild-type strain. In comparison, Wang et al. (2016) used 71 μM DPA and a three-stage mutagenesis breeding, isolating a colony with 4.7% astaxanthin (DW), 1.7 times higher than the base strain, and almost the

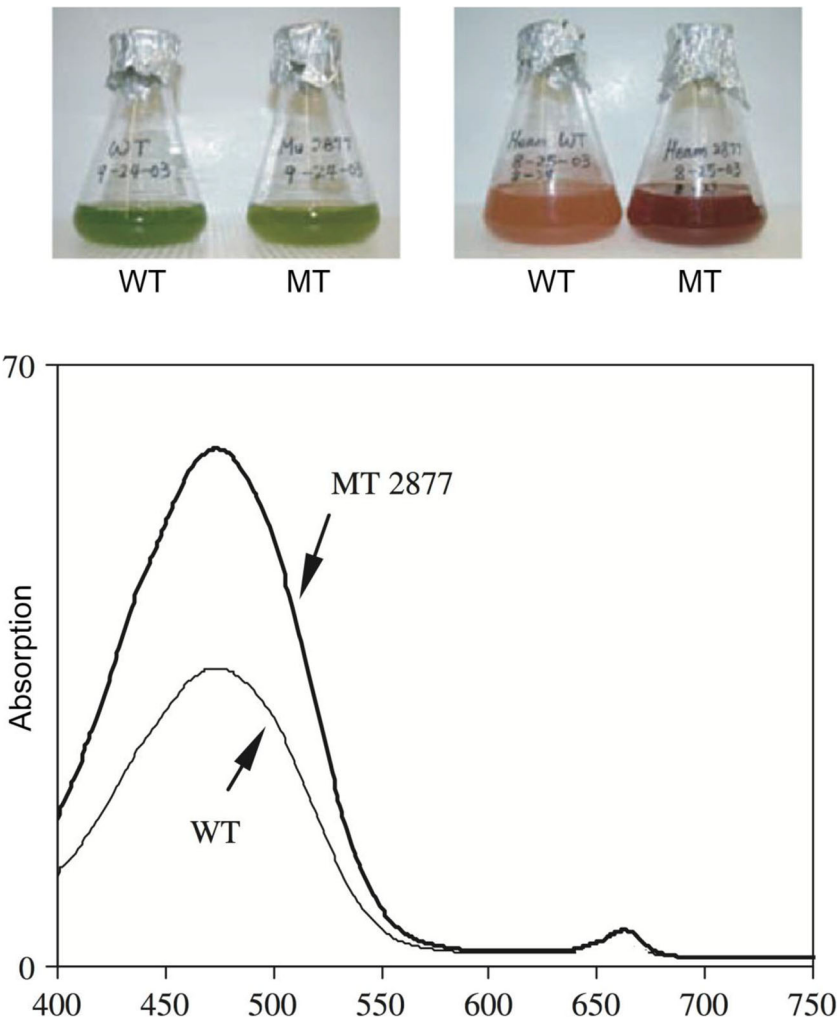

Fig. 2 Culture aspect of WT and a chemically induced mutant MT derived from the same strain **a** under favorable growth conditions and **b** under stress conditions, where a higher astaxanthin level is reflected by its darker red color. **c** WT strain reached ca 2% astaxanthin DW, while the mutant reached almost double concentration, as can be evidenced by the methanol extracts absorption spectra. Reprinted from enhanced protection against oxidative stress in an astaxanthin-overproduction *Haematococcus* mutant (Chlorophyceae), Zhengyu Hu, Yantao Li et al. European Journal of Phycology 43(4), Nov 1, 2008. © 2008 British Phycological Society, reprinted by permission of Informa UK Limited, trading as Taylor & Francis Group, www.tandfonline.com on behalf of British Phycological Society

highest astaxanthin concentration reported for any *H. pluvialis* mutant (Table 2).

Even when Tripathi et al. (2001) obtained a strain that nearly triplicated the yields of the base strain, its productivity was much lower than the strains obtained by Wang et al. (2016), emphasizing the requirement of a previous selection step of high yield base strains with commercial utility. Furthermore, the use of DPA does not always guarantee the selection of overproducers as reported by some groups, who relied on other selection agents to obtain overproducers (Chen et al. 2003; Sandesh Kamath et al. 2008). Differences on the capacity to select enhanced cells could be attributed to different susceptibilities of unrelated strains. Nevertheless, Chen et al. (2003) and Wang et al. (2016) used the same strain, 712, but reported different results. Chen does not report any remarkable strain selected using this herbicide, while the latter was able to obtain high yield overproducers by using DPA at higher concentrations and a multi-stage selection system. Therefore, culture conditions and selection schemes are very important for successful overproducer isolation.

Another herbicide, glufosinate, has strong effects on plants, increasing ammonia levels and halting photosynthesis. It has also been reported to inhibit cell growth and induce astaxanthin accumulation on *H. pluvialis* cells (Aflalo et al. 1999). It has been used as a replacement of DPA for the isolation of overproducing strains, reaching a maximum of 3.9% astaxanthin DW (Sandesh Kamath et al. 2008). However, not every herbicide is suitable to successfully select mutants, as demonstrated in compounds like norflurazon and fluridone, which were tested on early reports and are no longer used (Tjahjono et al. 1994; Chen et al. 2003).

Non-herbicide mutant selection

The selection under different herbicides is not the only option to isolate overproducing strains. For instance, nicotine, an inhibitor of lycopene cyclase that avoids astaxanthin formation has also been used by Tripathi et al. (2001) and Chen et al. (2003) to select resistant strains with enhanced astaxanthin production. Using nicotine, Tjahjono et al. (1994) were able to obtain resistant, but not overproducer strains. Apparently, the difference in nicotine concentrations (100 μM vs 200 μM) could be responsible for the negative results (Chen et al. 2003). Compactin, an inhibitor of isoprenoid biosynthesis and consequently carotenoids, was used to isolate strains that doubled the astaxanthin content of original strains (Chumpolkulwong et al. 1997). The same results were replicated later, obtaining a strain that doubles the yield of the base strain (Tripathi et al. 2001). Carotenoid biosynthesis target sites for these compounds are presented in Fig. 1. Other researchers completely discarded the use of inhibitors by using a high stress condition of blue and white light to select strains that accumulate twice as much carotenoids than the base strain. These selected strains have a brown color on the green background of non-over carotenoid producers (Sun et al. 1998). Following a similar visual selection, mutagenized cells cultivated in liquid media, followed by light stress with sodium acetate and ferrous sulfate additions, resulted in strains that nearly doubled the astaxanthin production, as demonstrated by light microscopy and reverse phase high-performance liquid chromatography (Hu et al. 2008) (Fig. 2). Differential growth of partially deficient photosynthetic mutants under heterotrophic and autotrophic conditions has also been exploited to select highly photosensitive mutants with increased astaxanthin accumulation (Hong et al. 2012) (Fig. 3). The selection of cells that present altered growth rates after mutagenesis could be achieved by direct cell counting and optical density determination on microplates, as an alternative screening method to isolate overproducer cells (Liu et al. 2016). Even when the later authors did use DPA and nicotine to determine effects on photosynthesis, the inhibitors were not used for the mutant screening. In Hong et al. 2018, Hong et al. reported a new technique for colorimetric selection of *H. pluvialis* mutants using azide, a chemical that acted as an excellent astaxanthin inducer, followed by an oil-based astaxanthin quantification, offering a high-throughput screening strategy without complex pretreatment processes.

Genetic engineering

Some microalgae offer a great potential for genetic engineering in terms of production of recombinant proteins as well as high value compounds like carotenoids or PUFAs. Despite the breakthroughs done with the microalga *Chlamydomonas reinhardtii* as the model organism for doing genetic engineering, few efforts have been done in general with other microalgae. During the last decade, *H. pluvialis* has been a target for genetic engineering either by nuclear or chloroplast transformation (Steinbrenner and Sandmann 2006; Gutiérrez et al. 2012). Recently, Qiulan et al. (2018) published the first draft genome of *H. pluvialis*, a valuable genetic resource for understanding the genetic basis of astaxanthin production.

Nuclear transformation

Transformation experiments were mostly adopted from a biolistic approach. Teng et al. (2002) reported that transient expression of the *lacZ* gene could be observed in motile cells but not in non-motile cells. Later, the activity of the endogenous β-carotene ketolase (BKT) promoter was studied by transiently expressing the *lacZ* gene on *H. pluvialis* (Meng et al. 2005, Fig. 4). An alternative transformation method has been used through *Agrobacterium* (Kathiresan and Sarada 2009; Kathiresan et al. 2009) but was not repeated successfully by others researchers (Fig. 5).

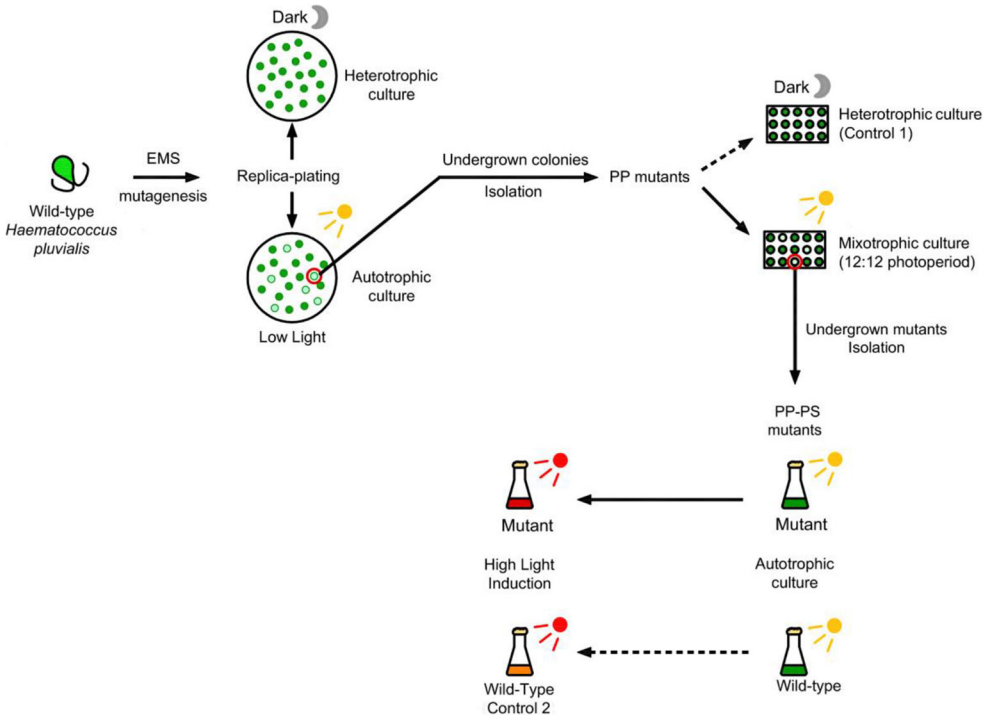

Fig. 3 General scheme for the isolation of highly photo-inducible, partially photosynthesis-deficient and photosensitive *H. pluvialis* mutants, which presented a lower photosystem II efficiency but higher astaxanthin productivity than WT cells. EMS-treated cells were grown sequentially in heterotrophic, autotrophic, mixotrophic, autotrophic, and inductive autotrophic conditions. During culture partially photosynthesis-deficient (PP), partially photosynthesis-deficient and photosensitive (PP–PS), and highly photo-inducible PP–PS mutants were isolated. Reprinted from Process Biochemistry, 47(12), Min-Eui Hong, Seung Phill Choi, Youn-Il Park, Young-Kee Kim, Won Seok Chang, Byung Woo Kim, Sang Jun Sim, Astaxanthin production by a highly photosensitive *Haematococcus* mutant, pp. 1972–1979, © 2012, with permission from Elsevier

Nowadays, an advanced shuttle-vector has been developed for efficient nuclear transformation based on an endogenous dominant marker that allows the production of safe transgenic microalgae strains without foreign DNA sequences (Sharon-Gojman et al. 2015). All previous research proved the possibility to genetically enhance *H. pluvialis*; however, they were not focused on astaxanthin enhancement. The first effort to improve astaxanthin accumulation by nuclear transformation was carried out with a mutated phytoene desaturase gene (*pds*) that confers resistance to norflurazon (Steinbrenner and Sandmann 2006), showing 26% higher astaxanthin levels after 48 h of high light exposure as compared with the wild type. Additionally, cloning an extra copy of the

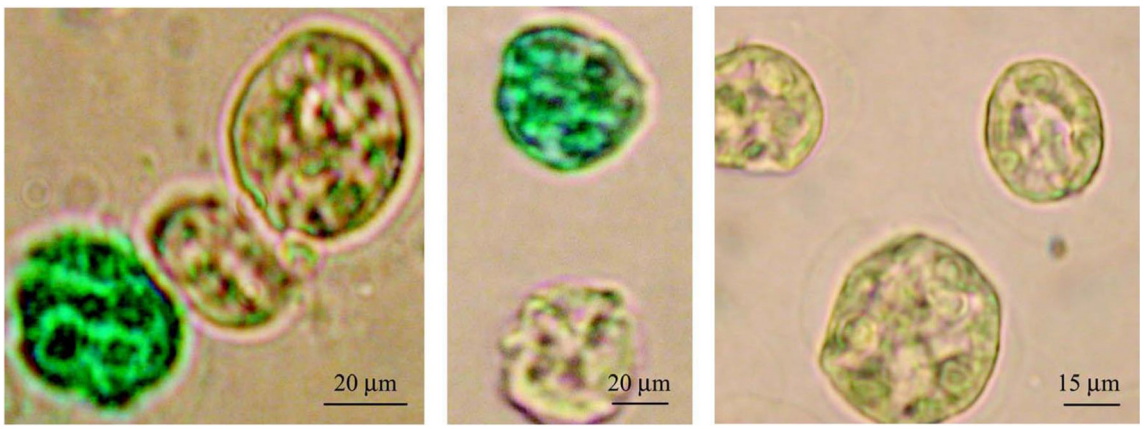

Fig. 4 Early proof of transient transformation on *H. pluvialis*, green-blue cells are stained by β-galactosidase activity derived by *lacZ* exogenous gene. **a** *lacZ* expression under control of *H. pluvialis bkt* promoter region. **b** *lacZ* expression under control of SV40 promoter-enhancer. **c** Negative control showing no β-galactosidase activity. Figure from Meng, Chun-Xiao; Teng, Chang-Ying, Cloning and Characterization of β-Carotene Ketolase Gene Promoter in *Haematococcus pluvialis*, Acta Biochimica et Biophysica Sinica, 2005, 37(4), 270–275, by permission of Oxford University Press

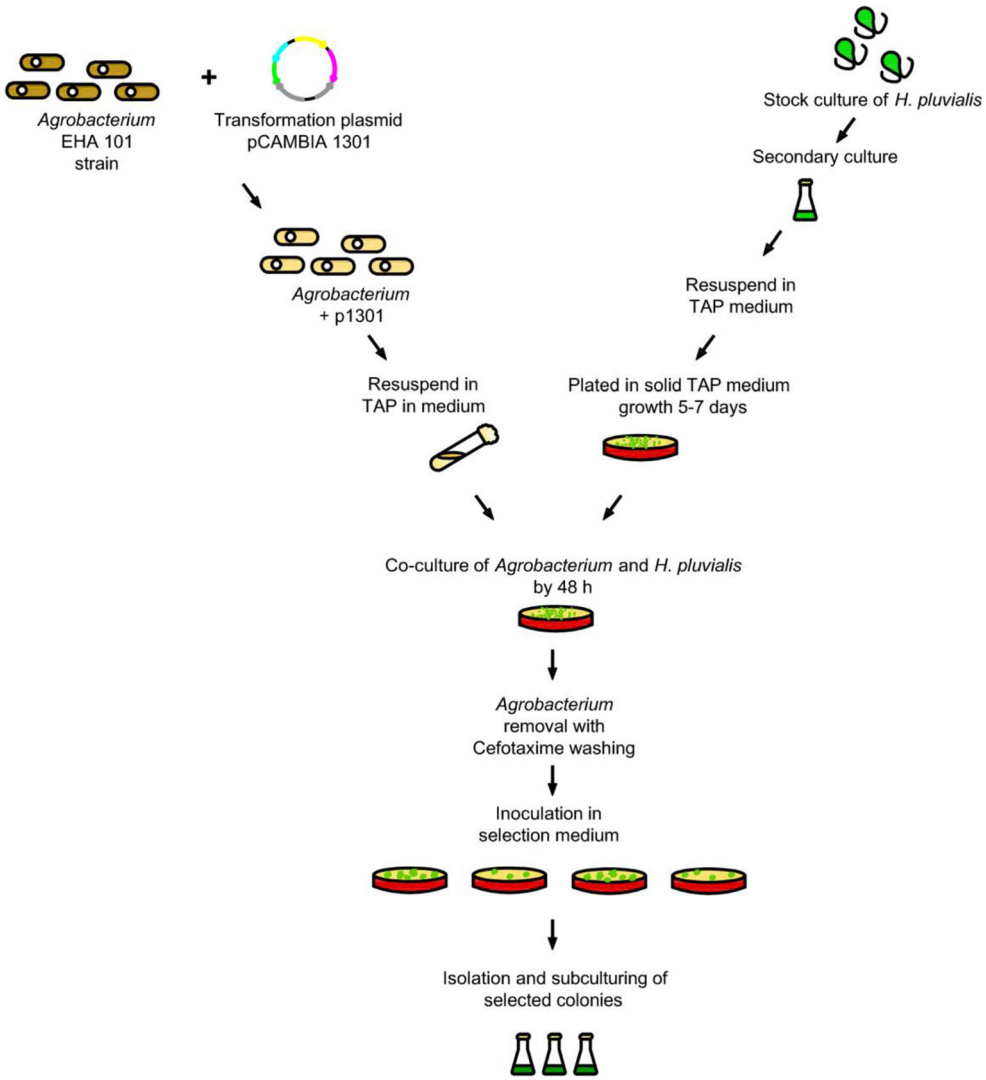

Fig. 5 Illustration showing the *H. pluvialis* transformation mediated by *Agrobacterium tumefaciens*, a common natural vector widely used for plant transformation. The co-cultivation step brings both cell types in close proximity, where previously transformed *Agrobacterium* transfers the genetic construct to the microalgae. Further selection of transformed strains is performed on antibiotic containing media, with resistance provided by the genetic construct previously transferred. TAP, Tris acetate phosphate media. Reprinted by permission from Springer Nature, Journal of Applied Phycology 21, Towards genetic improvement of commercially important microalga *Haematococcus pluvialis* for biotech applications, Shanmugam Kathiresan et al., © 2009

bkt gene directly into the nucleus allowed a four-fold increase in astaxanthin content under stress conditions (Kathiresan et al. 2015). Recently, the modification of *H. pluvialis* trophic capabilities has been achieved by nuclear transformation with the hexose uptake protein (HUP1) gene of the green microalga *Parachlorella kessleri*. The transformed *H. pluvialis* strains proliferate under very low illumination on nutrient media supplemented with glucose, and produce astaxanthin in total darkness under special conditions, or with subsequent exposure to increasing illumination levels, providing a new way to supply nutrients for astaxanthin development on low-light conditions (Waissman-Levy et al. 2019).

Although most genes and enzymes for algae-specific carotenogenic pathways are still unknown, a number of them from *H. pluvialis* have been cloned and sequenced (Takaichi 2011). A summary of different transformation efforts and their respective efficiencies is presented in Table 3.

Chloroplast transformation

Since most of the carotenogenic pathway occurs in the chloroplast of green algae, the optimization of carotenoid production can be undertaken by metabolic engineering, either by the expression of genes directly inserted in the chloroplast or by nuclear expression followed by gene product translocation to the chloroplast. Expression of genes from the chloroplast genome guarantees the expression in the right place and is undoubtedly the best strategy in terms of protein accumulation since it allows control of exogenous DNA insertion. On the other hand, nuclear expression is associated with silencing and positional effects that could lead to lower protein expression (Pérez-España et al. 2016; Henríquez et al. 2016; Siddiqui et al. 2019). By using next generation sequencing, Bauman et al. (2018) revealed an extremely large 1.35-megabase chloroplast genome for *H. pluvialis*, reported as the largest assembled chloroplast genome of any plant or alga known to date.

Table 3 Summary of different genetic transformation approaches reported to date for *H. pluvialis*. Transformation efficiency is provided when available

Reference	Stability and type	Transform. method	Promoter	Reporter/sel. genes	Selective conditions	Transformation efficiency	Observations
Teng et al. (2002)	Transient	Biolistic	SV40	*lacZ*	Visual–Blue colored cells under microscope	4-5 × 10^{-5} cell μg^{-1} DNA[a]	First report of *H. pluvialis* transformation. No transformation observed on bombardment pressures higher than 900 PSI.
Meng et al. (2005)	Transient	Biolistic	Endogenous BKT	*lacZ*	Visual–Blue color under microscope	Not informed	Study aimed at BKT promoter characterization with sequential deletion.
Steinbrenner and Sandmann (2006)	Stable - nuclear	Biolistic	Endogenous PDS	Modified endogenous PDS L504R	Growth on bleaching agent norflurazon at 5 mM	1 × 10^{-6} cell μg^{-1} DNA (100 CFU on 10^8 cells) 2 × 10^{-6} cell μg^{-1} DNA on additional work[b]	Test the use of modified *pds* as a dominant endogenous selection marker. Visible red phenotype only after 8 h of high light exposure, and 26% higher astaxanthin than base strain for P3 transformed strain.
Kathiresan and Sarada (2009)	Stable - nuclear	*Agrobacterium* -mediated	CaMV35S	*hpt*	Growth on hygromycin at 2–10 mg L^{-1}	100 CFU on 10^6 co-cultured cells	Initial studies to establish *Agrobacterium* mediated transformation of *H. pluvialis*, including compatible culture media, antibiotic susceptibility of both cell types and detailed co-culture procedure.
Kathiresan et al. (2009)	Stable - nuclear	*Agrobacterium* -mediated	CaMV 35S	*uidA* GFP *hpt*	Growth on hygromycin at 2–10 mg L^{-1}	100–150 CFU on 10^6 co-cultured cells.	Robust method for *H. pluvialis* transformation. Resistance selection due to *hpt* gene and direct visualization GFP protein/GUS activity demonstrated.
Gutierrez et al. (2012)	Stable - chloroplast	Biolistic	*rbcL*	*aadA*	Growth on spectinomycin at 200 μg mL^{-1}	2,1 × 10^{-5} cell μg^{-1} DNA (427 CFU on 2 × 10^7 cells)	Only report of chloroplast transformation. Chloroplast *rbcL* promoter and 3′ UTR sequence used for strong transcription. Aimed at expression of heterologous genes for protein production.
Sharon-Gojman et al. (2015)	Stable - nuclear	Biolistic	Endogenous PDS	Modified endogenous PDS L504R	Growth on norflurazon at 2–5 μM	2 × 10^{-5} cell μg^{-1} DNA (111 CFU on 6 × 10^6 cells)	Use modified *pds* as endogenous selection marker, developing vectors capable of insert two additional genes at high efficiency.
Kathiresan et al. (2015)	Stable - nuclear	*Agrobacterium* -mediated	CaMV 35S	*bkt* *hpt*	Growth on hygromycin at 10 mg L^{-1}	Not informed	Overexpression of *bkt* gene increased not only BKT but also PSY, PDS, LCY and BKH, and levels of astaxanthin and other carotenoids were enhanced on transformed cells, both untreated and stressed to induce carotene formation.
Galarza et al. (2018)	Stable chloroplast	Biolistic	Endogenous PDS nuclear gene	*aadA*	Growth on spectinomycin at 200 μg mL^{-1}	8.5 cell μg^{-1} DNA	First report of plastid transformation of a microalgae with its endogenous pds nuclear gene. 67% higher astaxanthin accumulation in transformed strains when compared with wild-type.
Waissmann-Levy et al. (2019)	Stable - nuclear	Biolistic	Endogenous PDS	Mutated m*PDS*	Growth on 2 μM norflurazon	Not informed	Incorporation of *HUP1* gene from *Parachlorella kessleri* allows heterotrophic growth on media supplemented with glucose in very low light (5 μmol photons m^{-2} s^{-1}) or total darkness.

[a] Number of transformed cells determined under microscope, not necessarily represents real transformation efficiency since no CFU were determined on this work

[b] Transformation efficiency experimentally determined with the same pPlat-pds vector as reported by the later research of Sharon-Gojman et al. (2015) under new experimental conditions

Gene and promoter abbreviations: *rbcL* chloroplast gene ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), *lacZ* β-galactosidase gene, *bkt* β-carotene ketolase, *pds* phytoene desaturase, *PDS L504R* phytoene desaturase modified at aminoacidic position 504 (leucine changed to arginine, confers resistance to norflurazon), *CaMV35S* Cauliflower mosaic virus promoter, *Hpt* hygromycin phosphotransferase (resistance to hygromycin), *uidA* β-glucuronidase (GUS staining), *GFP* green fluorescent protein, *aadA* adenylyl transferase (resistance to spectinomycin), *psy* phytoene synthase, *lcy* lycopene cyclase, *bkh* β-carotene hydroxylase, *HUP1* hexose uptake protein

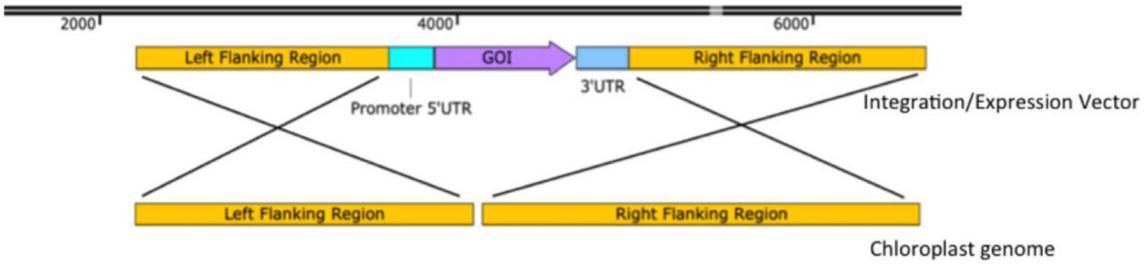

Fig. 6 Schematic presentation of an integration/expression vector. Transgene integration occurs by homologous recombination between flanking regions of chloroplast genome and vector. GOI, gene of interest. Crossed lines represent homologous recombination

Following the successful chloroplast transformation of *H. pluvialis* driven by the endogenous *rbcL* promoter (Gutiérrez et al. 2012, Fig. 6), the metabolic engineering study presented by Galarza et al. (2018) overexpressed the endogenous *pds* gene in order to achieve a higher astaxanthin accumulation. After transformation, astaxanthin accumulation per dry weight was enhanced up to 67%.

Polyploidy

Artificial induction of polyploidy is an interesting alternative for microalgae improvement, which may hold an enormous potential for increasing metabolite productivity. However, only a few studies have been carried out in this field. Ploidy levels refer to the number of chromosome sets present in a cell or individual. Considering "n" as the basic number, two sets are present on a diploid state (2n = 2x). The polyploidy state is the heritable condition, where more than two chromosome sets are present on a cell (Comai 2005). This is a very common condition in higher plants, with 30 to 80% of modern angiosperm plants originating from a polyploid ancestor or showing some degree of polyploidy (Meyers and Levin 2006). In polyploids, the increased number of chromosomes cause changes on physiology, phenotype, and fertility (Levin 1983), with enlarged cell size and bigger nuclei, chloroplast, and mitochondria (Stebbins 1970; Levin 1983). Additionally, the increase on cell size is reflected on the presence of larger tissues and organs, a phenomenon known as the Gigas effect (Morgan et al. 2003).

Artificial polyploidy induction of microalgae

Most of the artificially polyploidized microalgae reports are directed to genetic and cytological studies. In *Chlamydomonas eugametos* and *C. reinhardtii,* colchicine was used to study inheritance mechanisms, producing diploids and higher ploidy cells from haploid strains (Wetherell and Krauss 1956). In *Chlorella* sp., colchicine-derived polyploids showed an increased number of nuclei and endoplasmic reticulum (Wanka 1968). Polyploidy was also present in the cyanobacterium *Anacystis nidulans* (Griese et al. 2011). In this species, individual polyploids (4n) showed a higher growth rate, and consequently a potential increase in productivity, when compared with the original strains (Vonshak and Richmond 1981). More recently, the anti-mitotic agent demecolcine, closely related to colchicine, was used to induce diploidy in *C. reinhardtii*. This resulted in bigger cells and enhanced abiotic stress resistance while being more productive than their haploid counterpart under these conditions, demonstrating an enhancement of the polyploid strains (Kwak et al. 2017). Concerning carotenogenesis in *H. pluvialis*, increasing the copy number of genes is expected to also increase the astaxanthin content (Lee and Zhang 1999). Latest research indicate that *Haematococcus* presents a cell cycle that includes multiple DNA replication stages, being effectively polynuclear and polyploid in its latter aplanospore stage, but recovering its individual diploid stage at the end of multiple cytokinesis that produces flagellate zoospores (Reinecke et al. 2018).

Nonetheless, the only available reference mentioning polyploidization for increasing productivity in this species reported an increased growth rate, but a lower astaxanthin production per cell. The study was only focused on protein expression profiles, not giving further details about the methodology used to induce polyploidy (Kim 2006). However, it represents an early attempt at using polyploidy for *Haematococcus* improvement. In a more recent attempt to enhance astaxanthin content, our group isolated one polyploidized strain of *H. pluvialis* that had bigger aplanospores and nearly 50% more astaxanthin accumulated in DW than its original strain under laboratory conditions and semi-industrial set-ups (Fig. 7, unpublished data). This provided further evidence of the utility of this approach.

Artificial polyploidy induction with protoplast fusion

Alternative methods to generate polyploidy without using anti-mitotic agents are available. The cell wall of flagellated *Haematococcus* can be digested by proteinase K treatment for protoplast preparation and it is possible to regenerate the cell wall

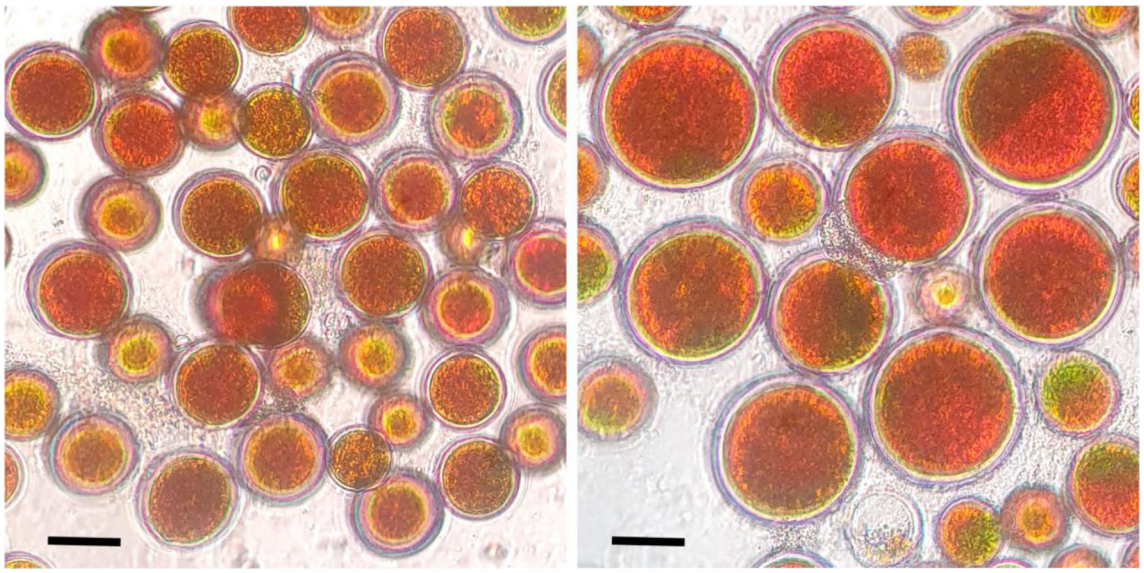

Fig. 7 Light micrographs of *H. pluvialis* base strain (left) and polyploid strain (right) derived from the same base strain subjected to colchicine treatment, showing an increase in the cell size and ploidy levels representative of the gigas effect from polyploids (right). Bar represents 25 μm. Both images are taken from late aplanospore stage and show astaxanthin accumulation. Images from unpublished results from the authors

from these cells (Tjahjono et al. 1994). Under appropriate conditions, these protoplasts can be fused, resulting in strains that present cells with twice as much volume and with three times higher antioxidant yields than the parental lines, clearly showing the Gigas effect, along with an enhancement of astaxanthin production (Tjahjono et al. 1994). Furthermore, *H. pluvialis* protoplasts can be fused with the chrysophyte *Ochromonas danica*, resulting in individuals with a mixture of metabolic capacities, as evidenced by their fatty acid profiles. They combine lipids only present in *Haematococcus* (C16:0 and C18:3n-6) or *O. danica* (C16:2 and C24:0). This combination also takes place in the interspecies fusants that show both fatty acid profiles, and combined growth abilities on a specific media, revealing genetic recombination between the two microalgal phyla. These heterofusants showed the presence of two unfused nuclei, lacking the capacity to produce astaxanthin and presenting an

Table 4 A summary of advantages and disadvantages when using different biotechnological approaches for *Haematococcus* improvement

Methods for improvement	Advantages	Disadvantages
Random mutagenesis	Technically easy to perform	Time consuming
	Does not require previous genome information	Potential for multiple unwanted mutations on strains
	Enhanced strains can be used for human consumption, treated as varieties—not regulated	Unstable or reversion of enhanced phenotype
Genetic engineering	Precise gene insertion/deletion could be achieved without disturbing additional genes	Previous genome information required
	Allow integration of key genes for enhancing metabolic pathways.	Genetically engineered organisms or products not allowed for human consumption on several countries
	Can be directed to chloroplast or nucleus	Nuclear genetic engineering is unstable
Artificial polyploidization	Enhanced strains can be used for human consumption, treated as varieties—not regulated	Requires of careful testing of adequate concentration and times for polyploidization agent exposure
	Can be used for further enhancement of strains generated by other techniques	Requires extensive screening to isolate overproducing strains
	Great potential for high gains due to the Gigas effect	Protoplasts can be difficult to manipulate
	Possibility of interspecies fusion for new traits and secondary metabolites	

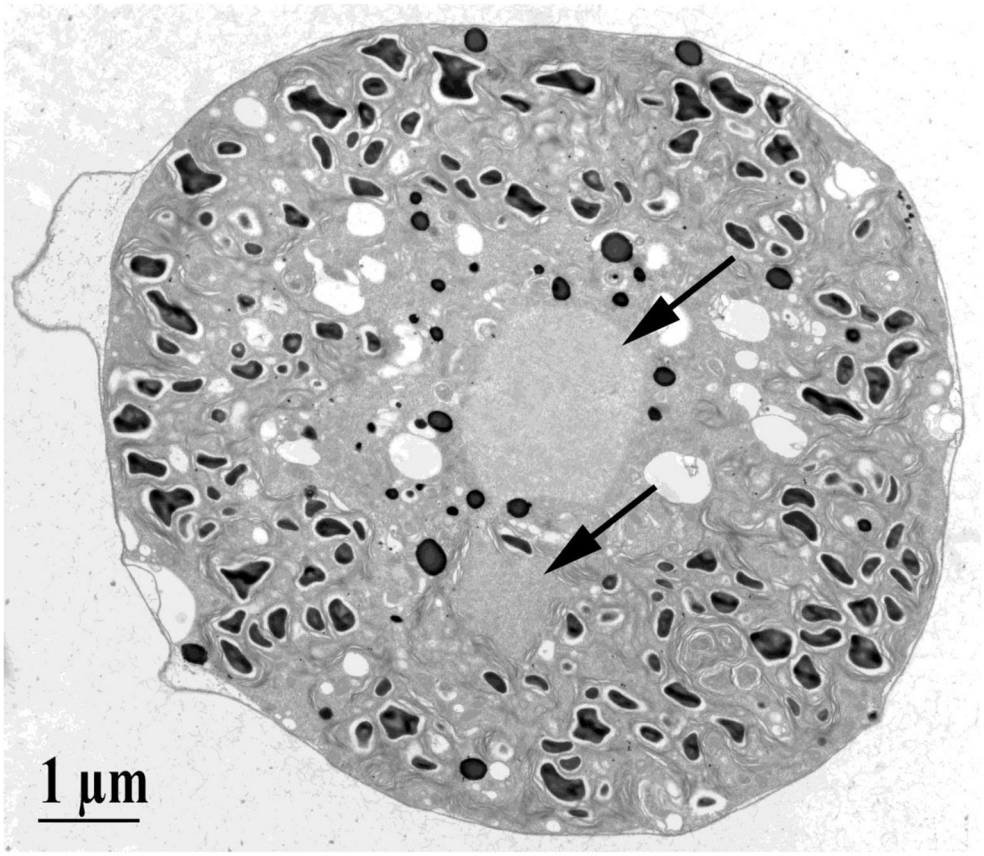

Fig. 8 Transmission electron micrography of a fusant obtained between *Haematococcus pluvialis* and *Ochromonas danica*, showing the two unfused nuclei present on each cell indicated by arrows. Figure reproduced from Protoplast fusion and genetic recombination between *Ochromonas danica* (Chrysophyta) and *Haematococcus pluvialis* (Chlorophyta), Abd El-Fatah Abomohra, Mostafa El-Sheekh, et al., Phycologia 55(1), 2016, reprinted by permission of the publisher (Taylor & Francis Ltd., http://www.tandfonline.com)

always-green color (Abomohra et al. 2016, Fig. 8). In this case, a heterokaryotic cell was obtained instead of a true allopolyploid, since there are two different nuclei on each cell. A graphic comparison of these methods of modification is shown in Fig. 9. The advantages and drawbacks of biotechnological approaches for astaxanthin yield improvement in *H. pluvialis* are presented on Table 4.

Alternatives to Haematococcus pluvialis *for astaxanthin production*

Even though *H. pluvialis* is the most efficient producer of natural astaxanthin, its culture presents several problems, such as slow growth rate and low biomass when compared with other microalgae (Lorenz and Cysewski 2000; Olaizola 2000). *Chromochloris zofingiensis* can be used as an alternative producer since it has faster growth rate. Moreover, it can grow under heterotrophic conditions using glucose as energy source (Sun et al. 2008; Liu et al. 2014). Nevertheless, *C. zofingiensis* shows very limited astaxanthin productivity when compared with *H. pluvialis*. For example, the less productive strain of *H. pluvialis*, CCAP34/8, produces 1.1% astaxanthin DW (77 mg L^{-1}), while the best reported *C. zofingiensis* strain (CCAP211/14), produces 0.37% astaxanthin DW and 25 mg L^{-1}, both under photoautotrophic batch culture conditions (Del Campo et al. 2004; García-Malea López et al. 2006). Additionally, astaxanthin from *C. zofingiensis* consists in a mixture with 50% of other keto-carotenoids, not allowed for human consumption in USA, Europe and Australia (Liu et al. 2014). Furthermore, it is not recognized as GRAS (Yang et al. 2016), hindering its use for human consumption. A second alternative consists in the heterologous expression of the carotenogenic pathway in other hosts, since there is wide knowledge of the genes involved in astaxanthin biosynthesis. However, a highly engineered strain of *Escherichia coli*, BW-ASTA, only yielded 0.14% DW astaxanthin and required IPTG, an expensive inducer, hindering the commercial mass-scale production in this heterologous system (Lemuth et al. 2011).

Conclusion and perspectives

Even when *H. pluvialis* is the best natural source of astaxanthin known to date, its productivity still requires improvement. This challenge has been addressed from different biotechnological approaches. Classical mutagenesis has well-established protocols and is easy to perform but presents some drawbacks, since it is time-consuming, and its results are inherently unpredictable. Due to its random nature, there is no control over random changes at a genetic level, probably

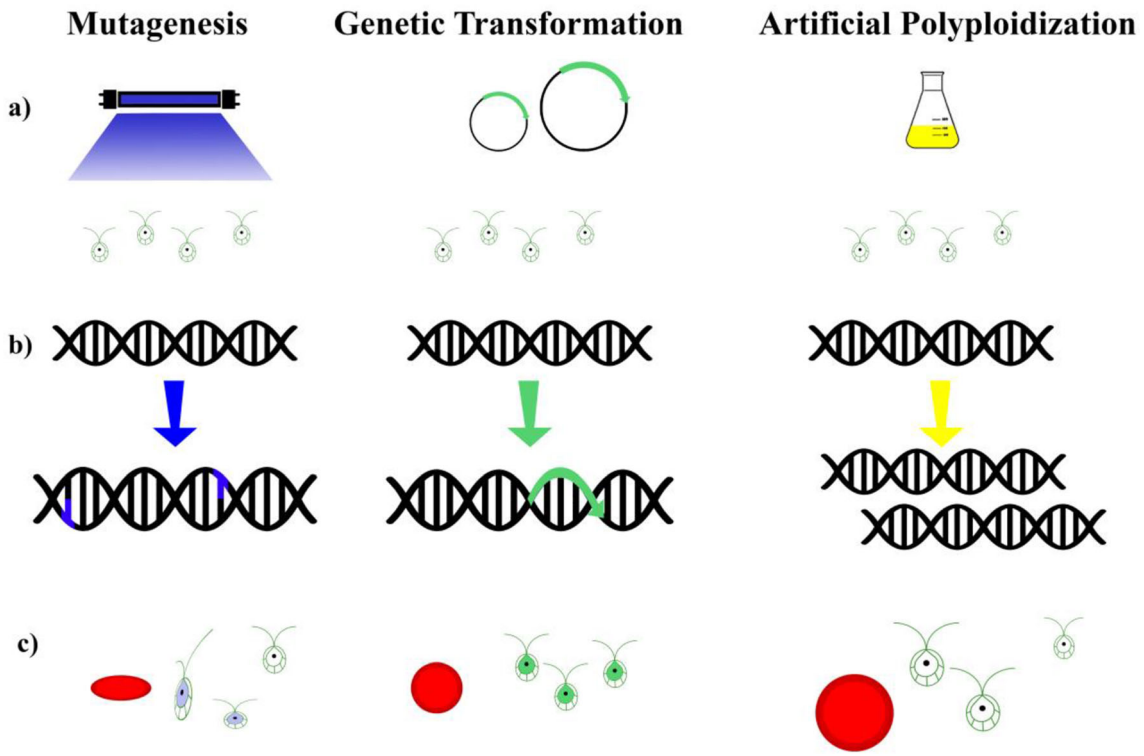

Fig. 9 Comparison of the three methods for genetic improvement on *Haematococcus pluvialis*. **a** Modification inducer, including UV radiation or chemicals usually employed for random mutagenesis, DNA vectors with gene construct for genetic transformation, and chemical polyploidization agent, respectively. **b** DNA change resulting on each case, random mutation, DNA integration, or whole DNA duplication, respectively. **c** Final effects of modification, mutant cells with distinctive morphology or growth and pigment accumulation, modified organisms containing exogenous or rearranged DNA, and bigger cells with higher pigment production, respectively

presenting multiple mutations that may have impact on growth and reproduction stages, including non-flagellated or unstable cells (Gómez et al. 2013), revertant mutants from DES or UV-treated cells (Wang et al. 2016), and UV and EMS mutants loss under continuous selection (Chumpolkulwong et al. 1997). The aforementioned data indicates that even though random mutagenesis is simple to perform, the resulting strains must be carefully tested to ensure they maintain the improved properties. Additionally, it is important to use a strain with the highest astaxanthin accumulation capability as a base for mutagenesis, since several studies that show increments that doubled the initial strain productivity are still behind the results of non-improved highly productive strains. As it is mentioned in the reported work of Tripathi et al. (2001), a 225% increase on astaxanthin yield was reached, but the original *H. pluvialis* strain only produced 0.43% on DW, well behind the 6% yields reported by some industries for commercial use. Additionally, the reported increase was achieved in mixotrophic conditions, further increasing the costs under a future industrial set-up. In absolute terms, several strains obtained by mutagenesis, presented in Table 2, displayed yields very close or higher than 4% DW, but since there is no information available in literature, these strains are not currently used for industrial production. However, it is still possible that some mutant strain is used under industrial secret, which we are not aware of.

Genetic engineering of carotenoid metabolic pathways has been employed in several species to increase yields, but presents specific challenges, such as detailed knowledge of carotenoid biosynthesis pathways, availability of nuclear and chloroplast genomes, and enzyme cellular localization knowledge. Even when *H. pluvialis* has been transformed by different techniques, there are only a few examples of astaxanthin enhancement in this microalga. In the first case, the use of a modified version of the *pds* gene for nuclear transformation, conferring resistance to a bleaching agent, increased the astaxanthin content of transformed strains (Steinbrenner and Sandmann 2006). Additionally, the overexpression of the *bkt* gene by nuclear transformation enhanced pigment production, as demonstrated by Kathiresan et al. (2015), and astaxanthin yield, by the insertion of the endogenous nuclear *pds* gene into the chloroplast (Galarza et al. 2018). On the other hand, Waissmann-Levy et al. (2019) stably integrated the gene *HUP1* from *P. kessleri* into *H. pluvialis*, allowing hexose uptake and the capacity to survive with minimal or no light at all when growing in glucose-supplemented media. As the preceding examples demonstrate, both nuclear and chloroplast genomes of *H. pluvialis* have been genetically manipulated by either biolistic

approaches or *Agrobacterium* mediated transformation, with variable transformation efficiencies (Table 3). Recently, the release of both chloroplast and nuclear genomes for *H. pluvialis* opened new opportunities for research and genetic engineering of this microalga. Only the chloroplast genome of this microalga is 1.35 Mb in size, being the largest assembled chloroplast to date (Bauman et al. 2018). Also, their nuclear genome is huge, reported to be 669 Mb and containing 18.545 genes in the first draft of Luo et al. (2019), one of the biggest sizes in currently sequenced green algae. The presence of six copies of the *bkt* gene in *H. pluvialis* nuclear genome, while only one copy is present in close relative species such as *Volvox carteri* or *C. reinhardtii*, might contribute to the high productivity of astaxanthin in this species (Luo et al. 2019). The three types of *bkt* genes in *H. pluvialis*, *bkt*1, *bkt*2, and *bkt*3, are isoform copies. According to the latest genome assembly of this species by Luo et al. (2019), *bkt* experienced multiple gene duplications during genome evolution, with one group containing *bkt*1, while the other group contains *bkt*2 and *bkt*3. The bkt gene family expansion may contribute to the high astaxanthin yield of *H. pluvialis* and, along with transcriptomic data, reinforce the usefulness of these resources for *Haematococcus* enhancement. In addition, other researchers have developed specific expression vectors for either nuclear or chloroplast transformation (Gutiérrez et al. 2012; Sharon-Gojman et al. 2015) that may be useful to enhance astaxanthin accumulation. Finally, the Crispr/Cas9 system could also be adapted for gene editing in *H. pluvialis* to achieve further improvement of carotenoids (Nymark et al. 2016). Despite transformation is an excellent tool for *H. pluvialis* improvement, several restrictions still exist for the commercialization of natural products derived from genetically engineered organisms (GMO) in markets aimed to human consumption, since some consumers may have concerns about the safety of GMO-derived products. For this reason, additional regulations may be required for the introduction of GMO microalgae into global markets (Beacham et al. 2017). These restrictions need to be addressed before attempting a commercial use for genetically engineered strains.

Artificial polyploidization techniques have been used to enhance agricultural traits for several decades in superior plants, resulting in important gains due to the Gigas effect, leading to bigger cells and higher amounts of secondary metabolites. Despite these benefits, artificial polyploidization of microalgae has only a few appearances in literature. *H. pluvialis* is regarded as diploid in its zoospore stage, but it presents an unusual cell cycle with octaploid and even higher ploidy levels before reaching cytokinesis (Reinecke et al. 2018), offering an excellent starting material for a stable fixing of these high ploidy levels by means of artificial polyploidy induction. But the performance of *Haematococcus* cells with stable high ploidy levels are unknown and can be low, since it has been described that cells possessing higher DNA loads have a slower growth (possibly due to the requirement of longer times on the DNA synthesis and mitosis stages of the cell cycle; Doyle and Coate 2019). In the case of *H. pluvialis* treated with colchicine, the evidence points out the utility of this approach, with cells presenting higher growth rates, but lower astaxanthin accumulation and differential protein expression profile that could explain this discrepancy (Kim 2006). In the same context, our group has successfully obtained polyploidized strains that showed bigger cells and more astaxanthin at laboratory and industrial conditions (deposited on BEA repository and protected by Chilean and international patents, unpublished results). Protoplast fusion techniques also appeared to be useful for *H. pluvialis* astaxanthin enhancement, resulting in a strain with three-fold higher carotenoid accumulation under Fe^{2+} stress conditions, and 30% higher production of this antioxidant than the original wild type cells, notably surpassing the yields from the mutant strains used to make the fusants (Tjahjono et al. 1994). Additionally, the inter-species fusants could hold great potential, not only to obtain better astaxanthin yields or enhanced growth characteristics, but also to "assemble" novel metabolic pathways in *Haematococcus* fusants, mixing the biochemical capabilities of different microalgae to obtain novel strains. This was demonstrated by Abomohra et al. (2016), who attempted to produce new strains that combine fatty acids, with potential pharmaceutical use, with astaxanthin production. Even when this research failed to obtain this objective, it demonstrated the combination of different fatty acid profiles into one new organism. Therefore, it may be possible to use this strategy to mix different metabolic pathways of important commercial value for biorefineries, especially where the production of several high value chemicals is desirable to reduce costs and maximize the benefits of the cultures.

Most of the literature cited shows researchers who evaluate the performance of the modified strains under laboratory conditions, with culture volumes typically under 500 mL, very uniform illumination and temperature, and active aeration by shaking or air bubbling. These uniform growth conditions are seldom encountered in industrial setups, with wide variation in light intensities, reaching cells that are near the surface in open-ponds, or near artificial light sources in photobioreactors. The same is true for temperature variations that can affect the growth of *Haematococcus* cells. Additionally, the culture media used in laboratory-scale experiments tends to be very different from the ones used industrially. Usually, the purity of reagents varies for industrial conditions and will be of lower quality for cost reasons, with several impurities that could hamper the cell growth and hinder the yields obtained at a laboratory scale.

As a final remark, the continued enhancement of *H. pluvialis* astaxanthin aims for competitive industrial production. It may be achieved by more than one of the biotechnological alternatives described in this review, starting with the best naturally occurring strains, to

generate overproducing cells that can grow better in current industrial production systems, not requiring expensive chemical supplies or modification of the existing facilities. Furthermore, the ability to produce additional high-value compounds in addition to astaxanthin, by the integration of novel metabolic pathways by genetic engineering, will allow the integration into a biorefinery set-up, enhancing even more the economic value of novel *H. pluvialis* strains.

Acknowledgments The authors wish to acknowledge the comments of Jorge Pérez Pérez, production manager of Astax Chile SpA. (former Pigmentos Naturales S.A.). Their contributions helped to improve this manuscript.

Funding Authors would like to acknowledge the support of Agencia Nacional de Investigación y Desarrollo de Chile (ANID). This work was funded by the Chilean Fondo de Fomento al Desarrollo Científico y Tecnológico (FONDEF) under grant number ID15I-10014. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily attributed to the funding agencies.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Disclosure Patent related to the results described in this study have been submitted to National Institute of Intellectual Property (INAPI-CHILE) No 3422-2017 and PCT No PCT/CL2018/050002. The *H. pluvialis* polyploid strain was deposited No 3373451 at the Banco Español de Algas (BEA), Gran Canaria, Spain.

References

Abomohra AEF, El-Sheekh M, Dieter H (2016) Protoplast fusion and genetic recombination between *Ochromonas danica* (Chrysophyta) and *Haematococcus pluvialis* (Chlorophyta). Phycologia 55:65–71

Acién FG, Molina E, Reis A, Torzillo G, Zittelli GC, Sepúlveda C, Masojídek J (2017) Photobioreactors for the production of microalgae. In: Gonzalez-Fernandez C, Muñoz R (eds) Microalgae-Based Biofuels and Bioproducts. Woodhead Publishing Series in Energy. Woodhead Publishing, NY pp 1–44

Aflalo C, Bing W, Zarka A, Boussiba S (1999) The effect of the herbicide glufosinate (BASTA) on astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Z Naturforsch C 54:49–54

Ambati RR, Phang SM, Sarada R, Aswathanarayana RG (2014) Astaxanthin: sources, extraction, stability, biological activities and its commercial applications–a review. Mar Drugs 12:128–152

Bauer A, Minceva M (2019) Direct extraction of astaxanthin from the microalgae *Haematococcus pluvialis* using liquid–liquid chromatography. RSC Adv 9:22779–22789

Bauman N, Akella S, Hann E, Morey R, Schwartz AS, Brown R, Richardson TH (2018) Next-generation sequencing of *Haematococcus lacustris* reveals an extremely large 1.35-megabase chloroplast genome. Genome Announc 6. https://doi.org/10.1128/genomeA.00181-18

Beacham TA, Sweet JB, Allen MJ (2017) Large scale cultivation of genetically modified microalgae: a new era for environmental risk assessment. Algal Res 25:90–100

Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70:313–321

Borowitzka MA (2013) Energy from microalgae: a short history. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, pp 1–15

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol Plant 108:111–117

Boussiba S, Bing W, Yuan JP, Zarka A, Chen F (1999) Changes in pigments profile in the green alga *Haematococcus pluvialis* exposed to environmental stresses. Biotechnol Lett 21:601–604

Breitenbach J, Zhu C, Sandmann G (2001) Bleaching herbicide norflurazon inhibits phytoene desaturase by competition with the cofactors. J Agric Food Chem 49:5270–5272

Capelli B, Cysewski G (2012) The world's best kept health secret: natural astaxanthin. Cyanotech Corporation, Kailua-Kona (HI)

Capelli B, Bagchi D, Cysewski GR (2013) Synthetic astaxanthin is significantly inferior to algal-based astaxanthin as an antioxidant and may not be suitable as a human nutraceutical supplement. Nutrafoods. 12:145–152

Chamovitz D, Sandmann G, Hirschberg J (1993) Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis. J Biol Chem 268:17348–17353

Chekanov K, Lobakova E, Selyakh I, Semenova L, Sidorov R, Solovchenko A (2014) Accumulation of astaxanthin by a new *Haematococcus pluvialis* strain BM1 from the white sea coastal rocks (Russia). Mar Drugs 12:4504–4520

Chen Y, Li D, Lu W, Xing J, Hui B, Han Y (2003) Screening and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis*. Biotechnol Lett 25:527–529

Chen Z, Chen J, Liu J, Li L, Qin S, Huang Q (2020) Transcriptomic and metabolic analysis of an astaxanthin-hyperproducing *Haematococcus pluvialis* mutant obtained by low-temperature plasma (ltp) mutagenesis under high light irradiation. Algal Res 45: 101746

Cheng J, Li K, Yang Z, Zhou J, Cen K (2016) Enhancing the growth rate and astaxanthin yield of *Haematococcus pluvialis* by nuclear irradiation and high concentration of carbon dioxide stress. Bioresour Technol 204:49–54

Chew BP, Park JS (2004) Carotenoid action on the immune response. J Nutr 134:257S–261S

Chew BP, Park JS, Wong MW, Wong TS (1999) A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice *in vivo*. Anticancer Res 19:1849–1853

Choi HD, Youn YK, Shin WG (2011) Positive effects of astaxanthin on lipid profiles and oxidative stress in overweight subjects. Plant Foods Human Nutr 66:363–369

Christian D, Zhang J, Sawdon AJ, Peng CA (2018) Enhanced astaxanthin accumulation in *Haematococcus pluvialis* using high carbon dioxide concentration and light illumination. Bioresour Technol 256:548–551

Chumpolkulwong N, Kakizono T, Handa T, Nishio N (1997) Isolation and characterization of compactin resistant mutants of an astaxanthin synthesizing green-alga *Haematococcus pluvialis*. Biotechnol Lett 19:299–302

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6:836–846

Cordero F, Couso I, Leon R, Rodríguez H, Vargas M (2011) Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. Appl Microbiol Biotechnol. 91:341–351

Cort A, Ozturk N, Akpinar D, Unal M, Yucel G, Ciftcioglu A, Yargicoglu P, Aslan M (2010) Suppressive effect of astaxanthin on retinal injury

induced by elevated intraocular pressure. Regul Toxicol Pharm 58: 121–130

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 49:557–583

Curtain C (2000) Plant biotechnology- the growth of Australia's algal β-carotene industry. https://tspace.library.utoronto.ca/handle/1807/23481. Accessed 29 June 2020

Del Campo JA, Rodríguez H, Moreno J, Vargas MA, Rivas J, Guerrero MG (2004) Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biot 64:848–854

Dickinson S, Mientus M, Frey D, Amini-Hajibashi A, Ozturk S, Shaikh F, Sengupta D, El-Halwagi MM (2017) A review of biodiesel production from microalgae. Clean Technol Envir 19:637–668

Ding W, Zhao P, Peng J, Zhao J, Xu JW, Li T, Reiter RJ, Ma H, Yu X (2018) Melatonin enhances astaxanthin accumulation in the green microalga *Haematococcus pluvialis* by mechanisms possibly related to abiotic stress tolerance. Algal Res 33:256–265

Ding W, Peng J, Zhao Y, Zhao P, Xu JW, Li T, Yu X (2019) A strategy for boosting astaxanthin accumulation in green microalga *Haematococcus pluvialis* by using combined diethyl aminoethyl hexanoate and high light. J Appl Phycol 31:171–181

Doyle JJ, Coate JE (2019) Polyploidy, the nucleotype, and novelty: the impact of genome doubling on the biology of the cell. Int J Plant Sci 180:1–52

Eisenreich W, Rohdich F, Bacher A (2001) Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci 6:78–84

Enzing C, Nooijen A, Eggink G, Springer J, Wijffels RH (2012) Algae and genetic modification: research, production and risks. Amsterdam: Technopolis Group. https://library.wur.nl/WebQuery/wurpubs/428629. Accessed 5 July 2020

Essen LO, Klar T (2006) Light-driven DNA repair by photolyases. Cell Mol Life Sci 63:1266–1277

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281:237–240

Frost S (2008) Breaking News on Supplements & Nutrition. U.S. Eye Health Ingredients Markets, USA

Galarza JI, Gimpel JA, Rojas V, Arredondo-Vega BO, Henríquez V (2018) Over-accumulation of astaxanthin in *Haematococcus pluvialis* through chloroplast genetic engineering. Algal Res 31: 291–297

García-Malea López MC, Del Río Sánchez E, Casas López JL, Acíen Fernández FG, Fernández Sevilla JM, Rivas J, Guerrero MG, Molina Grima E (2006) Comparative analysis of the outdoor culture of *Haematococcus pluvialis* in tubular and bubble column photobioreactors. J Biotechnol 123:329–342

Gómez PI, Inostroza I, Pizarro M, Pérez J (2013) From genetic improvement to commercial-scale mass culture of a Chilean strain of the green microalga *Haematococcus pluvialis* with enhanced productivity of the red ketocarotenoid astaxanthin. AoB Plants 5: plt026

Gong M, Bassi A (2016) Carotenoids from microalgae: a review of recent developments. Biotechnol Adv 34:1396–1412

Griese M, Lange C, Soppa J (2011) Ploidy in cyanobacteria. FEMS Microbiol Lett 323:124–131

Grung M, D'Souza FML, Borowitzka MA, Liaaen-Jensen S (1992) Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3*S*,3'*S*)-astaxanthin esters. J Appl Phycol 4:165–171

Guedes AC, Amaro HM, Malcata FX (2011a) Microalgae as sources of carotenoids. Mar Drugs 9:625–644

Guedes AC, Amaro HM, Malcata FX (2011b) Microalgae as sources of high added-value compounds–a brief review of recent work. Biotechnol Prog 27:597–613

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends Biotechnol 21: 201–216

Gutiérrez CL, Gimpel J, Escobar C, Marshall SH, Henríquez V (2012) Chloroplast genetic tool for the green microalgae *Haematococcus pluvialis* (Chlorophyceae, Volvocales). J Phycol 48:976–983

Gwak Y, Hwang YS, Wang B, Kim M, Jeong J, Lee CG, Hu Q, Han D, Jin E (2014) Comparative analyses of lipidomes and transcriptomes reveal a concerted action of multiple defensive systems against photooxidative stress in *Haematococcus pluvialis*. J Exp Bot 65:4317–4334

Harith ZT, de Andrade Lima M, Charalampopoulos D, Chatzifragkou A (2020) Optimised production and extraction of astaxanthin from the yeast *Xanthophyllomyces dendrorhous*. Microorganisms 8:430

He B, Hou L, Dong M, Shi J, Huang X, Ding Y, Cong X, Zhang F, Zhang X, Zang X (2018) Transcriptome analysis in *Haematococcus pluvialis*: astaxanthin induction by high light with acetate and Fe^{2+}. Int J Mol Sci 19:175

Henríquez V, Escobar C, Galarza J, Gimpel J (2016) Carotenoids in microalgae. Subcell Biochem 79:219–237

Hoeffler JF, Hemmerlin A, Grosdemange-Billiard C, Bach TJ, Rohmer M (2002) Isoprenoid biosynthesis in higher plants and in *Escherichia coli*: on the branching in the methylerythritol phosphate pathway and the independent biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate. Biochem J 366:573–583

Holtin K, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K (2009) Determination of astaxanthin and astaxanthin esters in the microalgae *Haematococcus pluvialis* by LC(APCI)MS and characterization of predominant carotenoid isomers by NMR spectroscopy. Anal Bioanal Chem 395:1613–1622

Hong ME, Choi SP, Park YI, Kim YK, Chang WS, Kim BW, Sim SJ (2012) Astaxanthin production by a highly photosensitive *Haematococcus* mutant. Process Biochem 47:1972–1979

Hong ME, Choi HI, Kwak HS, Hwang SW, Sung YJ, Chang WS, Sim SJ (2018) Rapid selection of astaxanthin-hyperproducing *Haematococcus* mutant via azide-based colorimetric assay combined with oil-based astaxanthin extraction. Bioresour Technol 267:175–181

Hu Z, Li Y, Sommerfeld M, Chen F, Hu Q (2008) Enhanced protection against oxidative stress in an astaxanthin-overproduction *Haematococcus* mutant (Chlorophyceae). Eur J Phycol 43:365–376

Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, Kamiyama M, Itakura H, Yamamoto S, Kondo K (2000) Inhibition of low-density lipoprotein oxidation by astaxanthin. J Atheroscler Thromb 7:216–222

Kathiresan S, Sarada R (2009) Towards genetic improvement of commercially important microalga *Haematococcus pluvialis* for biotech applications. J Appl Phycol 21:553

Kathiresan S, Chandrashekar A, Ravishankar GA, Sarada R (2009) Agrobacterium-mediated transformation in the green alga *Haematococcus pluvialis* (Chlorophyceae, Volvocales). J Phycol 45:642–649

Kathiresan S, Chandrashekar A, Ravishankar GA, Sarada R (2015) Regulation of astaxanthin and its intermediates through cloning and genetic transformation of β-carotene ketolase in *Haematococcus pluvialis*. J Biotechnol 196–197:33–41

Kim B (2006) Comparison of protein expression profiles between the wild type *Haematococcus pluvialis* and its mutants. Master's Thesis, INHA University, South Korea

Kim JY, Lee C, Jeon MS, Park J, Choi YE (2018) Enhancement of microalga *Haematococcus pluvialis* growth and astaxanthin production by electrical treatment. Bioresour Technol 268:815–819

Kiperstok AC, Sebestyén P, Podola B, Melkonian M (2017) Biofilm cultivation of *Haematococcus pluvialis* enables a highly productive one-phase process for astaxanthin production using high light intensities. Algal Res 21:213–222

Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63

Kwak M, Park WK, Shin SE, Koh HG, Lee B, Jeong BR, Chang YK (2017) Improvement of biomass and lipid yield under stress conditions by using diploid strains of *Chlamydomonas reinhardtii*. Algal Res 26:180–118

Lee RE. (2008) Phycology. Cambridge University Press, Cambridge 2008

Lee Y, Zhang D. (1999) Production of astaxanthin by *Haematococcus* In: Cohen Z (Ed). Chemicals from microalgae. Taylor & Francis, Lodon pp.41–56

Lemuth K, Steuer K, Albermann C (2011) Engineering of a plasmid-free *Escherichia coli* strain for improved in vivo biosynthesis of astaxanthin. Microb Cell Fact 10:29

León R, Fernández E (2007) Nuclear transformation of eukaryotic microalgae: historical overview, achievements and problems. Adv Exp Med Biol 616:1–11

Levin DA (1983) Polyploidy and novelty in flowering plants. Am Nat 122:1–25

Li Y, Sommerfeld M, Chen F, Hu Q (2010) Effect of photon flux densities on regulation of carotenogenesis and cell viability of *Haematococcus pluvialis* (Chlorophyceae). J Appl Phycol 22:253–263

Li J, Zhu D, Niu J, Shen S, Wang G (2011) An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis*. Biotechnol Adv 29:568–574

Li Y, Cui D, Zhuo P, Zhang L, Sun X, Xu N (2019) A new approach to promote astaxanthin accumulation via Na_2WO_4 in *Haematococcus pluvialis*. J Oceanol Limnol 37:176–185

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50:47–65

Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant 101:643–652

Linden H (1999) Carotenoid hydroxylase from *Haematococcus pluvialis*: cDNA sequence, regulation and functional complementation. Biochim Biophys Acta 1446:203–212

Liu J (2010) Genetic engineering of *Chlorella zofingiensis* for enhanced astaxanthin biosynthesis and assessment of the algal oil for biodiesel production. Dissertation, University of Hong Kong http://hdl.handle.net/10722/131813

Liu J, Sun Z, Gerken H, Liu Z, Jiang Y, Chen F (2014) *Chlorella zofingiensis* as an alternative microalgal producer of astaxanthin: biology and industrial potential. Mar Drugs 12:3487–3515

Liu J, Chen J, Chen Z, Qin S, Huang Q (2016) Isolation and characterization of astaxanthin-hyperproducing mutants of *Haematococcus pluvialis* (Chlorophyceae) produced by dielectric barrier discharge plasma. Phycologia 55:650–658

Lorenz RT, Cysewski GR (2000) Commercial potential for *Haematococcus microalgae* as a natural source of astaxanthin. Trends Biotechnol 18:160–167

Lotan T, Hirschberg J (1995) Cloning and expression in *Escherichia coli* of the gene encoding β-C-4-oxygenase, that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. FEBS Lett 364:125–128

Luo Q, Bian C, Tao M, Huang Y, Zheng Y, Lv Y, Li J et al (2019) Genome and transcriptome sequencing of the astaxanthin-producing green microalga, *Haematococcus pluvialis*. Genome Biol Evol 11:166–173

Meng CX, Teng CY, Jiang P, Qin S, Tseng CK (2005) Cloning and characterization of β-carotene ketolase gene promoter in *Haematococcus pluvialis*. Acta Biochin Biophys Sinica 37:270–275

Meyers LA, Levin DA (2006) On the abundance of polyploids in flowering plants. Evolution 60:1198–1206

Mobin S, Alam F (2017) Some promising microalgal species for commercial applications: a review. Energy Procedia. 110:510–517

Moreno-Garcia L, Adjallé K, Barnabé S, Raghavan GSV (2017) Microalgae biomass production for a biorefinery system: recent advances and the way towards sustainability. Renew Sust Energ Rev 76:493–506

Morgan ER, Hofmann BL, Grant JE (2003) Production of tetraploid *Gentiana triflora* var. japonica 'royal blue' plants. N Z J Crop Hort 31:65–68

Muñoz R, Guieysse B (2006) Algal-bacterial processes for the treatment of hazardous contaminants: a review. Water Res 40:2799–2815

Naguib YMA (1998) A fluorometric method for measurement of peroxyl radical scavenging activities of lipophilic antioxidants. Anal Biochem 265:290–298

Nakada T, Ota S (2016) What is the correct name for the type of *Haematococcus* Flot. (Volvocales, Chlorophyceae)? Taxon 65:343–348

Nawrocki WJ, Tourasse NJ, Taly A, Rappaport F, Wollman FA (2015) The plastid terminal oxidase: its elusive function points to multiple contributions to plastid physiology. Annu Rev Plant Biol 66:49–74

Nguyen KD (2013) Astaxanthin: a comparative case of synthetic vs natural production. Chemical and biomolecular engineering publications and other works. http://trace.tennessee.edu/utk_chembiopubs/94 11

Nymark M, Sharma AK, Sparstad T, Bones AM, Winge P (2016) A CRISPR/Cas9 system adapted for gene editing in marine algae. Sci Rep 6:1–6

Olaizola M (2000) Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. J Appl Phycol 12:499–506

Østerlie M, Bjerkeng B, Liaaen-Jensen S (1999) Accumulation of astaxanthin all-*E*, 9*Z* and 13*Z* geometrical isomers and 3 and 3' *RS* optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. J Nutr 129:391–398

Palozza P, Torelli C, Boninsegna A, Simone R, Catalano A, Mele MC, Picci N (2009) Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells. Cancer Lett. 283:108–117

Park JS, Chyun JH, Kim YK, Line LL, Chew BP (2010) Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. Nutr Metab 7:18

Pérez-España V, López-Pérez P, Romero T, Peralta-Gil M, Durán-Figueroa N, Badillo-Corona A (2016) Transformation of chloroplasts of *Chlamydomonas* for the production of therapeutic proteins. In: Vasconcelos JR, Gerken H, Liu J, Sun Z (eds) Recent Advances in Microalgal Biotechnology. Omics International. https://doi.org/10.4172/978-1-63278-066-9-67

Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl Microbiol Biot 65:635–648

Reinecke DL, Castillo-Flores A, Boussiba S, Zarka A (2018) Polyploid polynuclear consecutive cell-cycle enables large genome-size in *Haematococcus pluvialis*. Algal Res 33:456–461

Renstrøm B, Borch G, Skulberg OM, Liaaen-Jensen S (1981) Optical Purity of (3*S*,3'*S*)-astaxanthin from *Haematococcus pluvialis*. Phytochemistry 20:2561–2564

Rise M, Cohen E, Vishkautsan M, Cojocaru M, Gottlieb HE, Arad SM (1994) Accumulation of secondary carotenoids in *Chlorella zofingiensis*. J Plant Physiol 144:287–292

Rohdich F, Hecht S, Gärtner K, Adam P, Krieger C, Amslinger S, Arigoni D, Bacher A, Eisenreich W (2002) Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of *isph* (*lytb*) protein. Proc Nat Acad Sci 99:1158–1163

Sandesh Kamath B, Vidhyavathi R, Sarada R, Ravishankar GA (2008) Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants Bioresour Technol 99:8667–8673

Sarada R, Tripathi U, Ravishankar GA (2002) Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. Process Biochem 37:623–627

Schmidt I, Schewe H, Gassel S, Jin C, Buckingham J, Hümbelin M, Sandmann G, Schrader J (2011) Biotechnological production of astaxanthin with *Phaffia rhodozyma/Xanthophyllomyces dendrorhous*. Appl Microbiol Biot 89:555–571

Shah M, Mahfuzur R, Liang Y, Cheng JJ, Daroch M (2016) Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. Front Plant Sci 7:531

Sharon-Gojman R, Maimon E, Leu S, Zarka A, Boussiba S (2015) Advanced methods for genetic engineering of *Haematococcus pluvialis* (Chlorophyceae, Volvocales). Algal Res 10:8–15

Siddiqui A, Wei Z, Boehm M, Ahmad N (2019) Engineering microalgae through chloroplast transformation to produce high-value industrial products. Biotechnol Appl Biochem 67:30–40

Singh RN, Sharma S (2012) Development of suitable photobioreactor for algae production – a review. Renew Sust Energ Rev 16:2347–2353

Sommer TR, D'Souza FML, Morrissy NM (1992) Pigmentation of adult rainbow trout, *Oncorhynchus mykiss*, using the green alga *Haematococcus pluvialis*. Aquaculture 106:63–74

Sousa I, Gouveia L, Batista A, Raymundo A, Bandarra N (2008). Microalgae in novel food products. In: Papadoupoulos K (ed) Food Chemistry Research Developments. Nova Science Publishers, Riejeka pp 75–112

Speranza L, Pesce M, Patruno A, Franceschelli S, de Lutiis MA, Grilli A, Felaco M (2012) Astaxanthin treatment reduced oxidative induced pro-inflammatory cytokines secretion in U937: SHP-1 as a novel biological target. Mar Drugs 10:890–899

Stebbins, GL (1970) Variation and evolution in plants: progress during the past twenty years. In: Hecht MK, Steere WC (eds) Essays in evolution and genetics in honor of Theodosius Dobzhansky. Springer, Boston, pp 173–208

Steinbrenner J, Linden H (2001) Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol 125:810–817

Steinbrenner J, Sandmann G (2006) Transformation of the green alga *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. Appl Environ Microbiol 72:7477–7484

Sun Z, Cunningham FX, Gantt E (1998) Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. Proc Nat Acad Sci 95: 11482–11488

Sun N, Wang Y, Li YT, Huang JC, Chen F (2008) Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). Process Biochem 43: 1288–1292

Sun H, Kong Q, Geng Z, Duan L, Yang M, Guan B (2015) Enhancement of cell biomass and cell activity of astaxanthin-rich *Haematococcus pluvialis*. Bioresour Technol 186:67–73

Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. Mar Drugs 9:1101–1118

Tanaka T, Shnimizu M, Moriwaki H (2012) Cancer chemoprevention by carotenoids. Molecules 17:3202–3242

Teng C, Qin S, Liu J, Yu D, Liang C, Tseng C (2002) Transient expression of *lacz* in bombarded unicellular green alga *Haematococcus pluvialis*. J Appl Phycol 14:497–500

Tjahjono AE, Kakizono T, Hayama Y, Nishio N, Nagai S (1994) Isolation of resistant mutants against carotenoid biosynthesis inhibitors for a green alga *Haematococcus pluvialis*, and their hybrid formation by protoplast fusion for breeding of higher astaxanthin producers. J Ferment Bioeng 77:352–357

Tominaga K, Hongo N, Karato M, Yamashita F (2012) Cosmetic benefits of astaxanthin on human subjects. Acta Biochim Pol 59:43–47

Tran N, Park JK, Lee CG (2009) Proteomics analysis of proteins in green alga *Haematococcus lacustris* (Chlorophyceae) expressed under combined stress of nitrogen starvation and high irradiance. Enzyme Microb Tech 45:241–246

Tripathi U, Venkateshwaran G, Sarada R, Ravishankar GA (2001) Studies on *Haematococcus pluvialis* for improved production of astaxanthin by mutagenesis. World J Microb Biot 17:143–148

Vidhyavathi R, Venkatachalam L, Sarada R, Ravishankar GA (2008) Regulation of carotenoid biosynthetic genes expression and carotenoid accumulation in the green alga *Haematococcus pluvialis* under nutrient stress conditions. J Exp Bot 59:1409–1418

Vonshak A, Richmond A (1981) Genome multiplication as related to the growth rate in blue-green algae *Anacystis nidulans*. Plant Cell Physiol 22:1367–1373

Waissman-Levy N, Leu S, Khozin-Goldberg I, Boussiba S (2019) Manipulation of trophic capacities in *Haematococcus pluvialis* enables low-light mediated growth on glucose and astaxanthin formation in the dark. Algal Res 40:101497

Wang J, Sommerfeld M, Hu Q (2009) Occurrence and environmental stress responses of two plastid terminal oxidases in *Haematococcus pluvialis* (Chlorophyceae). Planta 230:191–203

Wang N, Guan B, Kong Q, Sun H, Geng Z, Duan L (2016) Enhancement of astaxanthin production from *Haematococcus pluvialis* mutants by three-stage mutagenesis breeding. J Biotechnol 236:71–77

Wang F, Gao B, Wu M, Huang L, Zhang C (2019) A novel strategy for the hyper-production of astaxanthin from the newly isolated microalga *Haematococcus pluvialis* JNU35. Algal Res 39:101466

Wanka F (1968) Ultrastructural changes during normal and colchicine-inhibited cell division of *Chlorella*. Protoplasma 66:105–130

Wen X, Wang Z, Ding Y, Geng Y, Li Y (2020) Enhancing the production of astaxanthin by mixotrophic cultivation of *Haematococcus pluvialis* in open raceway ponds. Aquac Int 28:625–638

Wetherell DF, Krauss RW (1956) Colchicine-induced polyploidy in *Chlamydomonas*. Science 124:25–26

Yang B, Liu J, Jiang Y, Chen F (2016) *Chlorella* species as hosts for genetic engineering and expression of heterologous proteins: progress, challenge and perspective. Biotechnol J 11:1244–1261

Young A, Britton G (eds) (1993) Carotenoids in photosynthesis. Springer, Netherlands

Zhang BY, Geng YH, Li ZK, Hu HJ, Li YG (2009) Production of astaxanthin from *Haematococcus* in open pond by two-stage growth one-step process. Aquaculture 295:275–281

Zhang W, Wang J, Wang J, Liu T (2014) Attached cultivation of *Haematococcus pluvialis* for astaxanthin production. Bioresour Technol 158:329–335

Zhang D, Wan M, del Rio-Chanona E, Huang J, Wang W, Li Y, Vassiliadis VS (2016) Dynamic modelling of *Haematococcus pluvialis* photoinduction for astaxanthin production in both attached and suspended photobioreactors. Algal Res 13:69–78

Zhang L, Zhang C, Liu J, Yang N (2020) A strategy for stimulating astaxanthin and lipid production in *Haematococcus pluvialis* by exogenous glycerol application under low light. Algal Res 46: 101779

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.